EXPLORING THE DIVERSITY AND METABOLIC POTENTIAL OF ACTINOMYCETES FROM CASSAVA WASTEWATER

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Declaration

I do hereby declare that this thesis is my own work and there are no prior submissions of this report elsewhere and related work by others which served as a source of knowledge has been duly referenced.

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February 2021

Abstract

The processing of cassava results in the creation of a considerable amount of waste effluent. Understanding the indigenous microbial diversity proved to be a useful approach in investigating cassava effluent use for biodiesel production. We compared the diversity from cassava effluent using culture-dependent and culture-independent approaches using selective media and amplicon sequencing to target actinobacteria. Actinobacteria are producers of various useful metabolites, with some members in this phylum known for their oleaginicity. Arginine Glycerol Salt (AGS), Glycerine Asparagine Agar (GA) and Starch Casein Agar (SCA) were the most suitable media to enumerate the diversity in cassava effluent from this study. *Streptomyces*, *Micromonospora* and *Micrococcus* were the predominant actinobacteria detected by culture-dependent approach. In concurrence to culture-dependent approach, the culture-independent method also revealed various genera related to actinobacteria. In the culture-independent approach, *Bifidobacterium*, *Corynebacterium* and *Micromonospora* were found to be the dominant genera. The washing and pressing stages in cassava production had a more significant proportion of the actinobacteria from both methods adopted. Holistically, both approaches provided complementary information inferring the diversity within samples.

The functional potential of novel isolates from cassava waste effluent (*Streptomyces* and *Micromonospora*) was further explored for triacylglyceride (TAG) synthesis and accumulation. Phenotypic characteristics of isolates involving growth requirement, the enzymatic profile, resistance of isolates to various antibiotics, degradation of complex compounds, carbon and nitrogen utilization were explored to reveal the physiological properties useful in

biodiesel production. The fatty acid methyl ester (FAME) profiles of *Micromonospora* and *Streptomyces* represented a significant percentage of branched-chain fatty acids (BCFAs). Thin-layer chromatography, viable colony staining methods based on Nile Red, inferred TAG accumulation by novel Cassava wastewater (CWW) isolates. Further investigation into the key Kennedy pathway suggested that alternative pathways might be involved in TAG synthesis within *Micromonospora* and *Streptomyces* isolates. The presence of PAP multifunctional gene *lppα* (SCO1102) and *lppβ* (SCO1753) involved in Diacylglycerol (DAG) synthesis within *Streptomyces* CWW isolate and *Streptomyces albidoflavus* support this hypothesis.

List of presentations

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Marcus G. Awoniyi and Nagamani Bora. Exploring the diversity and metabolic potential of actinomycetes from cassava wastewater. Presented at the Microbiology Society Annual Conference, 8 – 12th April 2019, Belfast, Northern Ireland, UK.

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Acronyms and abbreviations

ACC	acetyl coenzyme A (CoA) carboxylase
AGPAT	Acylglycerol-3-phosphate-O-acyltransferase
AGS	Arginine glycerol salt agar
ATF	Acyl transferase
BCFAs	branched-chain fatty acids
BOD	Biological oxygen demand
CFE	Cassava Fermentation effluent
CME	Cassava Matna effluent
CoA	coenzyme A
COD	Chemical oxygen demand
CPE	Cassava pressing effluent
CWE	Cassava wastewater effluent
CWW	Cassava wastewater
DAG	Diacylglycerol
DGAT	Diacylglycerol-acyltransferase
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
FabA	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase
FabD	malonyl-CoA:ACP transacylase
FabF	3-oxoacyl-[acyl-carrier-protein] synthase 2
FabG	β-Ketoacyl-ACP reductase

FabH	β-Ketoacyl-ACP synthase III
FabI	enoyl-ACP reductase
FabZ	β-hydroxyacyl-ACP dehydratases
FAME	Fatty acid methyl ester
FAS	Fatty acid synthesis
FISH	Fluorescence in situ hybridisation
G3P	Glycerol-3- phosphate
GA	Glycerine Asparagine agar
GC	Gas chromatography
GHG	Greenhouse gas
GPAT	glycerol-3-phosphate-O-acyltransferase
GYM	Glucose yeast malt
HCN	Hydrogen cyanide
HVA	Humic vitamin agar
KEGG	Kyoto Encyclopedia of Genes and Genomes
mcrA	Methyl-coenzyme M reductase
NOx	Oxides of Nitrogen
OsO_4	Osmium tetroxide
OTU	Operational taxonomic unit
PAP	Phosphatidic acid phosphatase
PAP	phosphatidic acid phosphatase
PCoA	principal coordinate analysis
PCR	Polymerase chain reaction
PDAT	Phospholipid: diacylglycerol acyltransferase
PHA	polyhydroxyalkanoates

PPD	Post-harvest physiological deterioration
qPCR	Quantitative PCR
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal RNA
REP	Repetitive extragenic palindromic
SCA	Starch casein agar
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
SSU	Small subunit
TAE	Tris-acetate-EDTA
TAG	Triacylglycerol
Taq	*Thermus aquaticus*
TGGE	Temperature gradient gel electrophoresis
TLC	Thin layer chromatography
T-RFLP	Terminal restriction fragment length polymorphisms
TSA	Tryptone soya agar
UPGMA	Unweighted Pair-group Method with Arithmetic Mean
YMA	Yeast Malt Agar

CHAPTER 1: Background

1.1. Background

The world's current energy demand is mainly met by using conventional energy resources such as coal, natural gas, and petroleum. Fossil fuel consumption, particularly gasoline and diesel, is primarily in the industrial, domestic, transport and agricultural sectors. It is estimated that the world's total reserve of coal, natural gas and oil was 1.06 billion tonnes, 196.9 trillion cubic metres and 244.1 billion tonnes, respectively as of 2019. These resources can only suffice and meet human consumption for another 132, 49.8 and 50 years, respectively (Dudley, 2019). This data suggests the probability of encountering an energy crisis soon owing to the depletion of natural resources. These fossil-based fuels are non-renewable resources. Their combustion results in the emission of greenhouse gases (GHG) leading adversely to global warming, and affecting human life and environment (Alalwan *et al.*, 2019). In the long run, its impact could pose a significant threat to the planet's life-supporting capacity (Fivga *et al.*, 2019). With the expected development of growing economies such as China and India, the world's energy utilisation is expected to increase, leading to inevitable environmental damage (Mata *et al.*, 2010). Hence, it becomes imperative to explore alternative energy sources that are sustainable in the interest of future needs, thereby reducing the dependency on fossil fuels.

Currently, various alternatives to fossil energy such as wind, hydroelectric, photovoltaic, biofuel and solar energy are being investigated to address this global issue and develop sustainable solutions that can be implemented (Mata *et al.*, 2010). Government has introduced various policies and support regimes to promote these alternatives' into our daily energy usage. Fuel excise tax exemptions, mandates and targets, import tariffs, and direct production subsidies

are tools used by the government to achieve this objective (Fischer *et al.*, 2009). These alternative energy sources are not entirely immune to environmental sustainability issues (Hosseinzadeh-Bandbafha *et al.*, 2018).

1.2. Biofuel as an alternative source of energy

Biofuel is defined as renewable fuel generated from organic materials (Zainura, 2013). The production of biofuel is considered to be a sustainable alternative to fossil fuels (Zainura, 2013). Worldwide, the development and growth of biofuel production has been rapid over the last few years, with a constant annual increase leading to an estimated 95 million tonnes of oil equivalent in 2018. This is a 9.7% biofuel production increase from the previous years (Dudley, 2019).

Currently, there are over 1000 biofuel plants in operation in over 59 countries worldwide. The most significant biofuel production plants are localised in the USA and Brazil, contributing to half of the world's biofuel production (Dudley, 2019). Statistically, North America provides the world's largest biofuel production at 39 million tonnes of oil equivalent. Developments in biofuel production are still gaining momentum in other parts of the world, such as the Middle East and Africa, which are the least producing areas globally. Hence, there is a need to develop sustainable biofuel strategies in these demographic areas catering to demand and harnessing opportunities (Dudley, 2019).

Biofuels are either in the form of liquid (ethanol and biodiesel), solid (biochar) or gaseous (bio-hydrogen, bio-syngas and biogas) (Demirbas, 2009). Biofuels can be sourced from biomass, making them environmentally sustainable with added advantages, owing to their biodegradability and low GHG emissions (Demirbas, 2009). Over the years, different types of biofuels have evolved from one generation to the next based on the source of raw material available. The

first generation biofuels were sourced from edible food crops such as sugarcane, grains or seeds. The use of first-generation biofuels had gradually led to the food vs fuel debate, highlighting sustainability issues of water and land resources used in biofuel production. These issues have become more pressing owing to the recent food crises and increasing food prices (Alexander & Hurt, 2007; Doornbosch & Steenblik, 2007; Rulli *et al.*, 2016). Eventually, biofuels evolved into the second, third and fourth generation based on the availability of substrate and technologies. The second generation involves the use of waste vegetable oils and lignocellulosic biomass. The use of microalgae in biofuel production is associated with the third generation. The development of syngas fermentation technology involving acetogens is owed to various metabolic engineering strategies leading to fourth generation biofuels (Alalwan *et al.*, 2019).

Biodiesel is one such biofuel gaining momentum for its sustainability. Its characteristic higher combustion efficiency, lower greenhouse gas emissions, biodegradability, availability of feedstocks for its production, and renewability supports its suitability over fossil diesel (Hosseinzadeh-Bandbafha *et al.*, 2018). Chemical structure of biodiesel has mono-alkyl esters of long-chain fatty acids. They are derived from the transesterification process in which triglycerides (TAG) in the presence of a catalyst, reacts with alcohol to produce fatty acid alkyl esters (Fig.1.1). Since the most prevalent alcohol used in biodiesel production is methanol, biodiesel is also referred to as fatty acid methyl esters (FAME) (Hoekman *et al.*, 2012).

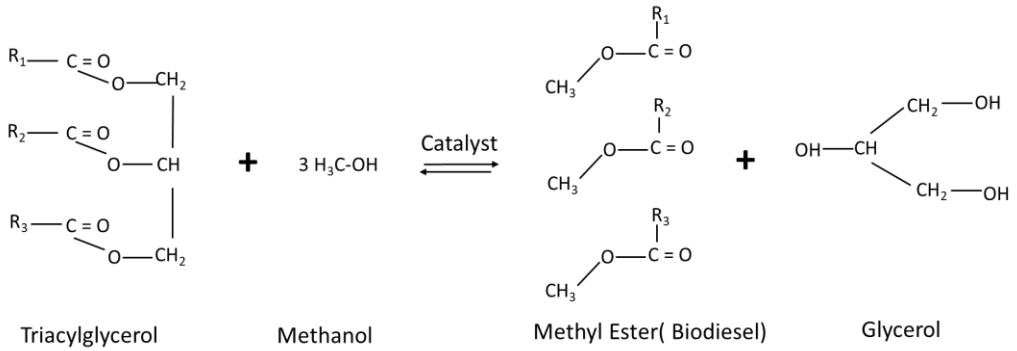


Figure 1.1: Transesterification of triacylglycerol. Catalyst (alkalis, acids or enzymes) R_1, R_2, and R_3 represents the Long chain hydrocarbons. Adapted from (Hotti & Hebbai, 2015)

So far, several feedstocks are used in biodiesel production. Table 1.1 highlights some of the key feedstocks used to date. Palm oil, rapeseed oil and soybean oil are the most dominant feedstocks in Asia, Europe and the United States, respectively. Vegetable oils with real potential, which are being explored, are sunflower, safflower, jatropha, coconut, canola and camelina (Atabani *et al*., 2012; Hoekman *et al*., 2012). The use of vegetable oils and animal fats remains a niche market in the production of biodiesel. This could be attributed to various reasons such as higher viscosity up to 20 times that of fossil fuel. However, the setbacks are incomplete combustion, poor fuel atomisation in engines, cost, low productivity and lack of market for the glycerin as a by-product with further impacts on the economic viability of this raw material (Carioca, 2010). The presence of free fatty acids in animal fats also increases the difficulty in the production process of biodiesel (Dias *et al*., 2008).

Algal lipid-based biodiesel has been in focus for the past few decades and is gaining momentum. It is regarded as one of the most sustainable feedstock alternatives. Microalgae's relatively fast doubling time, ability to utilise sunlight and growth using anthropogenic nutrients adds to microalgae's advantages as a

feedstock (Shuba & Kifle, 2018). Despite the benefits of microalgal lipids, the cost of production of algal-based biofuels is still too expensive. This is mainly due to the cost of oil extraction and subsequent processing (Wen & Johnson, 2009).

Table 1.1: Key feedstocks for biodiesel production. Adapted from (Ambat *et al.*, 2018; Atabani *et al.*, 2012; Hoekman *et al.*, 2012)

Edible oils	**Non-edible oils**	**Animal Fats**	**Other sources**
Canola oil	Karanja(Pongamia pinnata oil)	Waste fish oil	Bacteria
Coconut oil	Castor oil	Pine and kapok oil	Algae
Linseed oil	Rubber seed oil	Beef tallow	Switchgrass
Mustard oil	Chinese tallow seed oil	Mutton Fat	Tarpenes
Olive oil	Jatropha oil	Broiler chicken waste	Poplar
Palm oil	Sylbum marianum oil	Pork lard	Fungi
Peanut oil	Neem oil	Waste cooking oil	Miscanthus
Rapeseed oil	Cunaru		
Soybean oil	Mahua		
Camelina			
Sunflower oil			

Generally, although biodiesel would be the future alternative to fossil fuels, there are still some concerns on how sustainable this is. Further improvements to the processes and the emissions produced still need to be addressed. Bhaskar &

Babu (2015) compared the emission values from biodiesel and regular fossil diesel. Their report stated the lowered smoke density, carbon dioxide emission, and lower particle pollution as biodiesel's advantages over fossil diesel. However, the author noted increased nitrogen oxide (NOx) emission in biodiesel by an average of 6% for every 20% addition in blend when compared to fossil diesel. This Jiaqiang *et al.* (2017) in their review, suggest the use of additives and improved treatment of exhaust gas technologies as a possible solution to improve the issues raised.

Besides the above-mentioned environmental issues, there are still concerns about sustainable ways to produce biodiesel. During the cycle of biodiesel production, some non-renewable resources are still required. Fossil fuel requirements in powering farm equipment and the transport of crops used for biodiesel production also negatively affect sustainability. Synthetic fertilisers, which are used for growing crops, add to the cost of biodiesel production. Furthermore, they also contribute to N_2O emission (Pradhan *et al.*, 2012).

Economic sustainability issue concerning the cost and feedstock availability remains a pertinent challenge to the use of biodiesel. Price fluctuations in feedstock selection for biodiesel production remains a recurring challenge. The cost of feedstock could account for 75% of total biodiesel fuel cost (Atabani *et al.*, 2012). Mekhilef *et al.* (2011) discussed the price fluctuations in palm oil prices as an example and its impact on biodiesel production in Malaysia. They reported an increase in prices as high as fossil diesel at different time points. Therefore, the choice of feedstock can be a crucial factor in biodiesel generation. Hence, some researchers have suggested that the cost and feedstock sustainability issue could be resolved by utilising oleaginous microorganisms on

low cost and effective wastes (Chen *et al.*, 2018; Cho & Park, 2018; Qadeer *et al.*, 2018)

1.3. Biodiesel from oleaginous microorganisms - a lucrative option in biofuel development

Current research is centred on the use of oleaginous microorganisms in generating alternative biofuels, notably biodiesel. Several microorganisms belonging to the bacteria, fungi, yeast and microalgae have been highlighted to produce a significant amount of oil content exceeding 20% of their body mass weight (Shields-Menard *et al.*, 2018; Thevenieau & Nicaud, 2013). This group of oleaginous micro-organisms can accumulate triacylglycerol (TAG) similar in composition to plant oils (Martinez *et al.*, 2015). Historically, the consideration of the economics of microbial oils had dampened the feasibility of its industrial applications (Alvarez & Steinbüchel, 2002; Shields-Menard *et al.*, 2018). However, with the amelioration of current transesterification and fermentation technologies, microbial lipids can be generated in similar quantities compared to acres of agricultural land. These oleaginous microorganisms can play a predominant role in biodiesel production due to their fast growth rate and lipid productivity (Thevenieau & Nicaud, 2013). Furthermore, there are no weather requirements for the production of biodiesel from oleaginous microorganisms. This suggests they can be cultivated all year round for biodiesel (Thevenieau & Nicaud, 2013).

The nature of lipids produced vary between eukaryotes and prokaryotes. Algae, yeast and fungi produce polyunsaturated fatty acid triglycerides comparable to vegetable oils used in biodiesel production (Wood, 1988). According to

Sawangkeaw & Ngamprasertsith (2013), the productivity and lipid content of selected oleaginous microorganisms are presented in Table 1.2 below.

Table 1.2: Lipid content and productivity of selected oleaginous microorganisms. (Sawangkeaw & Ngamprasertsith, 2013)

Oleaginous Microorganism	**Lipid content (%w/w)**	**Productivity ($Kg/m^3/yr$)**	
		Biomass	**Lipid**
Yeast			
Candida curvata	29-58	691	315
Limpomyces starkeyi	61-68	636	410
Cryptococcus albidus	33-60	3362	2120
Fungi			
Mortierella isabelline	50-55	1276	679
Aspergillus oryzoe	18-57	377	215
Cunninghamella echinulata	35-58	232	134
Microalgae			
Chlorella zofingiensis	52	216	112
Scenedesmus obliquus	13-58	153	54
Chaetoceos muelleri	15-60	1065	404
Bacteria			
Rhodococcus opacus	24-26	N/R	N/R
Acinetobacter calcoacetius	27-38	N/R	N/R
Bacillus alcalophilus	18-24	N/R	N/R

Several biological systems have been explored to understand the nature and production of lipids from oleaginous organisms useful in biodiesel production. In yeast, Bonturi *et al.* (2015) investigated the effectiveness of lipid extraction strategies from oleaginous *Rhodosporidium toruloides* and *Lipomyces starkeyi.* Authors reported reasonable lipid yields (39% w/w) from the Folch and the Bligh & Dyer methods using both strains of oleaginous yeast. More recently, Annamalai *et al.* (2018) investigated lipid production from waste office paper using oleaginous yeast *Cryptococcus curvatus*. Other researchers, Granger *et al.* (1993), Akhtar *et al.* (1998) and Easterling *et al.* (2009) demonstrated the potential of lipid production from oleaginous yeast.

In Fungi, Eroshin *et al.* (2000) reported lipid production of 60.4% from 18.8% dry biomass. This was achieved using *Mortierella alpina* LPM 301 in a batch media cultivation containing glucose and potassium nitrate or urea as the nitrogen source. Other works have been carried out by Papanikolaou *et al.* (2017), Aki *et al.* (2001), Khot *et al.* (2012) and Harde *et al.* (2016) also inferring the production of lipids from fungi.

In microalgae, Miao & Wu (2004) reported an increased yield of 57.9% (3.4 times higher than autotropic cells) bio-lipids produced by *Chlorella protothecoides,* a heterotropic microalgae, by manipulating metabolic pathways. Semiao (2013) work on *Chlorella protothecoides* supports the premonition of substantial lipid production from oleaginous microalgae. Liu *et al.* (2008) demonstrated an increase in the lipid productivity of *Chlorella vulgaris* by supplementing media with five levels of Fe^{3+} concentration.

Bacteria represent a distinct group of oleaginous organisms with high cell growth rate using simple cultivation strategies. They are not usually lipid-

accumulating microorganisms as they produce polyhydroxyalkanoic acids when a carbon source is limited as a means of energy storage (Martinez *et al.*, 2015). Interestingly, some species of actinomycetes have been shown to accumulate triacylglycerols (Martinez *et al.*, 2015; Shields-Menard *et al.*, 2018). Some microbes belonging to the *Bacillus* genus were also found to produce oil content (Cho & Park, 2018; Subramaniam *et al.*, 2010).

1.3.1. Actinobacteria as oleaginous microorganisms

Actinobacteria are Gram-positive filamentous organisms with high G+C content (Nakashima *et al.*, 2005). Actinobacteria produce mycelium, which is a characteristic of filamentous fungi (Barka *et al.*, 2016). The comparison of actinobacteria with fungi is largely superficial. Like other bacteria, they possess a peptidoglycan cell wall containing its nucleoid and are susceptible to antibiotics. Physiologically, actinobacteria are aerobic with some exceptions (Barka *et al.*, 2016). They can be chemoautotrophic or heterotrophic. They can acclimatize to various environments, which accounts for them being ubiquitous (Barka *et al.*, 2016). They are mostly mesophilic, with the ability to tolerate temperature ranges between 25^{o}C-30^{o}C. Thermophilic actinobacteria have also been reported with growth between 50^{o}C to 60^{o}C (Edwards, 1993). Actinobacteria grow best within a pH range of 6 and 9, although some strains of *Streptomyces* have been reported to thrive even on a lower pH of 3.5 (Kim *et al.*, 2003). Members of actinobacteria phylum exhibit distinguished metabolic diversity, hence are of significant medical and industrial importance (Nakashima *et al.*, 2005).

Amongst the 18 significant lineages of the bacteria domain, actinobacteria constitute one of the largest taxonomic units (Ludwig *et al.*, 2012). The

metabolic, physiological and morphological diversity of genera within the phylum is enormous. The delineation of this phylum up to genus level is based on the 16S rRNA gene sequencing. Additional housekeeping gene such as *ssgB* and *rpoB* have more recently been used to discriminate between closely related genera (Barka *et al.*, 2016). The classification of actinobacteria based on the morphological, chemotaxonomic and metabolic characteristics has been extensively reviewed (Barka *et al.*, 2016). Morphological classification involving the use of microscopic morphology and chemotaxonomic classification utilising an organism's chemical composition has been used in the delineation into genus and species level (Barka *et al.*, 2016). Lipid composition, protein, sugars, menaquinones and cell wall amino acids are some markers used in chemotaxonomic classification. More recently, the classification using whole genome-based approaches has been used to classify actinobacteria (Girard *et al.*, 2013).

The actinobacteria phylum host a number of industrially significant oleaginous bacteria. Voss & Steinbuchel (2001) reported the ability of *Rhodococcus opacus* PD630 to produce fatty acid content as much as 51.9% of the cell dry weight when grown in fed-batch mode on sugar beet molasses and sucrose media. This report indicates the potential of TAG accumulation from *Rhodococcus opacus* PD630 using low-cost crops. The production of TAGs by *Rhodococcus opacus* remains the most widely described so far. Le *et al.* (2017), Mahan *et al.* (2017), He *et al.* (2017) have discussed extensively the importance of *Rhodococcus opacus* as a potential source for biodiesel production.

Gouda *et al.* (2008) investigated the capability of *Gordonia spp*. and *Rhodococcus opacus* PD630 to accumulate TAG using gluconate and different

agro-industrial wastes generated from the industries in Egypt as a carbon source. In comparison, significant lipid accumulation was observed in *R. opacus* (about 80%) at the beginning of stationary phase whilst it was at the end in *Gordonia spp* (about 72%). In addition, when tested with other agro-industrial wastes, both organisms showed accumulation of over 50% lipids suggesting the suitability of some genera belonging to actinomycet*e*s to utilise low-cost wastes in lipid accumulation.

Röttig *et al.* (2017) described *Streptomyces jeddahensis* as an oleaginous bacterium isolated from desert soil. The novel *Streptomyces* strain, was able to accumulate 65 % of its cell dry weight as fatty acids with ante iso-$C_{15:0}$, iso-$_{C15:0}$, $C_{16:0}$ and iso-$C_{16:0}$ as the predominant fatty acids. This strain was able to grow on a broad range of carbon sources at temperatures up to 50^{o}C..

Shields-Menard *et al.* (2015) investigated *Rhodococcus rhodochrous* for their ability to accumulate lipids and found about 50% accumulation of dry cell mass as lipids when cultured on glucose. Amara *et al.* (2016) discussed the importance of *Rhodococcus jostii* as a potential feedstock for biodiesel production. The authors also elucidated the TAG pathway in mycolic acid-containing actinomycetes.

Alvarez *et al.* (1997) demonstrated in their studies about other genera in actinomycetes such as *Norcardia* and *Rhodococcus* that can produce triacylglycerol under nitrogen limiting conditions. *Rhodococcus opacus, Rhodococcus jostii, Rhodococcus fascians, Streptomyces coelicolor, Streptomyces albus, Streptomyces griseus* amongst other actinomycetes have also been reported to produce a significant amount of triacylglycerols (Alvarez, 2010; Packter & Olukoshi, 1995).

1.3.2. Triacylglycerol (TAG) biosynthesis in oleaginous organisms

Triacylglycerols are water-insoluble triesters of glycerol bound with three fatty acid molecules (Shields-Menard *et al.*, 2018). TAGs possess high calorific values exceeding those of proteins and carbohydrates. Therefore, this characteristic feature makes them efficient energy stores as they release more energy when oxidised (Shields-Menard *et al.*, 2018). The accumulation of lipids in oleaginous microorganisms usually occurs after phospholipid synthesis during the stationary phase. The accumulation occurs during excess carbon and limited nutrient conditions. Various studies have reported that nitrogen limitation is responsible for the accumulation of lipids by oleaginous microorganisms (Alvarez & Steinbüchel, 2002; Alvarez *et al.*, 1997; Packter & Olukoshi, 1995; Ratledge, 2004).

In bacteria, the synthesis of TAG occurs via the Kennedy pathway (Fig. 1.2). Herein, glycerol-3- phosphate (G3P) serves as a glycerol source for acyl-CoA-dependent acylation to produce TAG. G3P from glycolysis is catalysed by glycerol-3-phosphate-O-acyltransferase (GPAT) to produce acylglycerol-3-phosphate. The synthesis of acylglycerol-3-phosphate by GPAT is the first acylation step. The second acylation step is catalysed by acylglycerol-3-phosphate-O-acyltransferase (AGPAT). The corresponding Phosphatidic acid produced is dephosphorylated by a phosphatidic acid phosphatase (PAP) resulting in the generation of diacylglycerol (DAG). The final acylation of the resulting DAG is catalysed by diacylglycerol-acyltransferase enzyme (DGAT) to produce TAG (Alvarez, 2016; Comba *et al.*, 2014; Kalscheuer & Steinbüchel, 2003).The esterification of these fatty acid with glycerol backbone occurs at the *sn*-1, *sn*-2, and *sn*-3 positions (Kalscheuer & Steinbüchel, 2003).

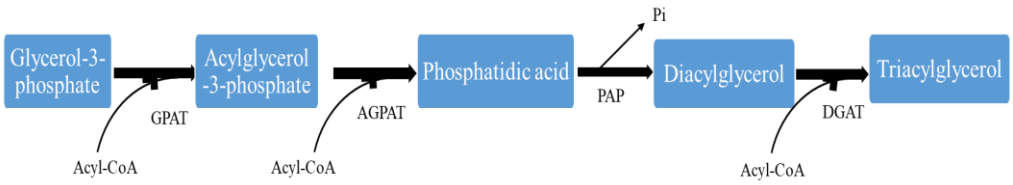


Figure 1.2: Schematic of the Kennedy pathway of TAG biosynthesis. Abbreviations: FAS (Fatty acid synthesis. DGAT (diacylglycerol acyltransferase), GPAT (glycerol-3-phosphate O-acyltransferase), AGPAT (acyl-glycerol-3-phosphate acyltransferase), PAP (phosphatidic acid phosphatase). Adapted from (Alvarez, 2016 ; Amara *et al*., 2016)

Despite the overwhelming benefits of using oleaginous microorganisms as production platforms for biodiesel production, the cost is high (Thevenieau & Nicaud, 2013) in terms of growth media and other substrates used. Hence, to sustainably utilise these oleaginous microorganisms for TAG production, alternative viable, cost-effective feedstocks need to be considered. Performance in terms of productivity and yield in bioprocess has to be further considered in choosing oleaginous microorganisms as production hosts (Thevenieau & Nicaud, 2013).

1.4. Cassava waste as an alternative substrate for the growth of oleaginous bacteria in TAG production

1.4.1. Cassava

Cassava (*Manihot esculanta* Crantz) also referred to as *tapioca* in France and *yucca* in Spanish is a perennial, root tuber crop belonging to the Euphorbiaceae family. It is one amongst the 100 different species of the genus *Manihot* (Parmar *et al.*, 2017). A native of South America, cassava is now widely grown in tropical

and subtropical parts of the world. This shrubby perennial crop can grow up to 6-8 feet tall and possess subterranean lateral storage organs in the form of starchy roots (Parmar *et al.*, 2017; Uchechukwu-Agua *et al.*, 2015). Cassava also has roots which are drought-resistant, offering flexibility in harvesting (Uchechukwu-Agua *et al.*, 2015). An image of a cassava root, post-harvest is shown in Fig.1.3.



Figure 1.3: Cassava roots post-harvest
Source: https://www.britannica.com/plant/cassava/images-videos

1.4.1.1. Global significance

Globally cassava cultivation , was estimated to be over 275 million tonnes in 2017. Africa contributes approximately 57% of total world production, with Nigeria being the largest cassava producer worldwide (FAO, 2018). On average, cassava production is estimated to require over 25 million hectares worldwide. Figure 1.4: Global cassava production in 2017 below illustrates the global production of cassava.

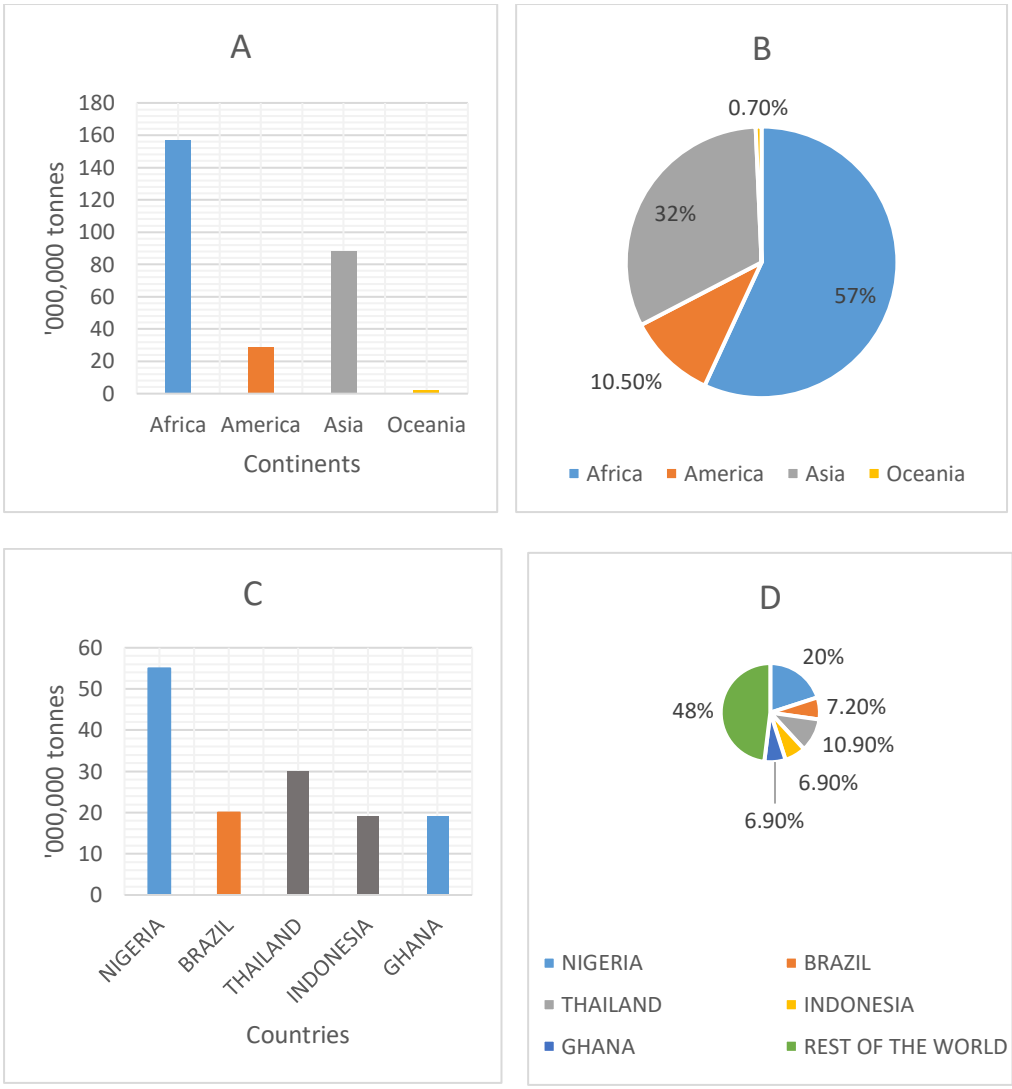


Figure 1.4: Global cassava production in 2017. A) Leading continents producing cassava. B) Percentage share of cassava produced in the world. C) Top cassava producing countries. D) Percentage share of cassava produced by top producers (adapted from FAO, 2018)

The cultivation of cassava is predominantly significant due to its starchy roots (Agwa *et al.*, 2014). It is estimated that cassava supplies households a starch content between 200–500 cal/day in most developing countries (Sánchez *et al.*, 2006). They are also considered to be a good source of Nicotinic acid, thiamine and riboflavin (Zhu, 2015). Hence, it is considered to be the 5^{th} most crucial calorie source and one of the fastest expanding staple crops worldwide besides

maize, rice, wheat and potato (Parmar *et al.*, 2017). In addition to its starch content, it is advantageous in terms of being a sturdy crop, with high yields and propagation, enhancing its economic significance (Oboh, 2005). Taiwo (2006) highlighted the importance of cassava cultivation as a potential commercial product both from a domestic and industrial perspective. The sustainability of cassava was examined, and the potential impact of cassava for economic growth was highlighted. Apata *et al.* (2018) discussed the importance of cassava as a staple food crop in Nigeria. The authors investigated cassava's economic impact in a sample rural area and concluded that cassava could be a wealth generation opportunity for most farmers.

Despite the characteristic importance of cassava, it contains a high amount of cyanogenic glucosides, i.e. lotaustalin and linamarin, which produce hydrogen cyanide (HCN). The production of HCN occurs when the plant tissue is disrupted during processing and harvesting. These cyanogenic glycosides produce HCN when hydrolysed by the endogenous enzyme linamarase (Arotupin, 2007). However, this HCN can be significantly reduced during the processing of cassava (Onabolu *et al.*, 2001).

1.4.1.2. Post-harvest handling of cassava

Storage, processing, and packaging of roots are the key strategies in handling cassava following harvest. Various traditional and modern methods were adopted in post-harvest storage. Storage of crops in-ground is the earliest and simplest traditional storage methods. However, this method is not as effective, as the lignification of storage root can occur because of the extended storage period (Uchechukwu-Agua *et al.*, 2015). Furthermore, some properties and features of the crop are lost. Burying and pilling of the root were also explored

which are not completely reliable and can cause crop deterioration. Storage in plastic bags, boxes and field clamping are some of the improved methods of cassava storage. Advanced techniques involving refrigerators and paraffin wax are other storage methods were explored in post-harvest cassava storage (Reilly *et al.*, 2003; Uchechukwu-Agua *et al.*, 2015) to alleviate the issues from traditional storage methods.

The processing of cassava is required almost immediately after harvest. They can only be stored between 2-7 days due to its rapid post-harvest physiological deterioration (PPD) (Parmar *et al.*, 2017; Sánchez *et al.*, 2006). Post-harvest physiological deterioration can be regarded as a non-microbial primary deterioration which is swiftly followed by microbial rotting. In cassava, this is one of the most pertinent deterioration of the tuber crop (Saravanan *et al.*, 2016). Damage to the structure of cassava can also hasten the PPD of the tuber crop. The first sign of PPD is usually visible on the xylem within 24 hours as a form of blue streaks. These streaks then turn black within 4-6 days (Parmar *et al.*, 2017). Cassava PPD can also be attributed to its high moisture content (60 to 75 %, wet basis) and respiratory rate (≈23 μL CO_2/kg-h at 25 °C) (Uchechukwu-Agua *et al.*, 2015). Cassava roots and leaves also contain HCN (120–1945 mg HCN equivalent/kg) which needs to be removed before they can be consumed (Hahn, 1992; Uchechukwu-Agua *et al.*, 2015). The intake of food or water containing a substantial amount of cyanide can cause acute intoxication and sometimes leading to death (Agwa *et al.*, 2014). Furthermore, cassava is not palatable in its raw form and has to be processed. Cassava processing into various forms mitigates against the low shelf life, transportation difficulties, cyanide content, and palatability (Hahn, 1992). The bulkiness of cassava due to

high moisture content is another reason for its processing. This adds to the transportation and market cost (Edhirej *et al.*, 2017; Hahn, 1992). Various studies on the processing of cassava have suggested the importance of cassava processing to enhance its usability. The importance of processing was extensively critiqued by Jansz & Uluwaduge (1997).

There are different methods for processing cassava. The techniques adopted for the processing of cassava were modified and improved over time. These techniques vary from milling, roasting, fermenting, soaking or seeping, pressing, drying, grating, slicing, steaming, peeling and boiling (Hahn, 1992). Figure 1.5: Different derivatives from cassava root shows the different products that can be obtained from cassava processing (Uchechukwu-Agua *et al.*, 2015).

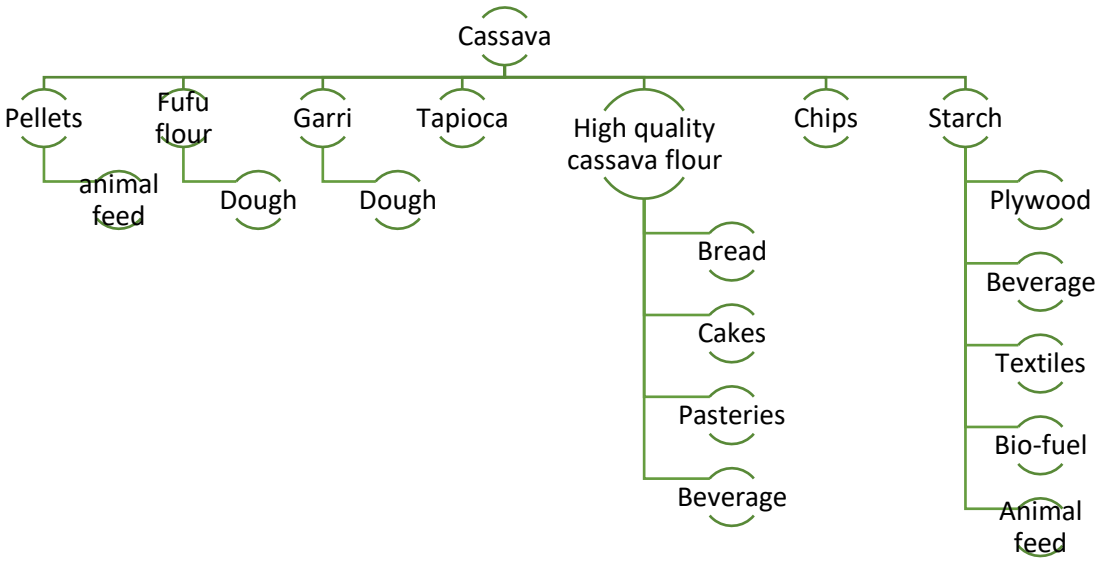


Figure 1.5: Different derivatives from cassava root (adapted from (Uchechukwu-Agua *et al.*, 2015)

1.4.1.3. Cassava production in Nigeria

Nigeria is the largest producer of cassava in the world. The cultivation of cassava is primarily due to the economic position of the nation. With the nation classified as a lower-middle-income country, households resort to consuming low-cost staple foods with high caloric values (Onyemauwa, 2010).

Cassava processing can lead to aesthetic nuisance and environmental pollution. There are several waste products from cassava processing. Cassava peel and cassava effluent are the two primary waste products generated (Oboh, 2005). These wastes are generated from either the initial stage of processing or at several stages later during the process. During the initial processing of cassava, the outer layer of the cassava root is removed. This peel contains the thin outer, brown and thick parenchymatous inner covering, which accounts for 20-35% of tuber weight (Uzoije & Egwuonwu, 2011).

In Nigeria, it is estimated that about 450,000 tons of cassava peels are generated annually (Agwa *et al.*, 2014). These peels obtained from cassava processing are usually indiscriminately discarded or allowed to rot on fields (Oboh, 2005), leading to significant environmental damage. Cassava wastewater or effluent is the second primary waste generated from cassava processing. It is estimated that the $23m^3$ of water is required per tonne of cassava processed (Okunade & Adekalu, 2013). This is lost further down the process chain. Washing of the tuber and squeezing of cassava paste accounts for the major consumption and loss of water causing several environmental concerns (Ubalua, 2007).

This effluent contains a host of inert materials, high contaminating load of chemical oxygen demand (COD), biological oxygen demand (BOD) and cyanide which could pose grave environmental problems when they are not

adequately discarded (Oghenejoboh, 2015). Furthermore, the study on the effect of discharge of cassava wastewater on soil carried out by Izonfuo *et al.* (2013) elucidates the alteration of soil's physicochemical properties. Oghenejoboh (2015), also concluded that the dissolved oxygen (DO) in pond water invariably depletes due to cassava wastewater discharge into ponds. In addition to depleted dissolved oxygen, Arimoro *et al.* (2008) reported an elevation in nitrate values, biological oxygen demand and a reduction in pH values of tropical streams in Southern Nigeria, which have been affected by an indiscriminate discharge of cassava wastewater. Cyanide is an important contaminant present in both wastes from cassava processing (Agwa *et al.*, 2014). It was inferred by Bengtsson & Triet (1994) that the average cyanide content in untreated cassava effluent varies between 3.2 to 3.5mg CN/L. The presence of cyanide in cassava waste prevents the activities of biological agents in its degradation (Olayiwola, 2013). Cyanide has been implicated in various health and environmental problems caused by improper disposal of cassava waste (Okunade & Adekalu, 2013). It has been reported that even a low concentration of cyanide consumed can have a deleterious effect on humans (Onabolu *et al.*, 2001). Diseases such as Cretinism Syndrome, Endemic Goiter and Leber' Optical Atrophy have been suggested to be caused by cyanide (Agwa *et al.*, 2014). Oghenejoboh (2015) also inferred an increase in pond water acidity due to high cyanide content from cassava waste. Despite the dangers, cassava wastewater is often discarded into roadside ditches or onto fields in Nigeria, where they are allowed to flow into water bodies or percolate into the ground (Agwa *et al.*, 2014).

1.4.1.4. Cassava waste value-added products

To mitigate the indiscriminate discharges of waste from cassava, there is an immediate need for safe waste disposal options. Alternatively, cassava waste effluent can be used in the generation of different value-added products. The burying of sealed bags containing solid waste residues in pits and ground is currently utilised to create a value-added product from this waste. The anaerobic conditions created in these bags can allow the growth of lactic acid bacteria during fermentation. Fermentation of solid residues such as cassava peel can enhance these waste's digestibility, easing its utilisation as poultry and pigs feed. Tweyongyere & Katongole (2002) demonstrated in their article, the potential for cassava peels to be used safely as animal feed. They reported reduced cyanide content at various time intervals by way of processing. Therein, it could also be concluded that cyanide content could be significantly reduced during fermentation of cassava peels. This will decrease the negative impact of this waste if they are to be discarded.

The use of cassava waste effluent is still a niche area, which needs exploration. There is potential to generate value-added products from cassava waste effluent such as biosurfactants. Nitschke & Pastore (2006) described the synthesis of biosurfactants by *Bacillus subtilis* LB5a using cassava waste effluent as a substrate. Costa *et al.* (2010) reported rhamnolipid applications produced from *Pseudomonas aeruginosa* L2-1 as surfactants when cassava effluent was used as a substrate for growth. Cassava waste effluent has also been suggested to support other bacterial strains in their growth for various end purposes. Francisco Fábio Cavalcante Barros (2013) investigated enzyme production capability of *Bacillus subtilis* when grown on cassava wastewater. In his finding,

he concluded that the strains cultured on cassava wastewater were suitable amylase and protease producers and were a better option for the production of these enzymes than synthetic media.

1.4.2. Microbial diversity studies on cassava wastewater effluent

The study of microbial diversity has become imperative due to the significance of microorganisms in the earth's biota. Microorganisms underpin vital processes, maintain complex relationships between themselves and other higher forms of life. Furthermore, they represent a significant proportion of life's genetic diversity (Salmonová & Bunešová, 2017). Microbial diversity is immense, yet the majority still remains unearthed from different ecological niches. Currently, about 1.8 million eukaryotic and 7031 prokaryotic species have been described. At the same time, it is estimated that over five to ten folds more species are yet to be identified (Alain & Querellou, 2009). This gap in knowledge of existing earth microbiota is mostly due to the inability to culture most organisms using current techniques (Colwell, 1997). The assessment of microbial diversity describes the qualitative variations between organisms in a consortium. Knowledge of this diversity helps to exploit the untapped reservoir of microbial resources useful to man (Hill *et al.*, 2000). Over the last decade, different methods and approaches have been introduced to investigate complex microbial diversity. These methods are based on artificially recreating growth conditions to isolate target microbes or the direct investigation based on the gene sequence comparison (homologue, orthologue, or paralogue gene sequences) (Hill *et al.*, 2000).

1.4.2.1. Culture-dependent approach in diversity studies

The culture-dependent method involves the use of inoculum and incubation of growth media to obtain a pure culture of single species from environmental samples. These media are fortified with basic nutrients needed for the growth of the target microbial community. This approach has been the method of choice for over a century in bacteria isolation and detection. It provides benefits including purification of single strain and amplification of its genetic material. Thus, resulting in the characterisation of direct descendants (Alain & Querellou, 2009; Mandal *et al.*, 2011). A wide range of bacteria from environmental samples can be isolated by using selective media. A target group's isolation requires media containing specific nutrient composition, selective anti-microbial agent, and suitable energy source. In other words, the intrinsic selectivity of media defines the diversity of bacteria which can be obtained from different environmental niches (Staley & Konopka, 1985). However, the strategy is plagued with the growth of organisms that grow faster in the selected media culture. This, therefore, gives a false representation of the actual microbial community (Skinner *et al.*, 1952). Challenges hindering the success of this strategy have also been associated with the choice of growth medium. The choice of nutrients, concentrations of these nutrients and the prevention of co-precipitation of chemicals introduced require careful consideration. Thus, the design of growth media remains challenging. Furthermore, this method is time-consuming as it requires considerable amount of days to propagate isolates on the desired media. Despite the flaws associated with this method, cultivation methods still remains pivotal for obtaining pure isolate of commercial importance (Salmonová & Bunešová, 2017).

1.4.2.2. Culture-independent approach in diversity studies

Due to the limitations of the culture-dependent method of characterising microbial diversity, the culture-independent approach has gained prominence in characterising unknown and uncultivable microorganisms. Culture-Independent studies exploit the direct extraction, quantification and identification of nucleic acids from environmental samples (Kirk *et al.*, 2004). This method, particularly those involving polymerase chain reaction (PCR), mediated amplification of target genes such as clone libraries, quantitative PCR (qPCR), terminal restriction fragment length polymorphisms (T-RFLP), restriction fragment length polymorphisms (RFLP), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) and metagenomics have been important in the assessment of microbial communities. These techniques analyse the rDNA or rRNA which form a part of the ribosome. In bacteria, the 16S rDNA has proved to be most informative in the investigation of evolutionary relatedness. The small subunit (SSU) rDNA molecules are predominantly significant in these studies because they are universally found in all life forms. The amplification of SSU rDNA molecules using the PCR is relatively easy and can be rapidly sequenced. The 16S rDNA molecule possesses both variable and highly conserved regions. Therefore, based on the differential sequence evolution rate, phylogenetic relatedness can be determined at several hierarchical levels. Some protein-coding genes such as methyl-coenzyme M reductase (*mcr*A)(Vianna *et al.*, 2009), sulfate thioesterase/thiohydrolase (*sox*B) (Chen *et al.*, 2009), ammonia monooxygenase (*amo*A) (Chen *et al.*, 2009; Shravage *et al.*, 2007), ribulose1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Chen *et al.*, 2009), have also been utilised in the microbial community studies. Non-PCR

based approaches such as fluorescence in situ hybridisation (FISH) and Microarrays have also proved to be useful in probing microbial community dynamics and diversity.

Elijah *et al.* (2014) reported the presence of 26 bacterial species in cassava wastewater, utilising culture-based and non-culture-based approaches. In this finding, he observed *Lactobacillus fermentum* and *L. plantarum* were the most dominant species with 11.1% and 10.7% coverage, respectively. This finding supports the *Lactobacillus* predominance as reported by Avancini *et al.* (2007) in cassava waste. This study thus provides substantial information supporting the vast abundance of bacteria present in cassava wastewater. Work done on cassava wastewater has been centred mainly on elucidating cassava wastewater properties and the production of biomolecules using cassava waste as substrate (Nitschke *et al.*, 2004; Nitschke & Pastore, 2006) (Costa *et al.*, 2010).

Currently, to the best of our knowledge, there was no work done to date investigating microbial diversity from different processing effluents of cassava. The potential of oleaginous bacteria in the utilisation of cassava waste effluent for growth was also not reported so far.

1.4.3. Physiological and functional assessment of micro-organisms from cassava waste

Holistic knowledge of functional aspects of dominant microbes within cassava effluent is vital in inferring their potential, especially in biodiesel production. The use of phenotypic and genotypic methods based on a polyphasic approach for bacterial classification was adopted to assess the function of cassava isolates in this study. The phenotypic methods are those which do not involve DNA/RNA sequencing. This method is based on the morphological,

biochemical and physiological properties of isolates (Sarethy *et al.*, 2014). These properties include the potential of cassava organisms to grow at different temperature range, salinity, pH, growth in the presence of various nitrogen and carbon sources, production of multiple enzymes and growth in the presence of anti-microbial agents. This approach also provides information on the organism's characteristics (cell shape, dimension, motility, growth rate and spore formation). The genotypic methods utilise DNA or RNA techniques to provide information on an organism (Sarethy *et al.*, 2014). This genotypic method provides information on the potential of microbes corresponding mostly to the presence or absence of particular functional genes or pathway in the microbe's genome (Escalas *et al.*, 2019).

Besides the genome content in relation to gene, genotypic traits involving 16S rRNA gene copy number, effective genome size, number of genes per genome and GC content are some other properties which have been used to gain more knowledge on microbes (Barberán *et al.*, 2012; Fierer *et al.*, 2014; Goberna & Verdú, 2016).

Currently, there is a less understanding of diversity studies on cassava effluent and the potential of utilising such functional diversity in biodiesel production.

1.5. Aims and objectives

Overall aim

The aim of this research project is to investigate the metabolic potential of oleaginous actinomycetes in the production of biodiesel, utilising cassava wastewater as a substrate.

Specific objectives

- To utilise culture-dependent and independent strategies to investigate the microbial diversity in cassava wastewater effluent obtained from different processing points, with actinobacteria as the target phyla.
- Phenotypic characterisation and FAMEs analysis as a tool to reveal the characteristic features of novel oleaginous strains from cassava effluent
- To hypothesize the functional aspects of indigenous novel species obtained from the cassava effluent in the production of biodiesel
- To explore the TAG accumulation potential of novel species. highlighting the potential pathways that may play a role in the synthesis of TAG

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CHAPTER 2: Culture-dependent and independent approach to investigate the actinobacterial diversity in cassava wastewater effluent

2.1. Introduction

The processing of cassava results in the creation of a considerable amount of waste effluent. In Nigeria, cassava is a staple crop which is drought and pest resistance, making it lucrative in mass cultivation (Oboh, 2005). Hence, there is a large scale cultivation and subsequent processing for end-use. The waste generated in cassava processing is being indiscriminately discarded due to ineffective waste management (Oghenejoboh, 2015).

Actinobacteria have been widely reported for their oleaginous properties (Le *et al.*, 2017; Voss & Steinbuchel, 2001). The exploration of oleaginous actinobacteria for biodiesel production from cassava effluent is an aspect, which is yet underexplored. Therefore, one of the objectives of this study was to explore the microbial diversity in cassava wastewater effluent obtained from different processing sample points, with actinobacteria as the target group. This information can lead to further investigations into the role of cassava effluent in biodiesel production.

Previous research on cassava waste has been carried out by Elijah *et al.* (2014) and Miambi *et al.* (2003). Elijah *et al.* (2014) investigated the molecular characterisation of bacterial species from cassava wastewater in comparison with cassava peel. Miambi *et al.* (2003) investigated the diversity within fermented cassava dough using culture-dependent and independent approaches. *Lactobacillus*, *Pediococcus* species as well as species of *Clostridium*, *Propionibacterium* and *Bacillus* were the dominant genera observed.

The culture-dependent and independent approach we used in current research targets actinobacteria as the functional group for the end purpose.

2.2. Materials and methods

2.2.1. Cassava waste effluent sampling

Cassava wastewater (CWW) sample was collected from 4 cassava processing companies located in southwestern Nigeria. Fig.2.1 shows the geographical location of the sample collection. Samples were obtained from three companies producing cassava flakes (*Garri*) (Oxford Foods Ikare-akoko, Bilatrade food processing Ado-Ekiti and Reputable food and allied processing company Ado-Ekiti) and one company producing cassava flour (Matna foods Benin-Owo, Ondo State). Details of sample locations are shown in Table 2.1. Samples were aseptically collected in sterile tubes, frozen and transported to the University of Nottingham on dry ice. Upon arrival, pH was determined using the Mettler Toledo pH meter. Samples were aliquoted into 15ml tubes and stored at -20^{o}c freezer (Indesit) for further analysis.

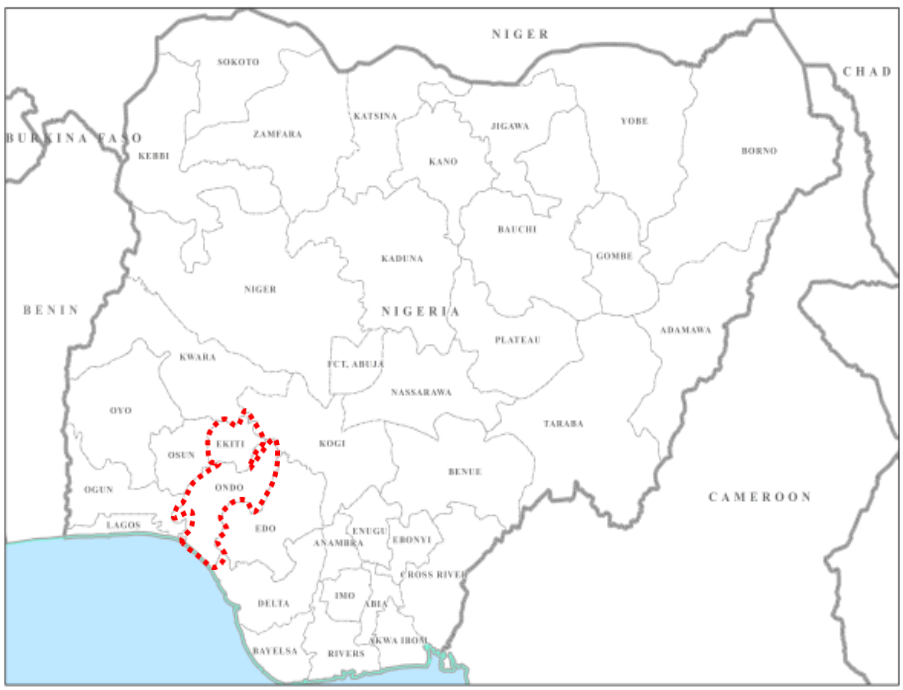


Figure 2.1: Geographical location of samples collected in Nigeria. *Adapted from https://maps.mapaction.org/dataset/206-2593/resource/28f4d75e-a8c9-4e33-b94f-4cbb8e37e610.*

Fig. 2.2 below illustrates the stages in which samples were collected from within the four companies.

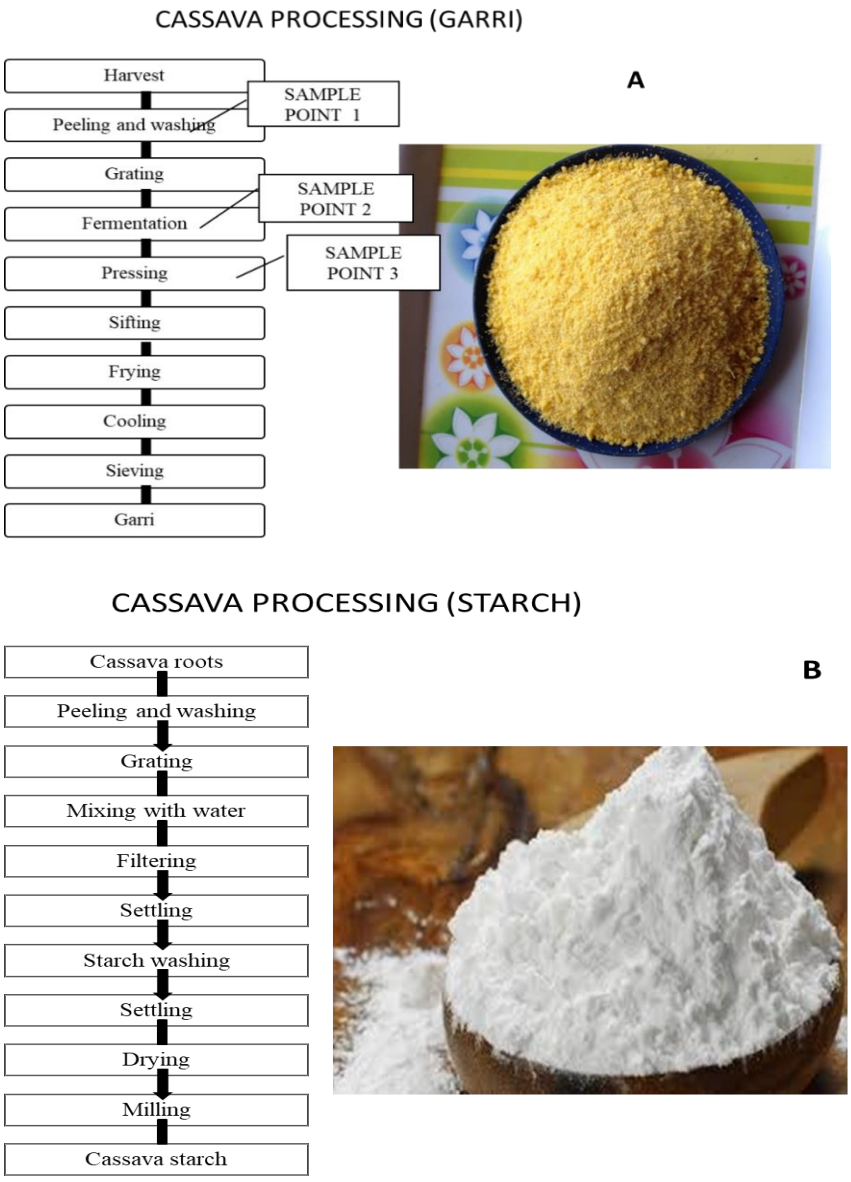


Figure 2.2: Cassava wastewater process showing sampling points. (A) Garri production process showing the sampling points. Wastewater samples were collected from the washing, fermentation and pressing stages of 3 separate cassava processing companies (Oxford Foods Ikare-akoko, Bilatrade food processing Ado-Ekiti, Reputable food and allied processing company Ado-Ekiti) (B) Cassava starch production: Wastewater sample was obtained from a pool of all production processing steps in Matna foods company.

Table 2.1: Cassava waste effluent sample collection points.

	Fermentation stage (CFE)	washing stage (CWE)	Pressing stage (CPE)	Culmination of all effluent (CME)
Oxford Foods Ikare-akoko (garri processing)	sample 1	sample 2	sample 3	-
Bilatrade food processing Ado-Ekiti (garri processing)	sample 5	sample 6	sample 7	-
Reputable food and allied processing company Ado-Ekiti (garri processing)	sample 8	sample 9	sample 10	-
Matna foods Benin-Owo, Ondo State (cassava starch processing)	-	-	-	sample 4

2.2.2. Culture-dependent analysis of cassava wastewater effluent

2.2.2.1. Selective isolation and genotypic identification of bacterial isolates.

Isolation studies were carried out using five different culture media such as Starch casein agar (SCA), Humic vitamin agar (HVA), Arginine glycerol salt agar (AGS) Yeast Malt Agar (YMA) and Glycerine Asparagine agar (GA) targeting oleaginous microorganisms belonging to actinobacteria. All samples from different companies were subjected to the same isolation procedures. Starch casein agar was prepared by dissolving 10 g of soluble starch, 2 g of K_2HPO_4, 2 g of KNO_3, 0.3 g of casein, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$ and 18 g of agar in 1000 ml water and pH was adjusted to pH 7.3 (Mohseni *et al.*, 2013). Humic Vitamin agar was prepared by dissolving 1 g Humic acid, 0.05 g MgSO4.7H_2O, 1.7 g KCl, 0.5 g Na_2HPO_4, 0.01g $FeSO_4$.7 H_2O, 1 g $CaCl_2$, B-vitamins (0.0005 g each of thiamine-HCl, riboflavin, Capantothenate, inositol, Niacin, pyridoxin, p-aminobenzoic acid, and 0.00025g of biotin), 18g agar, in water 1000 ml and pH was adjusted to 7.4 (Jiang *et al.*, 2013). Arginine glycerol salt was prepared by dissolving 1.0g arginine monohydrochloride, 12.50g glycerol, 0.001g MnSO4.$H_2$0, 0.001g $ZnSO_4$.7$H_2$0, 0.001g $CuSO_4$.5H20, 1.0g K_2HPO_4, 0.010g Fe_2 $(S0_4)_3$.6$H_2$0, 0.5g $MgSO_4$.7$H_2$0, 1.0g NaCl, and 15.0g of agar, in 1000 ml water and pH was adjusted to 7.3. (El-Nakeeb & Lechevalier, 1963). GA agar was prepared by dissolving 1.0g Asparagine, 10g glycerol, 1g dipotassium phosphate, and 10g of agar in 1000ml distilled water, pH was adjusted to 7.3. 1ml trace salt solution (Trace salt solution: 0.1g FeSO4, 0.1g MnCl2, 0.1g ZnSO4) (Zhang & Zhang, 2011). Yeast Malt extract agar was prepared by dissolving 4g Yeast, 4g

Dextrose, 10g malt extract and 15g of agar in 1000 ml water and pH was adjusted to 7.3 (Jiang *et al.*, 2013)

Antibacterial and antifungal supplementation of post sterilised media was carried out by supplementing media with 50µg/ml Nalidixic acid, 50µg/ml Novobiocin, 25µg/ml Nystatin and 5ml 0.1% Cyclohexamide. Serial dilutions of CWW sample were prepared by diluting 1 ml of CWW sample in 9 ml of ringer's solution (Bora *et al.*, 2015). Dilutions were made from 10^{0} to10^{-6}. Following dilution, 200 µl of each dilution was spread aseptically on triplicate plates in the class II microbiological safety cabinet (Bioquell, UK). Subsequently, plates were incubated at 28°C for three weeks (Sanyo Incubator). Single colonies from each dilution were further purified to get a single culture, by sub-culturing on Tryptone soya agar (TSA) (Oxoid, CM0131) and incubated further for 3 days.

2.2.2.2. DNA extraction from cassava effluent isolates

DNA was extracted from three-day-old biomass of purified single colonies grown in TSA broth (Oxoid, CM0129). Briefly, DNA extraction was carried out by following the protocol of Legras & Karst (2003). 2ml culture was centrifuged at 6000 rpm (5600 xg) for 5 minutes in an Eppendorf tube. Supernatant was discarded and pellet was re-suspended in 400 µl of lysis buffer (Tris 10mM, pH 7.6, EDTA 1 mM, NaCl 100 mM, Triton X-100, 2% w/v, sodium dodecyl sulphate (SDS) 1% w/v), 0.6g of glass beads and 400 µl of phenol/chloroform/iso-amyl alcohol (25/24/1 v/v). The mixture was vortexed for 4 minutes. Following vortexing, 200 µl of buffer Tris EDTA (pH 8.0) was added. The mixture was centrifuged at 6000 rpm (5600 xg) for 5 minutes. 400 µl of the aqueous layer was added to 500 µl chloroform isoamyl alcohol. The

mixture was agitated slightly and centrifuged for 2 minutes at 13,300 rpm (12,700 xg) . To the aqueous phase, two volume of 100% ethanol was added. Mixture was finally subjected to a 5minutes centrifugation at 13,300 rpm (12,700 xg). The nucleic acid pellet was dissolved in Qiagen (QIAquick PCR) elution buffer (30 µl).

2.2.2.3. De-replication and actinobacteria specific PCR amplification of isolates

2.2.2.3.1 Dereplication of isolates

To dereplicate similar clones of the same culture, repetitive extragenic palindromic (REP) PCR was carried out using REP primers RepIRI: iiiCGIGCICATCIGGC and Rep2I: iCGiCGTATCiGGCCTAC (De Bruijn, 1992). A 50 µl PCR reaction mixture containing 5 µl of 10X PCR Buffer (containing 15mM MgCl2), 1 µl of 200 µM dNTP, 2 µl of 20 µM of both primers, 1 µl of DNA template, 38.75 µl of nuclease-free water and 0.25 µl of 2.5unit *Taq* DNA polymerase (Bioline) was set up. The polymerase chain reaction was performed with initial denaturation for 10 minutes at 95^{o}C followed by 28 cycles of 1 minute denaturation at 95^{o}C, 1 minute annealing at 40^{o}C and 8 minutes extension at 65^{o}C. A final elongation step was done at 65^{o}C for 16 minutes. The amplified rep-PCR genomic fingerprint fragments were run on a 1.5% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 5 hours in 1x TAE (Bora *et al.*, 2015).

2.2.2.3.2 PCR amplification of 16S rRNA gene:

The de-replicated isolates were amplified by using Universal PCR primers: 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1525R, 5′-AAGGAGGTGATCCAGCC-3 (Downes *et al.*, 2000) generating 1500 bp

fragment from isolated genomic DNA. PCR amplification was carried out in a Techne TC-512 Thermal cycler. In all tubes, a 50 µl reaction mixture consisting of 5 µl of 10X PCR Buffer (containing 15mM MgCl2), 1 µl of 200 µM dNTP, 2 µl each from 20 µM of both primers, 1 µl of DNA template, 38.75µl of nuclease-free water and 0.25 µl of 2.5unit *Taq* DNA polymerase (Bioline) was set up. The polymerase chain reaction was performed with initial denaturation for 3 minutes at 95^{o}C followed by 38 cycles of 30s denaturation at 95^{o}C,30s annealing at 48^{o}C and 90s extension at 72^{o}C. A final elongation step was carried out at 72^{o}C for 10 minutes. Amplified PCR product was separated on a 1.5% agarose gel electrophoresis containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1X TAE buffer. Gel was scanned in a Gel Doc 1000 apparatus (UVP, Gel Doc-it imaging system). Amplified PCR product was purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. PCR product was checked once again for purity and yield on 1.5% agarose gel electrophoresis containing 5 µl ethidium bromide. DNA concentration was estimated using the Nanodrop (Nano Vue plus).

2.2.2.4. PCR purification, sequencing and analysis

Purified PCR product was sent for Sanger sequencing at Source Bioscience Genomics Service, UK. A BLAST (Basic Local Alignment Search Tool) analysis was carried out to compare gene sequences with nucleotide sequences from NCBI databases. Sequences with the highest similarity were obtained from the GenBank database and were aligned using ClustalW multisequence alignment program in the MEGA 7 (Kumar *et al.*, 2016). Phylogenetic relationship was inferred using neighbour-joining tool in MEGA 7 software

package (Saitou & Nei, 1987). Phylogenetic trees were constructed using 1000 bootstrap values.

2.2.3. Culture-independent analysis of cassava wastewater effluent

2.2.3.1. Sample preparation

Aliquot of samples from each stage of processing was obtained from -20^{o}C, thawed, and vortexed. A 4 ml suspension of each sample was centrifuged at 14000 rpm (13,440 xg) for 5 minutes for cell pelleting.

2.2.3.2. Community DNA extraction

Community DNA was extracted directly from the cassava effluent sample using the DNeasy Blood and Tissue extraction kit (Qiagen, USA). Protocol was followed per manufacturer's instructions. Samples were re-suspended in 200µl 1X Phosphate buffered saline (PBS). Following suspension in PBS, 20µl proteinase K was added. Buffer AL (200 µl) was added, and tubes were vortexed thoroughly for 5 minutes. Tubes were incubated at 56^{o}C for 10 minutes. For each tube, 200 µl absolute ethanol was added and vortexed thoroughly for 5 minutes. Samples were transferred into spin columns provided by the manufacturers for binding the precipitated DNA to column. Samples were washed with 500 µl of two buffers AW1 and AW2 respectively before eluting in 35 µl of AE buffer. DNA was confirmed on a 1.5% agarose gel electrophoresis containing 5µl ethidium bromide. Gels were electrophoresed at 100 V in 1X TAE buffer for 40 minutes. Gel was scanned in a Gel Doc 1000 apparatus (UVP, Gel Doc-it imaging system) (Bora *et al.*, 2015).

2.2.3.3. Nested polymerase chain reaction with universal primers and actinobacterial specific primers.

Following DNA extraction from CWW, nested PCR using two primer sets, universal primers and actinobacterial specific primers were used to amplify the target gene fragment. Universal PCR primers: 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1525R, 5′-AAGGAGGTGATCCAGCC-3 (Downes *et al.*, 2000) were initially used to amplify 1500 bp fragment from CWW community DNA. PCR amplification was carried out in a Techne TC-512 Thermal cycler. A 50 µl reaction mixture was prepared consisting of 5 µl of 10X PCR Buffer (containing 15mM MgCl2), 1 µl of 200 µM dNTP, 2 µl each from 20 µM of both primers, 1 µl of DNA template, 38.75 µl of nuclease-free water and 0.25 µl of 2.5 units of *Taq* DNA polymerase. The polymerase chain reaction was performed with initial denaturation for 3 minutes at 95^{o}C followed by 38 cycles of 30s denaturation at 95^{o}C,30s annealing at 48^{o}C and 90s extension at 72^{o}C. A final elongation step was carried out at 72^{o}C for 10 minutes. Amplified PCR product was separated on a 1.5% agarose gel electrophoresis containing 5 µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1x TAE buffer. The gel was scanned in a Gel Doc 1000 apparatus (UVP, Gel Doc-it imaging system). Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. Purified PCR product was checked on an agarose gel as earlier stated. DNA concentration was estimated using the Nanodrop (Nano Vue plus).

Amplicon obtained from universal 16S rRNA primers was utilised as the template DNA for nested amplification of a 650bp fragment (Bora *et al*., 2015) using actinobacterial specific primers SC-ACT-

235:5′CGCGGCCTATCAGCTTGTTG 3′ and SC-ACT-878: 5′CCGTACTCCCCAGGCGGGG 3′ (Stach *et al.*, 2003). The reaction was carried out in a 50 µl reaction mixture containing 5 µl of 10X PCR Buffer (containing 15mM $MgCl_2$), 1 µl of 200 µM dNTP, 2 µl each from 20 µM of both primers, 1 µl of DNA template, 38.75 µl of nuclease-free water and 0.25 µl of 2.5 units of *Taq* DNA polymerase. Amplification was performed in a Techne TC-512 Thermal cycler with initial denaturation for 5 minutes at 95^{o}C followed by 30 cycles of 45s denaturation at 95^{o}C,45s annealing at 60^{o}C and 45s extension at 72^{o}C. A final elongation step was carried out at 72^{o}C for 10 minutes. Amplified PCR product was separated on an agarose gel at 100 V for 40 minutes in 1x TAE buffer. The gel was scanned in a Gel Doc 1000 apparatus (UVP, Gel Doc-it imaging system).

2.2.3.4. High-throughput sequencing and analysis

The universal 16S rRNA and actinobacterial amplicon were used separately to infer the diversity of CWW community. Template from 16S rRNA and 650 bp fragment amplified from actinobacterial specific primers was used as a template for amplicon sequencing. Primers 341F (5′-CCTACGGGNGGCWGCAG-3′) (Klindworth *et al.*, 2013) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Yergeau *et al.*, 2015) targeting the variable regions (V3-V4) were used for culture-independent analysis. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The Ion Plus Fragment Library Kit 48 reactions (Thermo Scientific) was used to generate sequencing libraries based on manufacturer's recommendations. The Qubit@ 2.0 Fluorometer (Thermo Scientific) was used to assess the quality of Library produced. The Ion S5TM XL platform was used to sequence libraries. A 400

bp/600 bp single-end reads was generated. Single-end reads were subjected to quality control. The Uparse software (Uparse v7.0.1001) was used in performing analysis of sequences using all effective tags. OTUs were assigned to sequences with ≥97% similarity. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species) with Threshold of 0.8~1. MUSCLE (Version 3.8.31) was used to obtain the phylogenetic relationship of all OTUs representative sequences by comparing multiple sequences rapidly. A standard of sequence number corresponding to the sample with the least sequences was used in normalising OTU abundance information. The output normalised data was subsequently used for alpha and beta diversity analysis. Two indices were used to identify community diversity: Shannon -the Shannon index; Simpson -the Simpson index. Beta diversity analysis was used to evaluate differences of samples in species complexity. Cluster analysis was preceded by principal coordinate analysis (PCoA) and Unweighted Pair-group Method with Arithmetic Mean (UPGMA).

2.3. Results

2.3.1. Cassava waste effluent sampling

Cassava effluent sample was obtained from companies as described in Table 2.1. The average pH for each sampling point was recorded as seen in Table 2.2 below. Three sample points for each company processing cassava flakes and one sample point for cassava starch was reflected.

Table 2.2: Average pH readings from each processing effluent.

Company	**CWW sample point**	**Average pH**
Oxford Foods Ikare-akoko (garri processing)	1 Washing	3.74±0.1
Oxford Foods Ikare-akoko (garri processing)	2 Fermentation	4.21±0.1
Oxford Foods Ikare-akoko (garri processing)	3 Pressing	3.50±0.1
Matna foods Benin-Owo, Ondo State (cassava starch processing)	4 Cassava starch effluent	4.04±0.09
Bilatrade food processing Ado-Ekiti (garri processing)	5 Washing	3.90±0.06
Bilatrade food processing Ado-Ekiti (garri processing)	6 Fermentation	3.85±0
Bilatrade food processing Ado-Ekiti (garri processing)	7 Pressing	3.76±0.1
Reputable food and allied processing company Ado-Ekiti (garri processing)	8 Washing	4.64± 0.7
Reputable food and allied processing company Ado-Ekiti (garri processing)	9Fermentation	4.47±0.6
Reputable food and allied processing company Ado-Ekiti (garri processing)	10 Pressing	4.32±0.5

2.3.2. Culture-dependent analysis

2.3.2.1. Isolation of indigenous microorganisms

Culture media were supplemented with Nalidixic, Novobiocin, Nystatin and Cyclohexamide as Antibiotics and Antifungal supplements with actinomycetes as the primary targets for isolation. Isolation on all agars showed sparse growth on the 10^{o} dilution for all media. Fig. 2.3 shows the colony growth data from each sampling points and on media used.

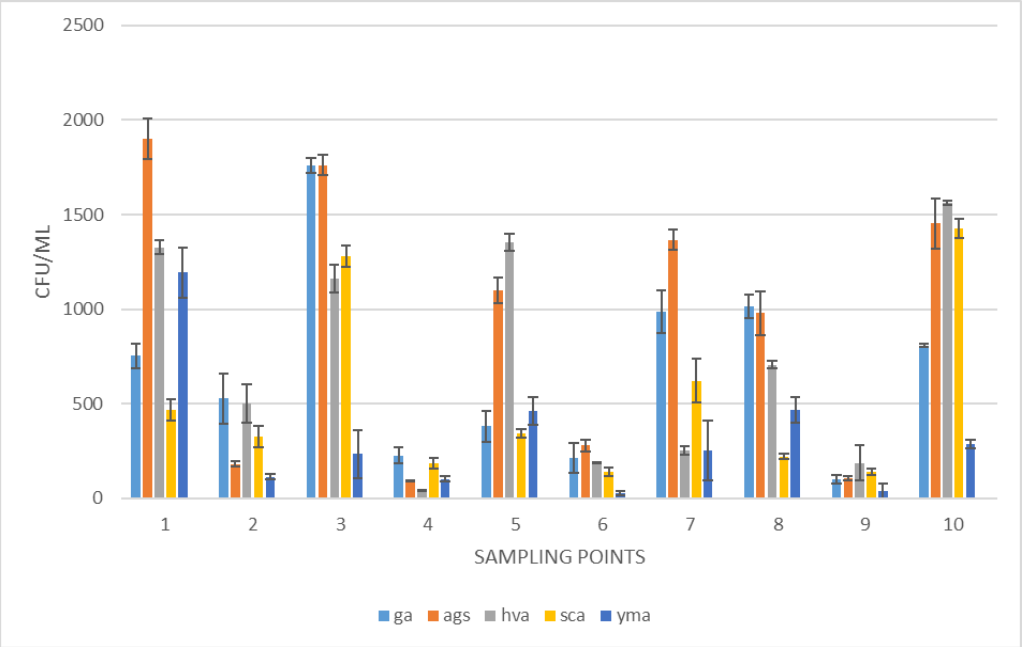


Figure 2.3: CFU/ml of bacteria from each sampling point on different agar media. SCA- Starch casein Agar, HVA- Humic Vitamin Agar, AGS- Arginine Glycerol Salt, YMA-Yeast Malt Agar, GA- Glycerine Asparagine agar. Key: (1 = Washing effluent from company 1(Oxford Foods), 2 = Fermentation effluent from Oxford Foods , 3 = Pressing effluent from Oxford Foods , 4 = Cassava starch effluent from Matna foods , 5 = Washing effluent from Bilatrade food processing company , 6 = Fermentation effluent from Bilatrade food processing company , 7 = Pressing effluent from Bilatrade food processing company , 8 = Washing effluent from Reputable food and allied processing , 9 = Fermentation effluent from Reputable food and allied processing , 10 = Pressing effluent from Reputable food and allied processing). Error bars depict standard deviation within samples.

AGS proved the most appropriate media for enumeration of bacterial isolates. This media provided the highest count of $4.6x10^4$ colony-forming unit across all sampling points in comparison with SCA, HVA, GA and YMA, which had 2.6 $x10^4$, 3.6 $x10^4$, 3.4 $x10^4$ and 1.6 $x10^4$ colony forming units per ml respectively. Sample point 3 (pressing stage) contained the highest count for indigenous bacteria with 3.1 $x10^4$. Sampling point 9 had the lowest colony-forming unit across all sampling points with 2.9 $x10^3$. Between the two cassava processes in which samples were collected, the cassava flakes process(garri) had a higher microbial load when compared with cassava starch processing. The average indigenous bacteria load was 1.7 $x10^4$ (cassava starch) and 3.2 $x10^4$ (cassava flake).

The trend in bacterial distribution amongst cassava flake processing was observed. As seen in Fig.2.4 below, the microbial load was relatively higher in the effluent from the pressing and washing stages. Also, for all companies 1,2 and 3 processing cassava flakes, the microbial load was lowest in the waste effluents obtained during fermentation.

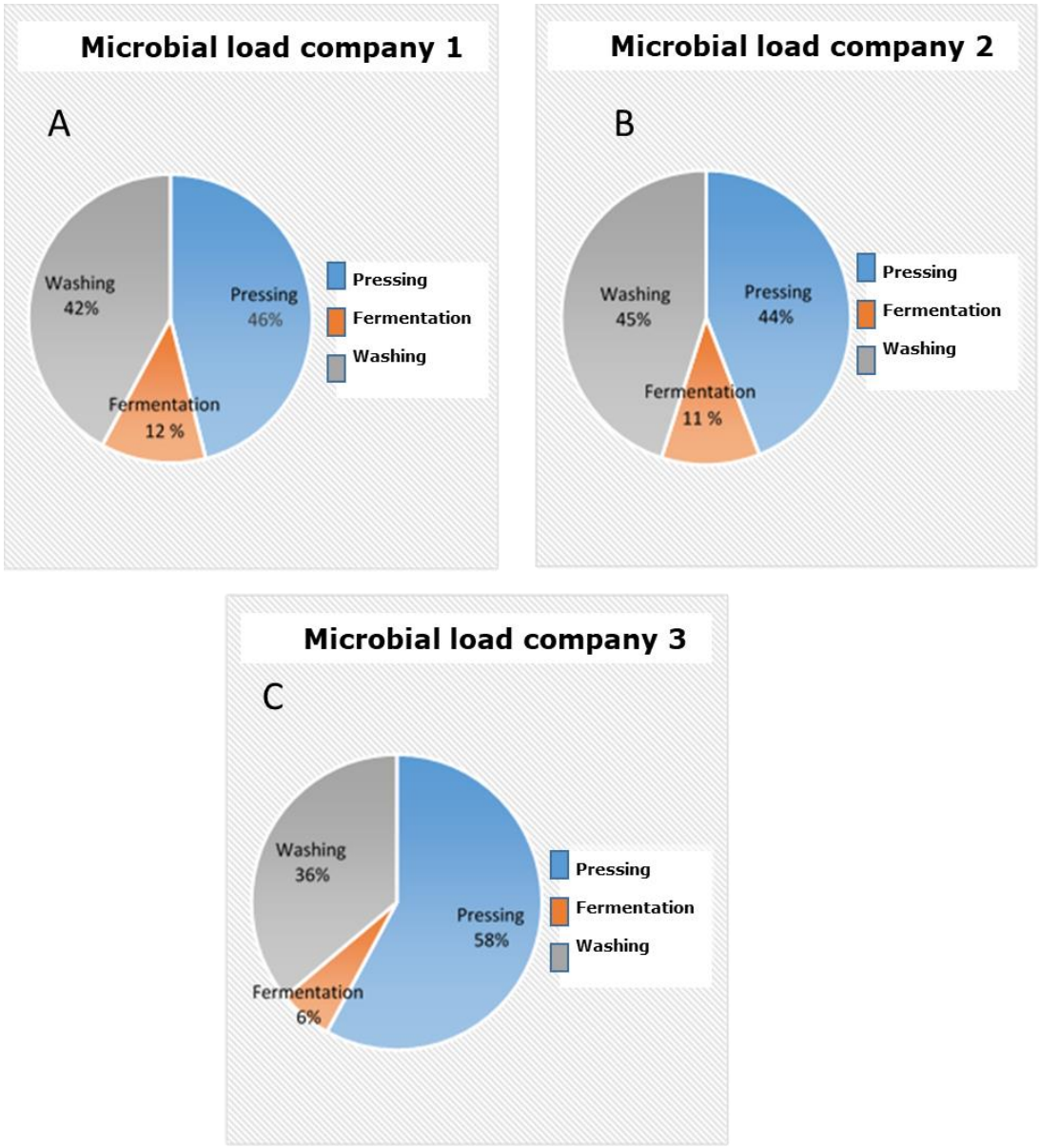


Figure 2.4: Distribution of bacteria load in companies processing cassava flake (Garri). A) Bacterial load company 1(Oxford Foods) (B) Bacteria load company 2 (Bilatrade food) (C) Bacteria load company 3(Reputable food and allied processing).

2.3.2.2. Dereplication of isolates from CWW and 16S sequence analysis

Preliminary dereplication based on morphological criterion and unique REP fingerprint patterns (Fig.2.5), 8 different isolates (Fig. 2.6) were chosen to be sequenced. These isolates were chosen as they possess unique REP fingerprints delineating isolates from other stains obtained from CWW. Isolated DNA was amplified using universal 16S rRNA primers to obtain a 1500 bp fragment. Post sequencing, blast search and alignment with related sequences from databases confirmed the identity with members of the *Streptomyces, Micrococcus, Micromonospora, Bacillus, Lactobacillus, Staphylococcus, Oceanobacillus and Pseudomonas*. Table 2.3 represents the results indicating the groups in which isolates were obtained. *Bacillus* was the most frequently encountered genus. This was represented in all sampling points irrespective of media used. *Micromonospora* was the most dominant genus retrieved from SCA present in 7 out of 10 samples investigated. Interestingly, although AGS media had the highest microbial load, there were no actinobacteria observed. GA indicated the highest actinobacterial diversity as it revealed the presence of *Micromonospora, Micrococcus* and *Streptomyces*.

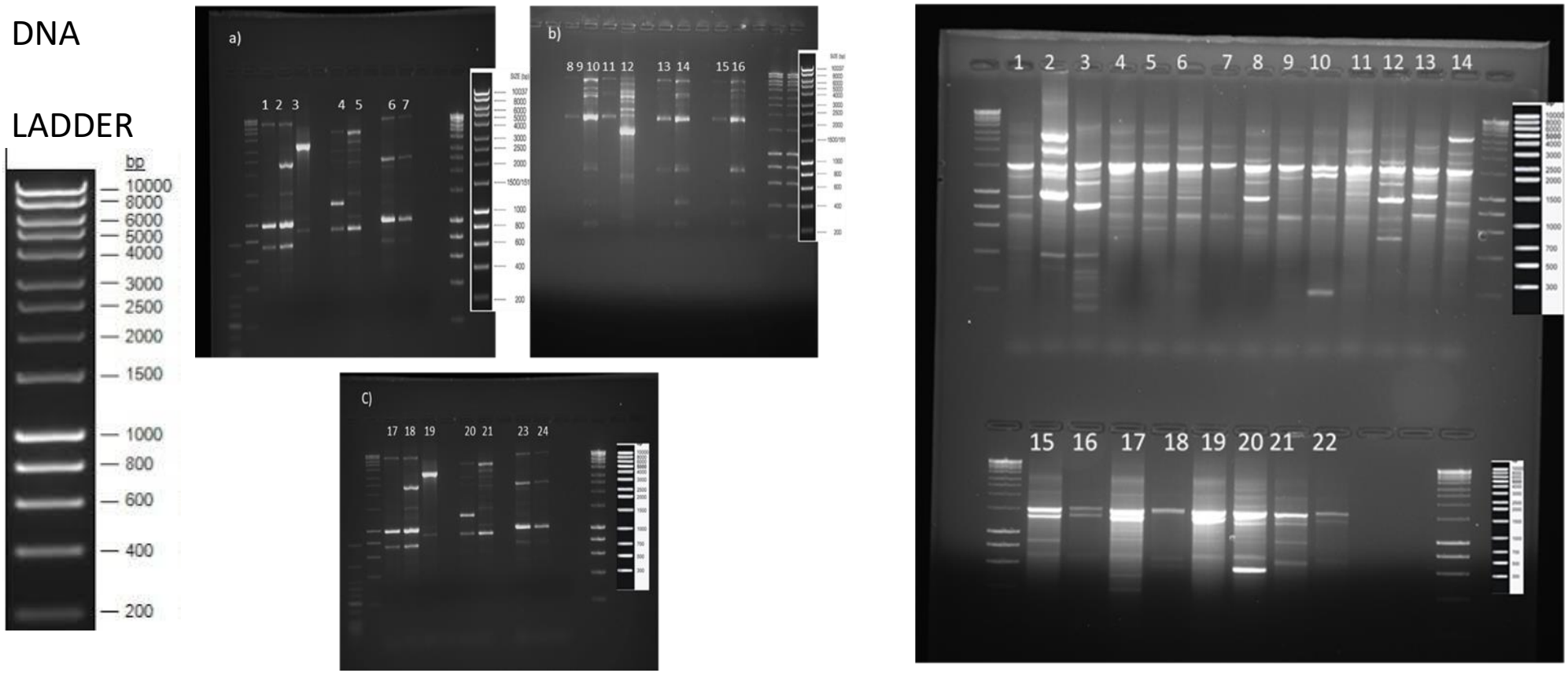


Figure 2.5: 1.5% Gel image showing REP PCR fingerprint patterns obtained from morphologically distinct isolates using RepIR and Rep2I primers. Image A,B,C illustrates REP fingerprint from distinct isolates of SCA, GA and AGS while image D illustrates REP fingerprint from YMA and HVA.

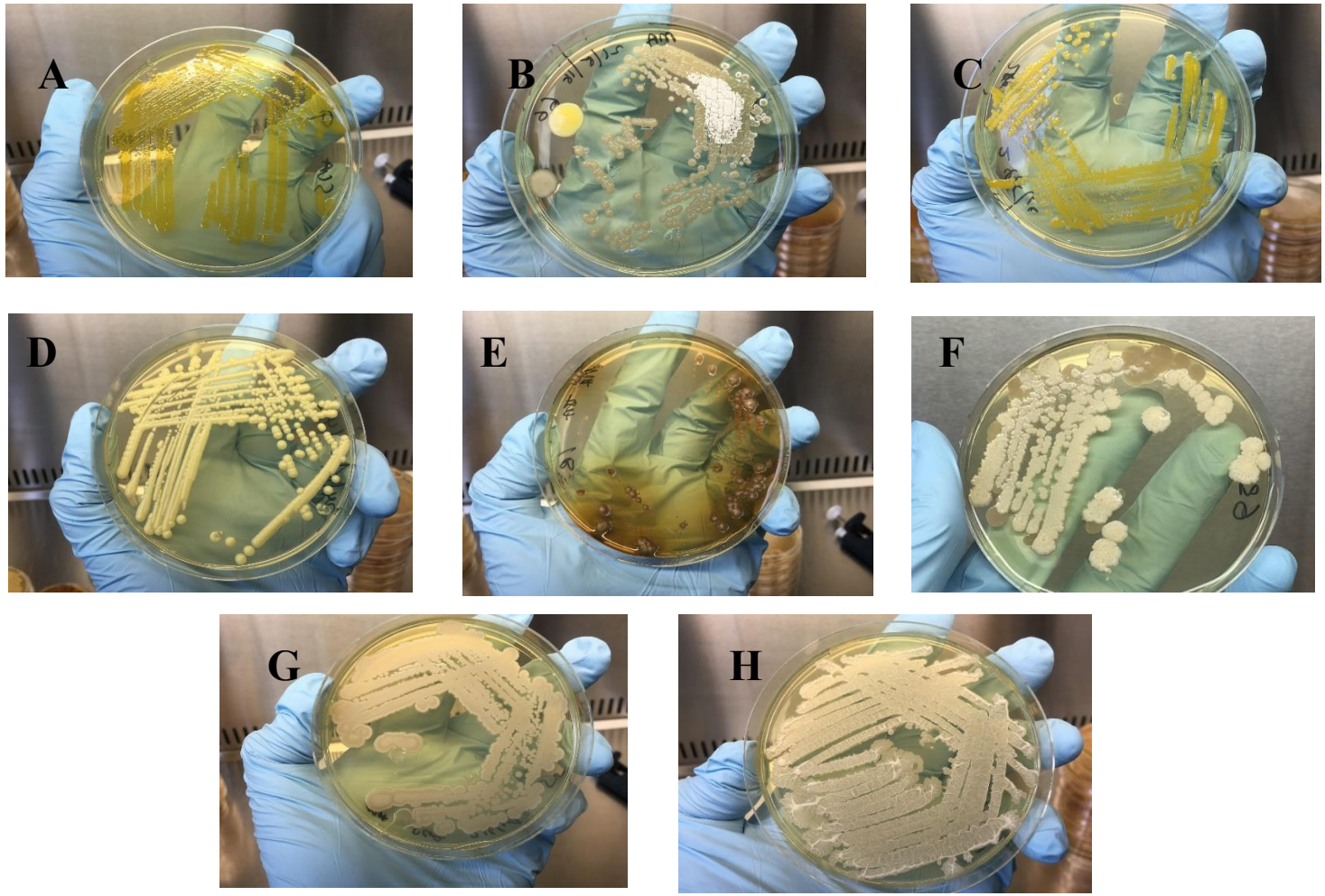


Figure 2.6: Image of agar plates showing the representative morphology of isolates sequenced. A) *Pseudomonas* B) *Streptomyces* C) *Staphylococcus* D) *Micrococcus* E) *Micromonospora* F) *Lactobacillus* G) *Oceanobacillus* H) *Bacillus*

Table 2.3: Representative origin of each genus from respective selective isolation media as identified by 16S rRNA sequence result

Samples	HVA	SCA	GA	YMA	AGS
1 Washing (Company 1)	*Bacillus*	*Micromonospora, Bacillus*	*Micromonospora, Bacillus*	*Bacillus*	*Bacillus*
2Fermentation (Company 1)	*Bacillus*	*Oceanobacillus, Bacillus,Micromonospora*	*Micrococcus, Bacillus*	*Micrococcus, Bacillus*	*Lactobacillus, Bacillus*
3 Pressing (Company 1)	*Bacillus*	*Micromonospora, Oceanobacillus, Bacillus*	*Micromonospora, Bacillus*	*Bacillus*	*Lactobacillus,Bacillus, Oceanobacillus*
4 Cassava starch effluent (Company 4)	*Bacillus*	*Micromonospora, Oceanobacillus, Staphylococcus, Bacillus*	*Bacillus*	*Micrococcus, Bacillus*	*Bacillus*
5 Washing (Company 2)	*Oceanobacillus, Bacillus*	*Micromonospora, Oceanobacillus,Bacillus*	*Bacillus*	*Bacillus*	*Lactobacillus, Bacillus*
6 Fermentation (Company 2)	*Bacillus*	*Oceanobacillus, Bacillus*	*Bacillus*	*Bacillus*	*Lactobacillus, Bacillus*
7 Pressing (Company 2)	*Bacillus*	*Bacillus*	*Bacillus*	*Micrococcus, Bacillus*	*Lactobacillus, Bacillus*
8 Washing (Company 3)	*Bacillus*	*Micromonospora, Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*
9Fermentation (Company 3)	*Bacillus*	*Pseudomonas, Bacillus*	*Bacillus*	*Bacillus*	*Lactobacillus, Bacillus*
10 Pressing (Company 3)	*Bacillus*	*Micromonospora, Pseudomonas, Lactobacillus, Bacillus*	*Streptomyces, Bacillus*	*Bacillus*	*Bacillus*

2.3.2.3. Phylogenetic analysis

16S rRNA gene-based phylogenetic analysis was carried out on selected type strain sequences obtained from the GenBank databases and all our actinobacterial isolates belonging to *Micrococcus*, *Micromonospora* and *Streptomyces* genera.

Three isolates (CMEYMATSA12B, CFEGATSA14B and 2CFEYMATSA17) from the consortium of microorganisms observed, showed similarities to *Micrococcus*. The phylogenetic positions of these isolates compared with the relevant type strain of *Micrococcus* is indicated in Fig. 2.7. However, there was no distinction between type strains and isolates based on information from the phylogenetic tree. All isolates clustered together in clads or subsets of type strains.

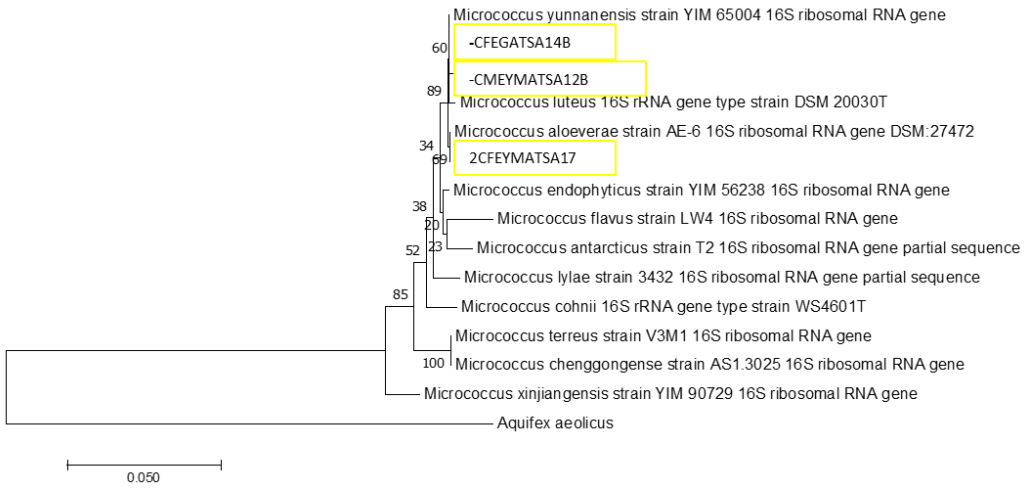


Figure 2.7: Phylogenetic tree showing evolutionary relationship amongst closely related isolates and published type strains of *Micrococcus* as inferred using the Neighbor-Joining method (Saitou & Nei, 1987).The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor

method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1136 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Fig. 2.8 shows the evolutionary relationships of isolates from this study and members belonging to the *Micromonospora* genus. All closely related isolates formed clads within the *Micromonospora* group. Within this group, all unknown isolates clustered distinctively from other type strains of *Micromonospora*. Closest relatives to CWW isolated strains were *Micromonospora cremea*, *Micromonospora coriariae*, *Micromonospora endolithica* and *Micromonospora cremea*. (See appendix for *Micromonospora* isolate sequence).

Unknown CWW isolate that showed maximum identity with related sequences of *Streptomyces* from GenBank using the BLAST program was compared with corresponding type strains of the genus. The sequences were aligned using ClustalW, and the evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). Fig. 2.9 below shows the evolutionary history as inferred using the Neighbour-Joining method (Saitou & Nei, 1987). (See appendix for *Streptomyces* isolate sequence).

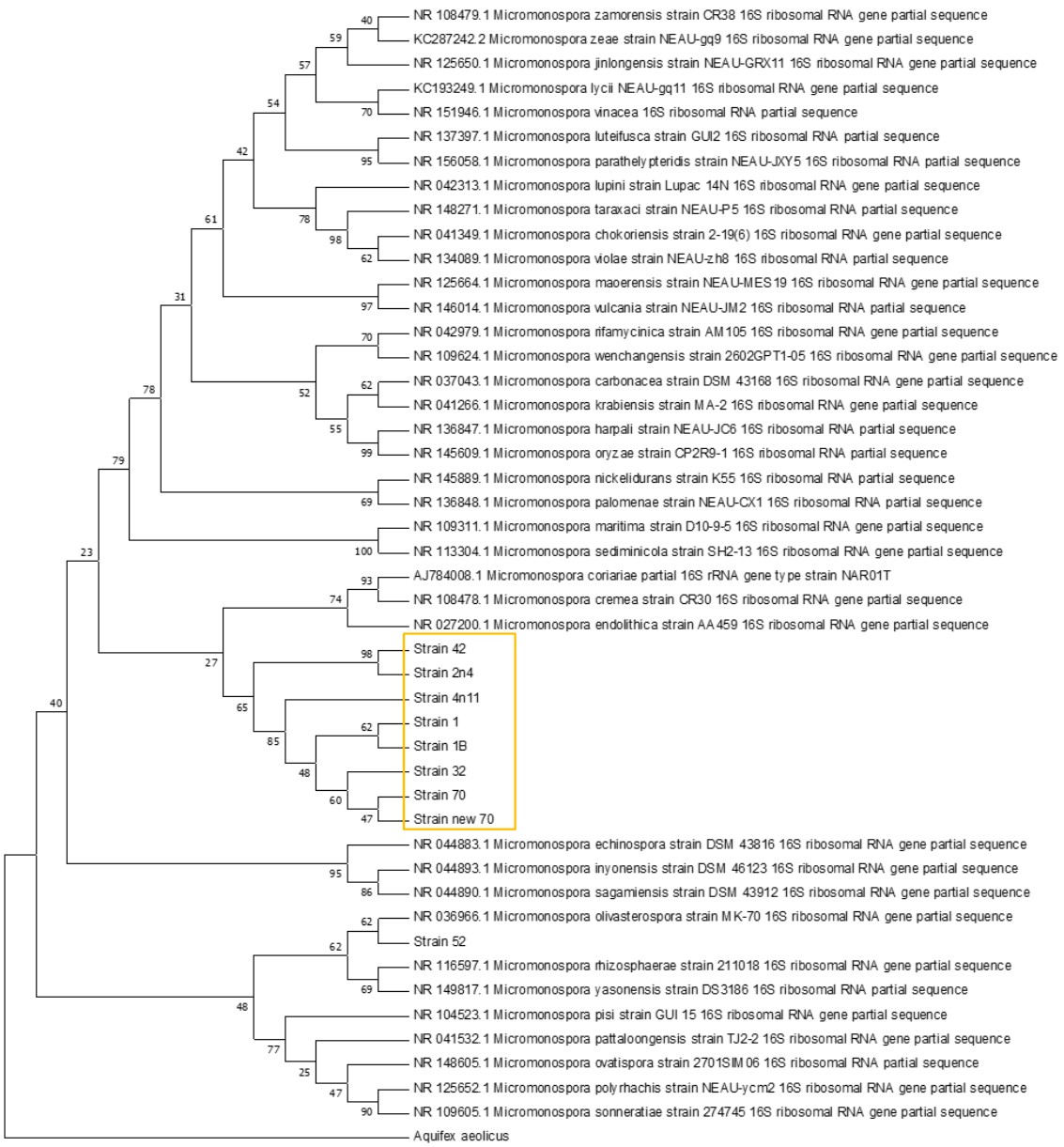


Figure 2.8: Phylogenetic tree showing the evolutionary relationship amongst CWW isolates and *Micromonospora* Type strains. Strain 42= 2CPEGATSA42 (Accession number: MWA26320), Strain 4n11= CMESCA4TSA11(Accession number:MWA26316), Strain 2n4 = 3CWEGATSA52 (Accession number: MWA26318), Strain 1= 3CPEGATSA1 (Accession number: MWA26314), Strain 1B=3CPEGATSA1B (Accession number: MWA26315), Strain 32= 2CFEGATSA32 (Accession number:MWA26321), Strain 70= 3CPEGATSA70 (Accession number: MWA26317). The optimal tree with the sum of branch length = 0.44201220 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed

using the Jukes-Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 82 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1272 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

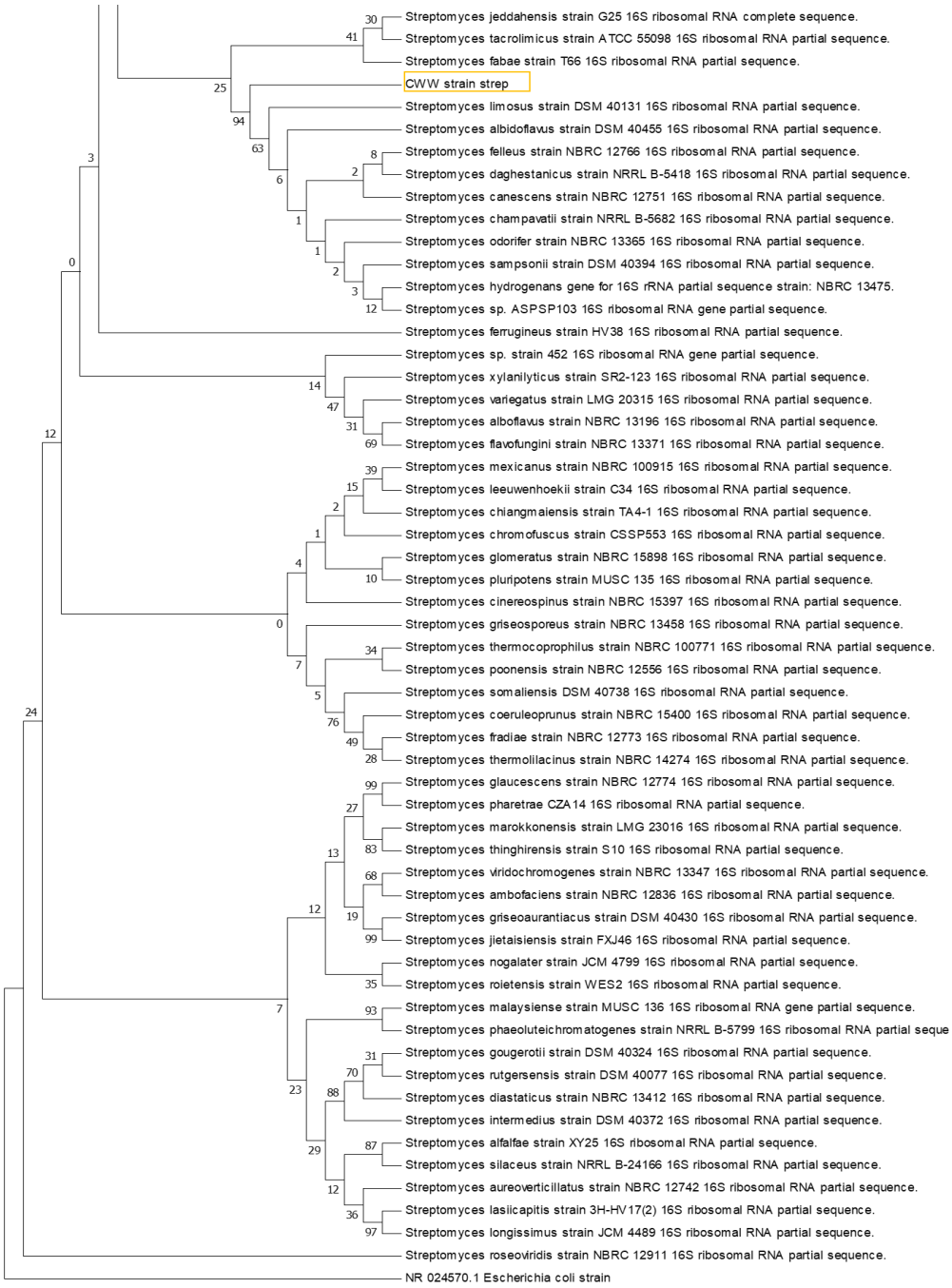


Figure 2.9: Partial phylogenetic tree showing the evolutionary relationship amongst isolates and *Streptomyces* type strains. CWW strep =3CPEGATSA66

(Accession number:MWA26323). The optimal tree with the sum of branch length = 0.44201220 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 82 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1272 positions in the final dataset. Evolutionary analysis was conducted in MEGA7 (Kumar *et al.*, 2016).

2.3.3. Culture-independent analysis of cassava wastewater effluent

2.3.3.1. ,Sample description and coding

For the purpose of analysing community results, samples were given a code as described in Table 2.4 below. These codes are used to describe the results.

Table 2.4: Description of sample and groups used in the analysis of community amplicon

Company	**Sample (Stage)**	**Group**	**Universal primer amplicon code**	**Group**	**Actinobacteria specific primer amplicon code**
Company 1 (Oxford Foods Ikare-akoko)	Sample 1 (Fermentation stage)	**Group A** (universal primer amplicon)	Ua.1	**Group E** (Actinobacteria primer amplicon)	Aa.1
	Sample 2 (Washing stage)		C1.2		C2.2
	Sample 3 (Pressing stage)		Ua.3		Aa.3
Company 2 (Bilatrade food processing)	Sample 5 (Fermentation stage)	**Group B** (universal primer amplicon)	C1.5	**Group F** (Actinobacteria primer amplicon)	C2.5
	Sample 6 (Washing stage)		C1.6		C2.6
	Sample 7 (Pressing stage)		C1.7		C2.7
Company 3 (Reputable food and allied processing)	Sample 8 (Fermentation stage)	**Group C** (universal primer amplicon)	C1.8	**Group G** (Actinobacteria primer amplicon)	C2.8
	Sample 9 (Washing stage)		C1.9		C2.9
	Sample 10 (Pressing stage)		N/A		Aa.10
Company 4 (Matna foods Benin-Owo, Ondo State)	Sample 4 (All effluent)	**Group D** (universal primer amplicon)	C1.4	**Group C2-D** (Actinobacteria primer amplicon)	C2.4

*Grouping is based on the company and the amplicon used in sequencing

2.3.3.2. Microbial diversity

Bacteria account for 85.632% of the total diversity, as seen in Fig. 2.10. Firmicutes, actinobacteria, proteobacteria are the key distinct phyla. A relatively lower percentage of cyanobacteria were also detected owing to the use of universal 16S rRNA gene. *Bifidobacterium*, *Corynebacterium*, *Micromonospora*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Enterobacter*, *Escherichia* and *Pectobacterium* were amongst the dominant genera present (Fig. 2.10). The distribution of sample operation taxonomic units (OTUs) within the observed phyla can be seen in Fig 2.11. Across all samples, sequence analysis of the 16S rRNA gene inferred dominant phyla as firmicutes (41.05%), actinobacteria (26.16%), Proteobacteria (6.67%) and cyanobacteria (2.326%).

The breakdown of OTU distribution within the different sample groups is illustrated in Fig.2.12. OTU abundance for actinobacteria was predominantly from groups E, F, G. These groups provided closely similar percentages, which made up the total actinobacteria diversity. From the distribution, group E provided the bulk percentage of *Bifidobacterium. Corynebacterium* and *Micromonospora* proportion in groups E, F, G are similar.

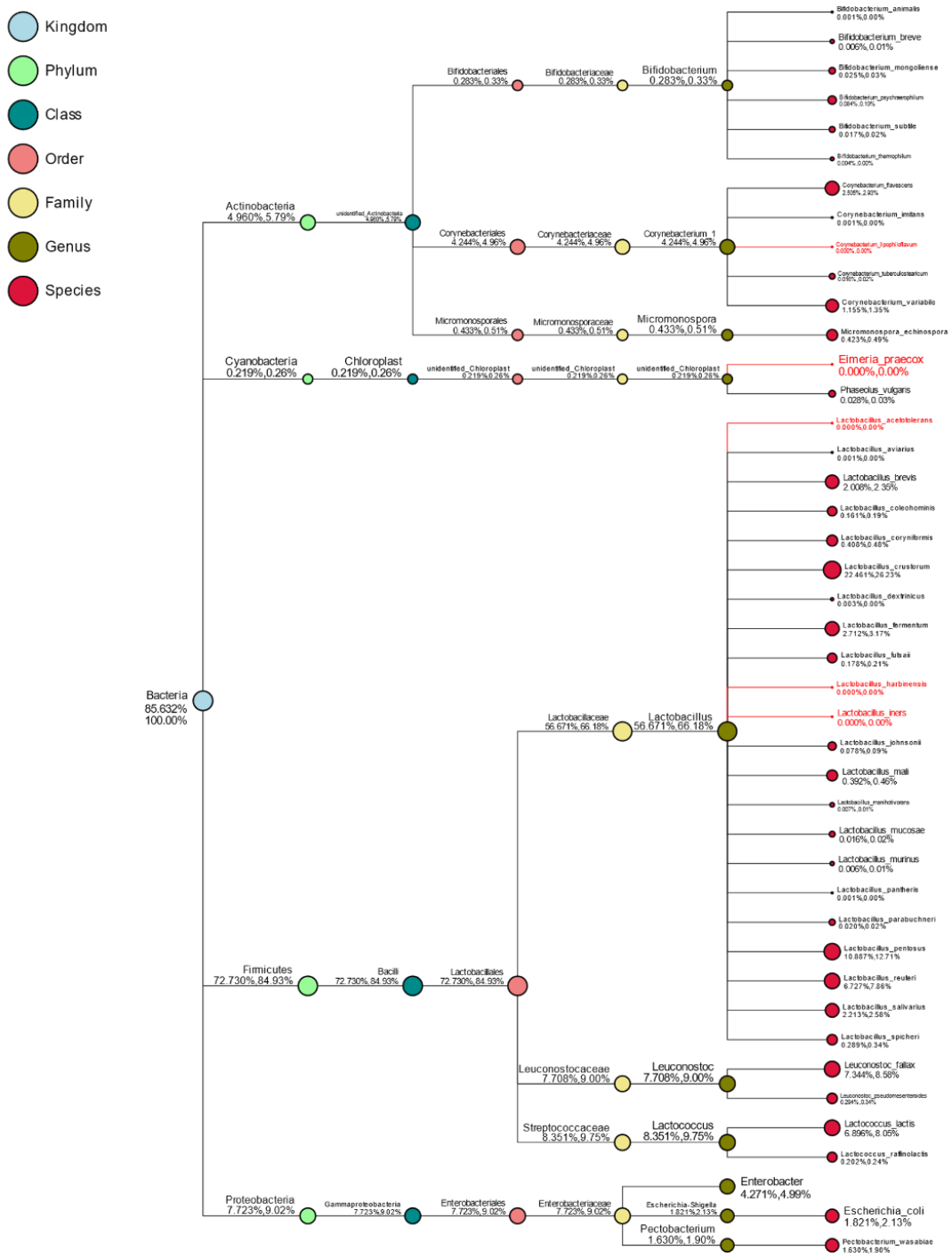


Figure 2.10: An overview taxonomy tree of OTUs from cassava wastewater. Sectors with different colours represent different groups. The size of the sector represents relative abundance. The first number below the taxonomic name represents the percentage in the whole taxon, while the second number represents the percentage in the selected taxon

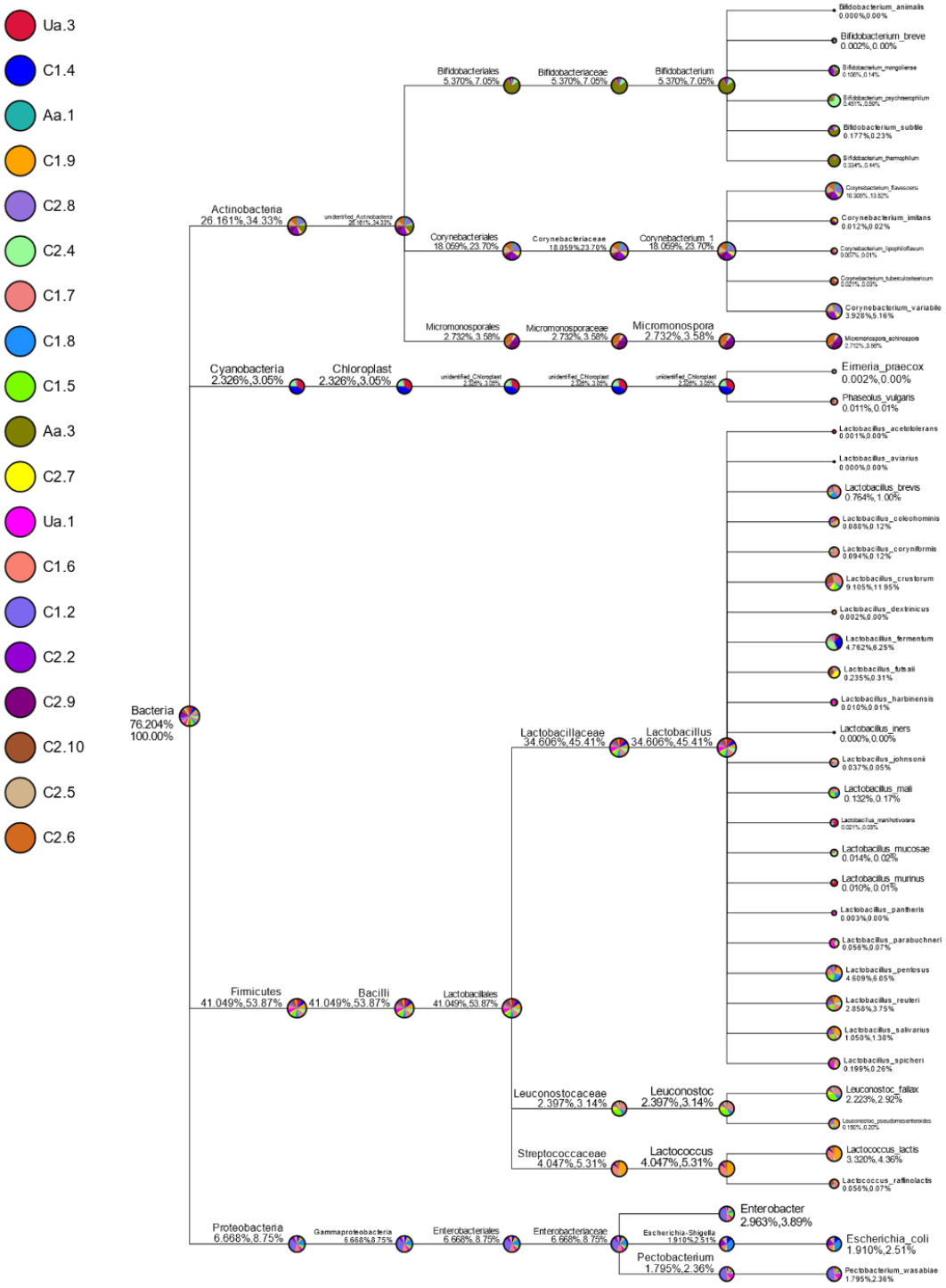


Figure 2.11: An overview of sample distribution in the top OTUs. Sectors with different colours represent different groups. The size of the sector represents relative abundance. The first number below the taxonomic name represents the percentage in the whole taxon, while the second number represents the percentage in the selected taxon

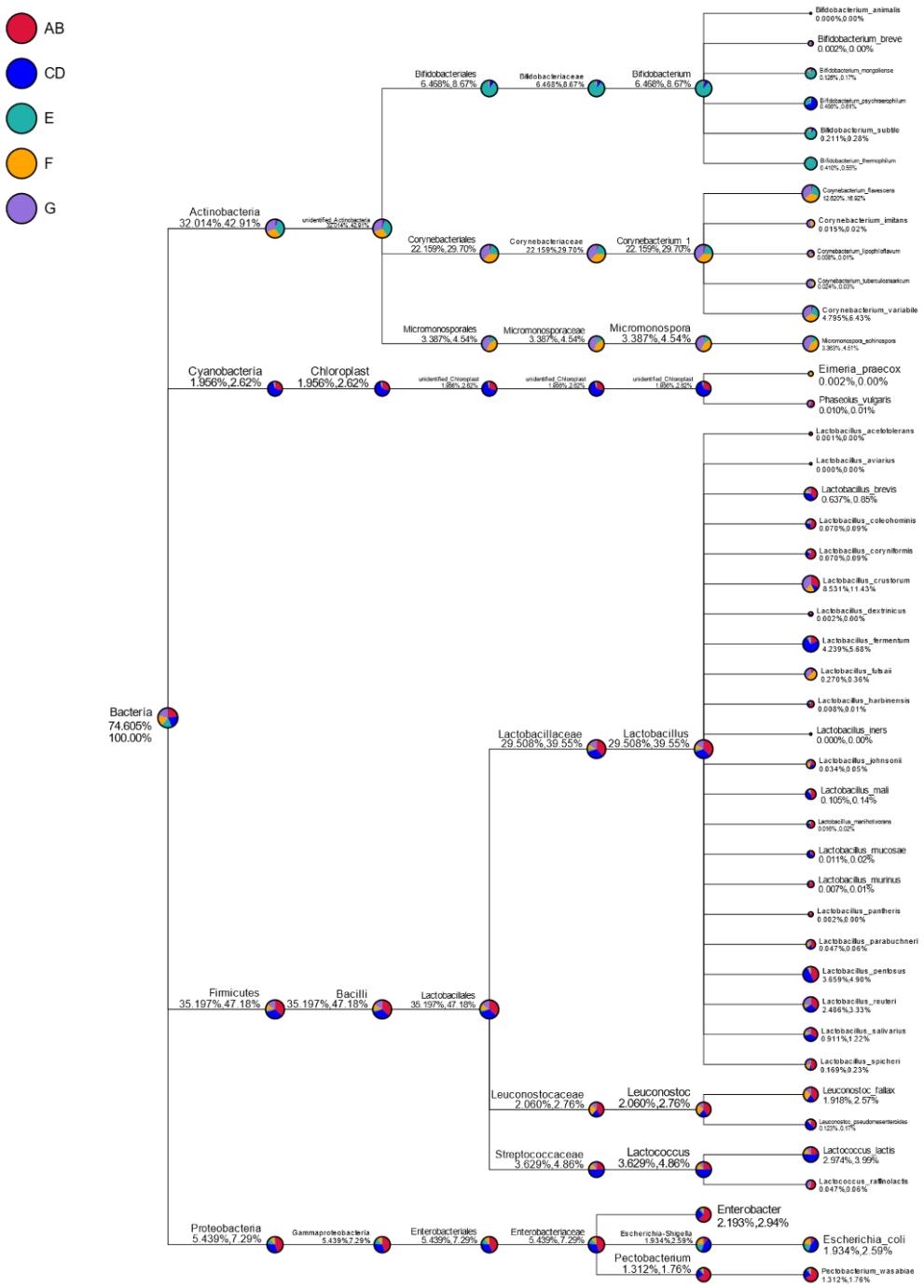


Figure 2.12: An overview of sample group distribution within top OTUs. In this figure, AB and CD represent the taxa from Universal primers from all companies and E, F, G represent Actinobacteria from all companies. Sectors with different colours represent different groups. The size of the sector represents relative abundance. The first number below the taxonomic name represents the percentage in the whole taxon, while the second number represents the percentage in the selected taxon.

An evolutionary tree illustrating the relative abundance of the top 100 genera observed across all sample can be seen in Fig. 2.13. This tree was constructed based on the representative sequence from all sample using both the Universal 16S rRNA gene amplicon and the actinobacteria specific amplicon. From the OTU annotation tree, *Lactobacillus* was the most dominant genera from all samples analysed. Other genera which had a significant representation within the construct were *Corynebacterium, Bifidobacterium, Lactococcus, Micromonospora and Enterobacteria*

The relative abundance of the top 100 genera observed across all groups is further represented in Fig. 2.14 . *Lactobacillus* was the most dominant genera in groups A, B, C, D. Group E was dominated by *Bifidobacterium* and *Corynebacterium*. Group F and G suggested the dominance of *Lactobacillus*, *Corynebacterium* and *Micromonospora*.

The distribution of species within each group is represented in Figs. 2.15,2.16,2.17,2.18 and 2.19. Within similar groups A and B, species belonging to genus *Corynebacterium* constitute the major actinobacteria observed. Species annotation from groups C and D indicated presence of species within *Micrococcus, Corynebacterium and Bifidobacterium.* The species annotation trees of group E,F,G suggested a host of actinobacterial species. Species belonging to *Bifidobacterium, Micromonospora, Actinomyces, Corynebacterium, Micrococcus and Cellulomonas* were common across three groups. From group F and G *Propionibacterium* was observed. *Dermatophilus* and *Glutamicibacter* were only observed in the tree annotation of group G.

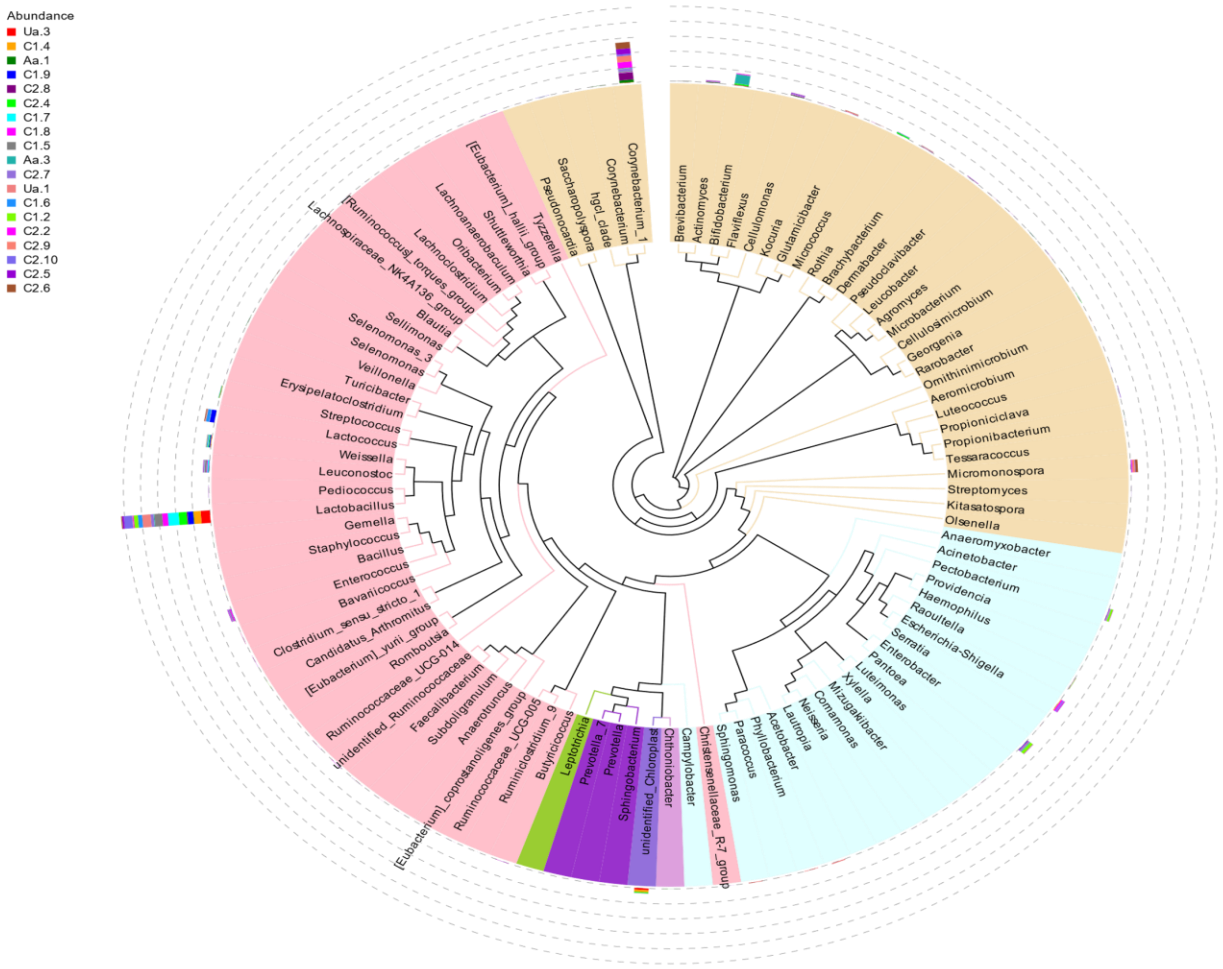


Figure 2.13: OTU annotation tree construct representing the relative abundance of bacteria across all 19 samples in this study. Different branch colours were indicative of the various phyla. The relative abundance of each genus was displayed outside the circle. Colour codes were attached to different samples

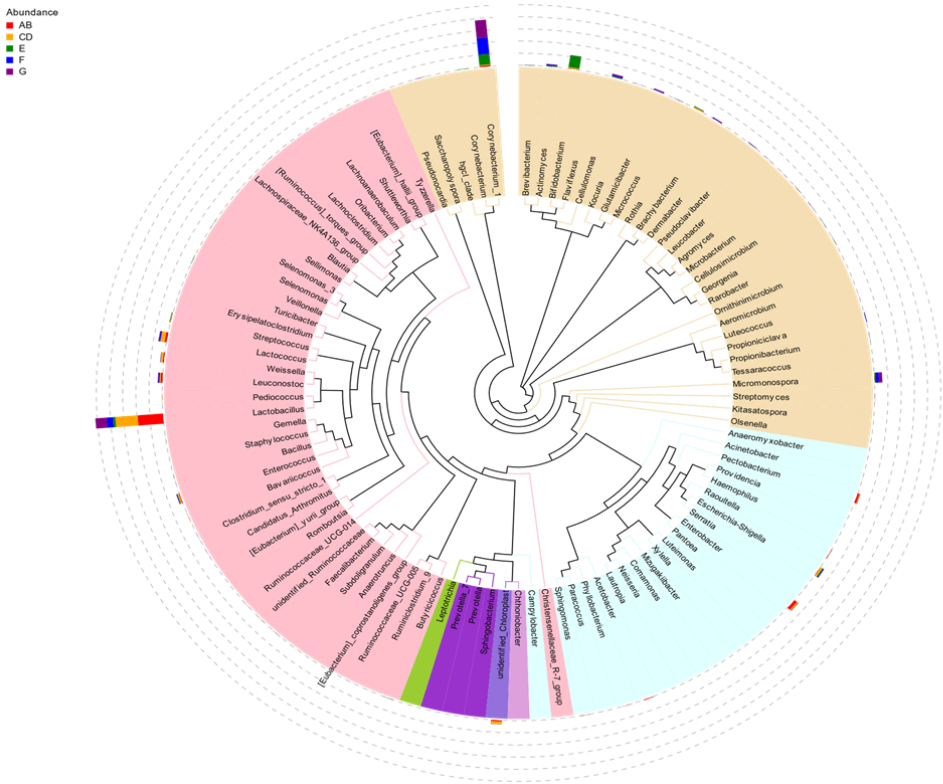


Figure 2.14: OTU annotation tree constructs representing the relative abundance of bacteria across all different groups in this study. Different branch colours were indicative of the various phyla. The relative abundance of each genus was displayed outside the circle. Colour codes were attached to different samples



Figure 2.15: GraPhlAn species annotation tree construct for group AB illustrating top species observed within group OTU.

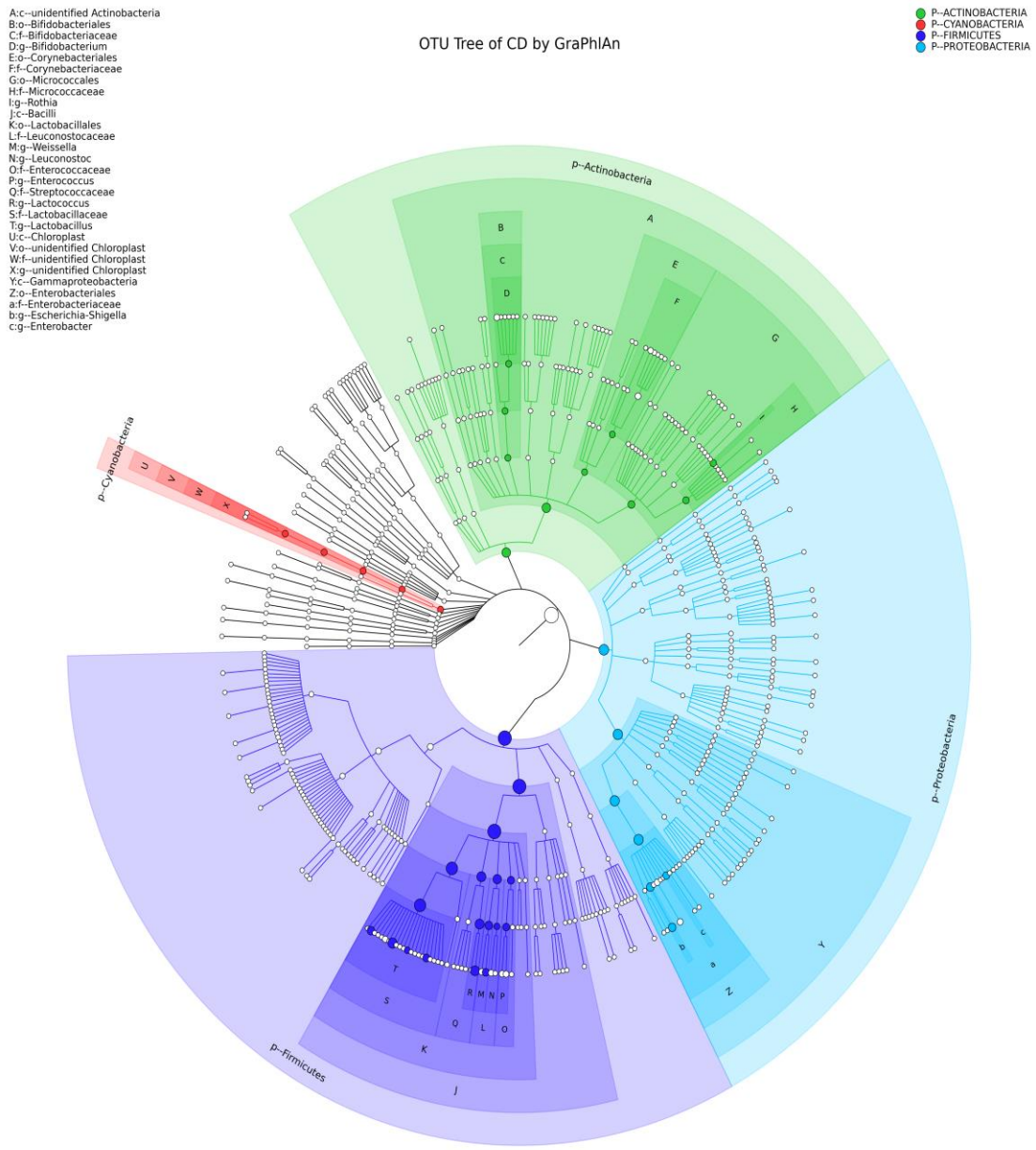


Figure 2.16: GraPhlAn species annotation tree construct for group CD illustrating top species observed within group OTU

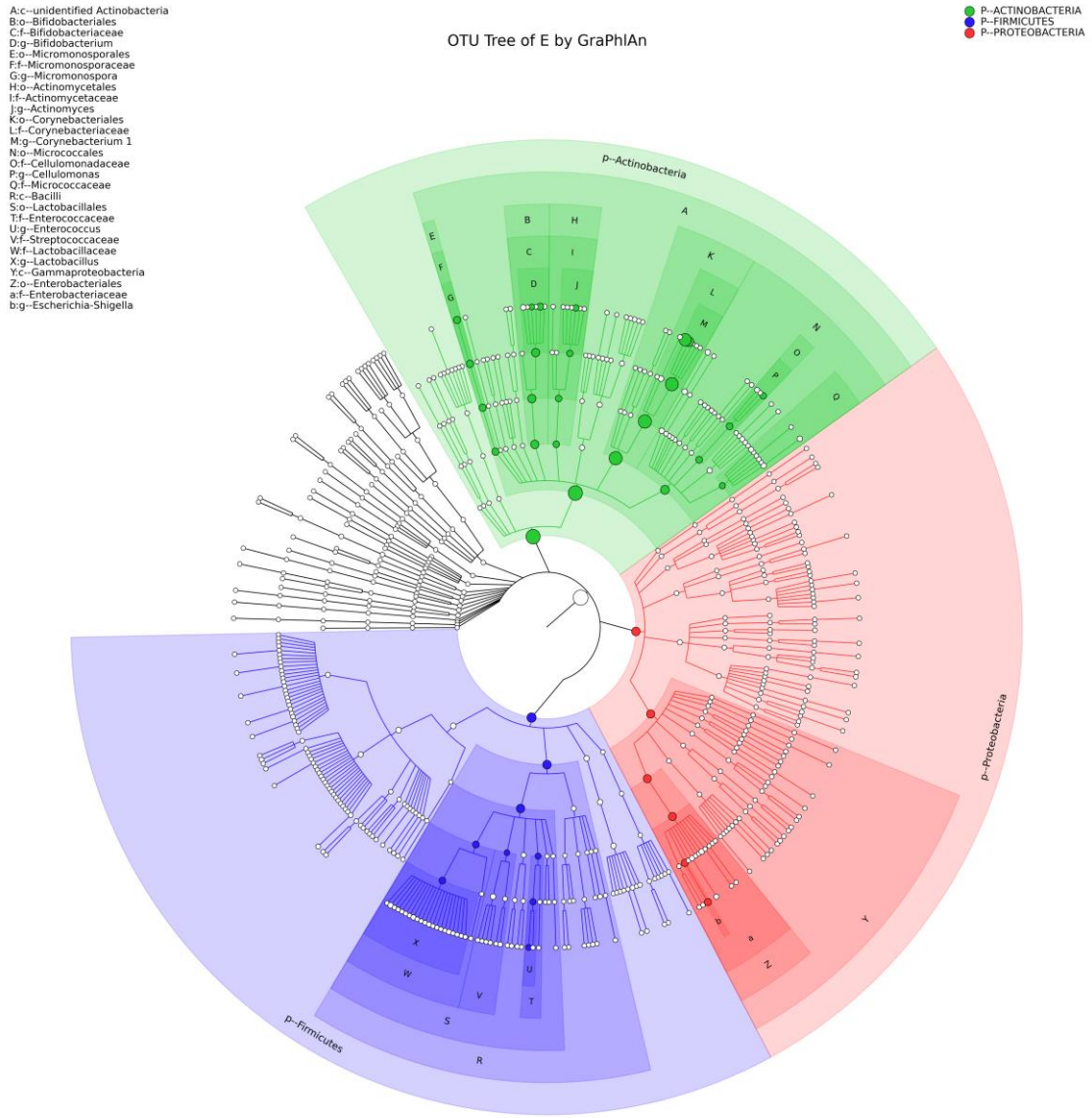


Figure 2.17: GraPhlAn species annotation tree construct for group E illustrating top species observed within group OTU.

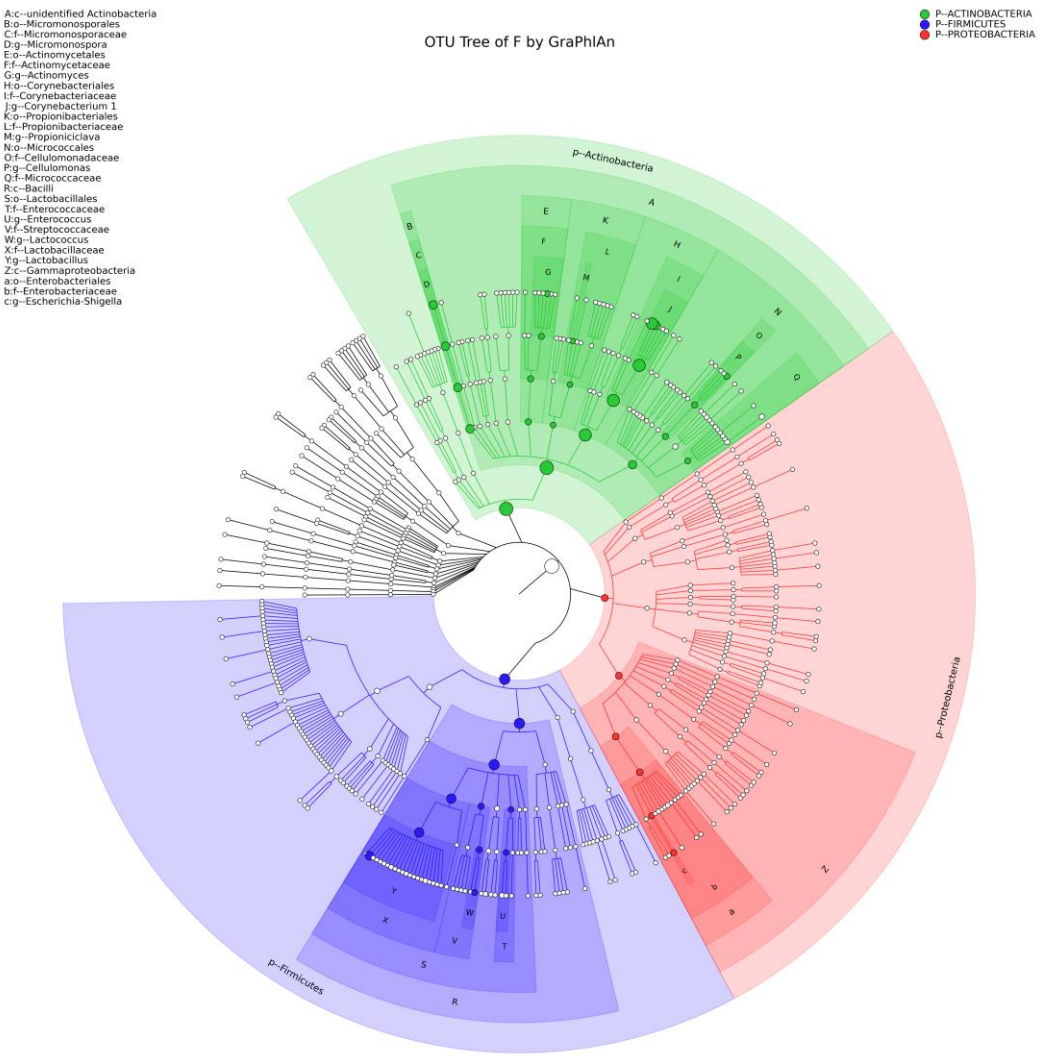


Figure 2.18: GraPhlAn species annotation tree construct for group F illustrating top species observed within group OTU

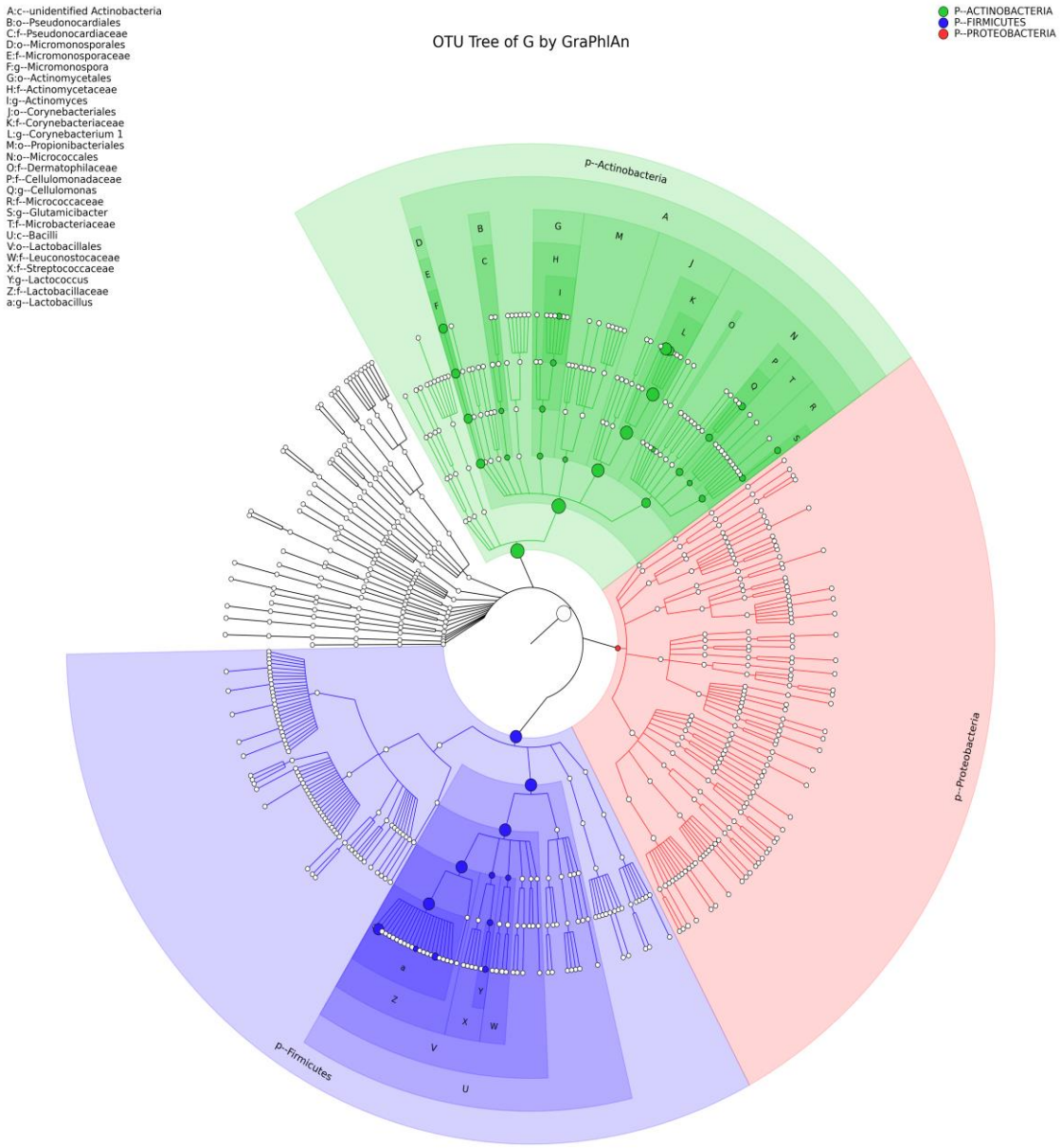


Figure 2.19: GraPhlAn species annotation tree construct for group G illustrating top species observed within group OTU

2.3.3.3. Analysis of phyla abundance

Based on annotation result, the most dominant phyla detected were, firmicutes, actinobacteria, proteobacteria and cyanobacteria across all sampling points using both universal and actinobacterial primers. The abundance of the top 10 phyla with the highest OTUs across samples is shown in Fig. 2.20 below.

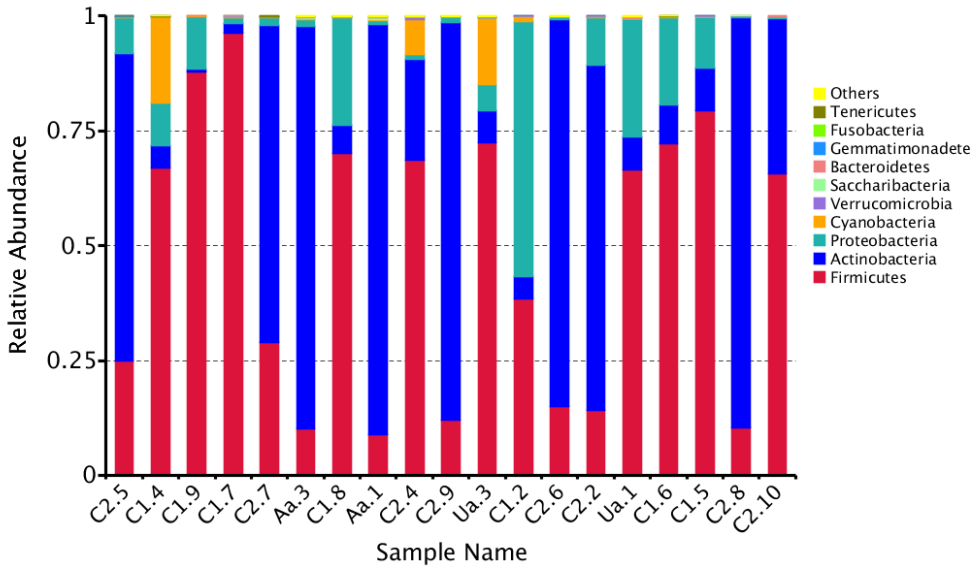


Figure 2.20: Phylum level composition of the bacterial community present in cassava effluent samples using both actinobacteria and universal primers. The relative abundance of the top 10 phyla is shown. "Others" represent other minor phyla.

Based on sequencing results from the 16S rRNA universal primer amplicon (Fig 2.20), Sample C1.7 (had the highest abundance of firmicutes while Sample C1.5 had the highest abundance of actinobacteria. Whilst Proteobacteria was most prominent in sample Ua.1, cyanobacteria were most represented in sample C1.4. Comparing the different stages across the companies based on the above gene sequence result, the washing stages (Samples C1.2, C1.6 and C1.8) were dominated by firmicutes except for sample C1.2 which had the highest proteobacteria OTUs. Across the fermentation stages (Sample Ua.1, C1.5 and

C1.8), the microflora proportion was predominantly firmicutes. The pressing stages (Ua.3 and C1.7), also presented a dominance of firmicutes.

Based on sequencing results from the 16S rRNA actinobacteria amplicon, actinobacteria was the dominant phylum except in Sample Aa.10, which had a higher abundance of firmicutes. Comparing the different stages across the companies based on this result, the washing stage (C2.2, C2.6 and C2.9) was most represented by the phylum actinobacteria. Firmicutes was still observed within all samples. Proteobacteria was also present, especially in sample C2.2 which had a significant proportion. From the Fermentation stage (Aa.1, C2.5, C2.8), actinobacteria abundance was followed by firmicutes. Sample C2.5 indicated a larger proportion of proteobacteria in relation to other samples. Across the pressing stage (Aa.3, C2.7 and Aa.10), there were significant proportions of actinobacteria and firmicutes. Sample Aa.10 illustrated more abundance of firmicutes than actinobacteria. The diversity amongst the 3 companies studied between washing, fermentation and pressing stages were constant. However, actinobacteria abundance was lowest in the pressing stage. This stage had the highest proportion of firmicutes. They were particularly illustrated in sample Aa.10.

Comparing samples from the cumulation of effluent from cassava starch (Matna cassava processing company), the abundance and distribution of indigenous microorganism in sample C1.4 (Sequenced from universal primer 16S rRNA amplicon) was between firmicutes (Highest), cyanobacteria, proteobacteria and actinobacteria (Least). Firmicutes was also most abundant in sample C2.4 (Sequenced from actinobacterial primer 16S rRNA amplicon) despite the amplification was targeting actinobacteria phylum. The distribution was from

firmicutes (Highest) to actinobacteria, cyanobacteria and proteobacteria (Least abundant).

2.3.3.4. Alpha diversity index analysis

Alpha diversity analysis was carried out to determine the species richness. The abundance of species within the microbial ecosystem was inferred using the rank abundance curve Fig. 2.21 and species accumulation box plot Fig. 2.22.

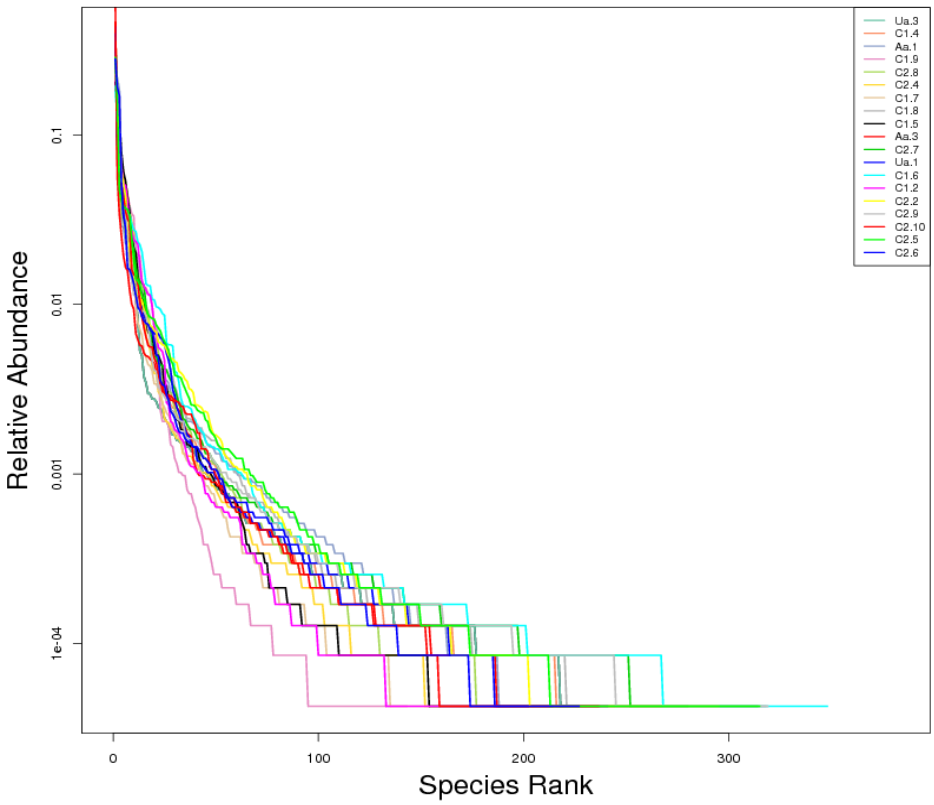


Figure 2.21: Rank abundance curve displaying relative species abundance within all samples.

The rank abundance curve for all 19 samples (Fig. 2.21) displays the relative species abundance and evenness of all samples. The rank abundance curve above indicated a difference in sample evenness and richness. Species evenness

was higher as seen from the curve in samples with higher species number and lowest in samples with lower number.

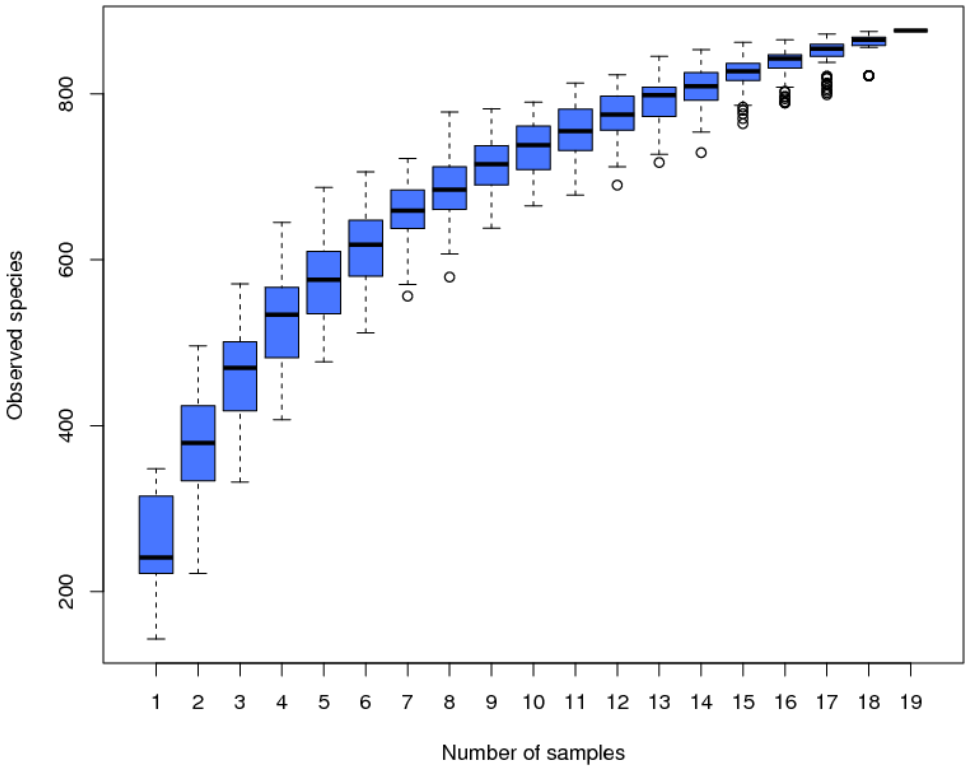


Figure 2.22: Species accumulation boxplot of the 19 samples. Boxplot illustrated the median species number, interquartile range and outliers estimated from permutations.

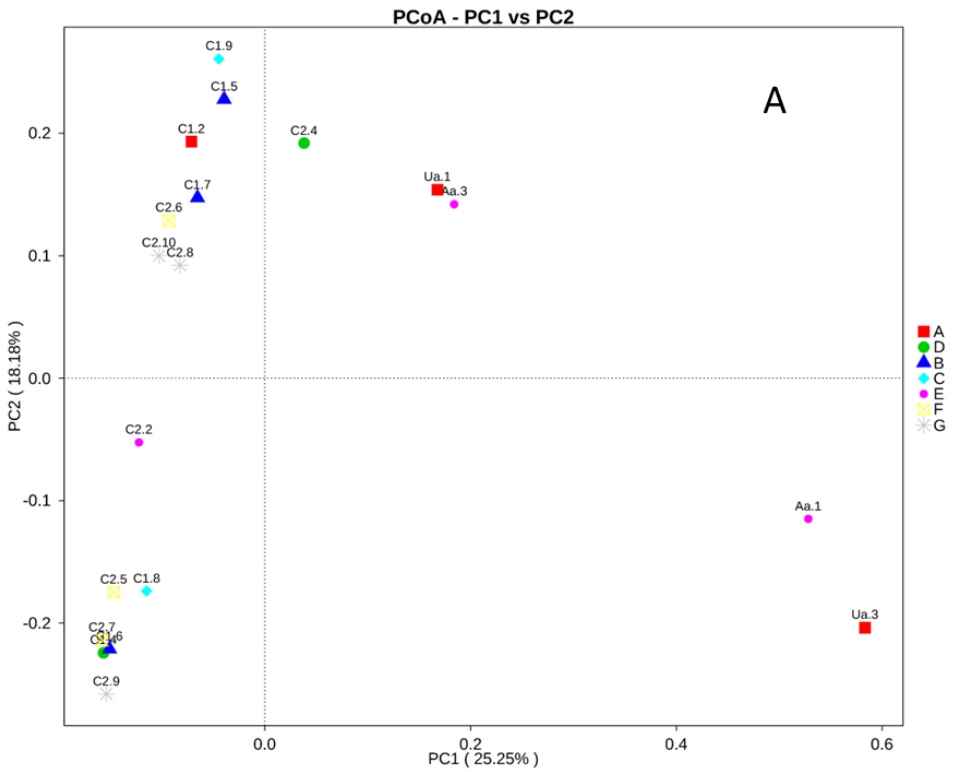
The species accumulation boxplot is another method of explaining the abundance and species evenness within samples. This boxplot illustrated the accumulative number of species in relation to increasing numbers of sample. As illustrated in Fig. 2.22, a rise in observed species was observed with the increasing sample number.

2.3.3.5. Beta diversity analysis

Beta diversity analysis was carried out to compare the different microbial communities present across all sampling points based on their composition (Lozupone & Knight, 2005). The weighted and unweighted Unifrac distance metrics were used to calculate the pairwise similarities between the samples. The results were visualised using the Principal coordinate analysis plot (PCoA) as seen on Fig. 2.23 and the Unweighted Pair-group Method with Arithmetic Means (UPGMA) Fig. 2.24.

2.3.3.5.1 Principal coordinate analysis

Principal coordinated analysis (PCoA) based on weighted Unifrac assesses the microbial community structure based on the abundance of OTU (Lozupone & Knight, 2005).



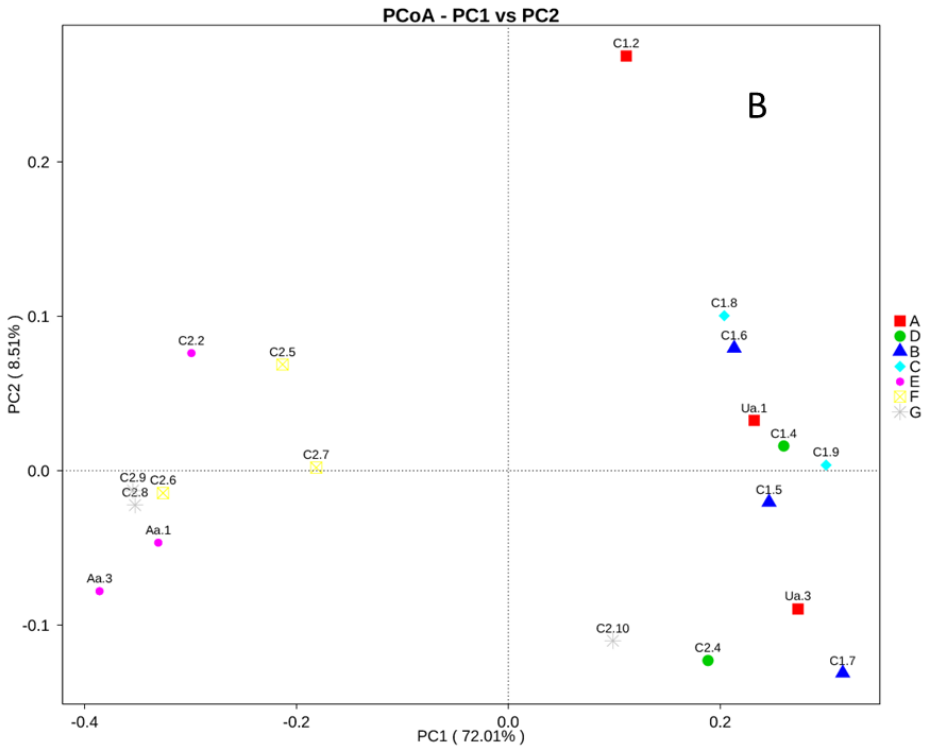


Figure 2.23: Principal coordinates analysis (PCoA) ordination illustrating the variation pattern between the different samples. A) based on un-weighted Unifrac metrics for all samples B) based on weighted Unifrac metrics for all samples

From the PCoA as seen on Fig. 2.23a, samples were clustered into three main regions of the plot. The position of each sample on the plot is a representative of the closeness of the most abundant microbial communities. The first cluster comprises samples C2.2, C2.5, C2.9, C2.6, C2.8, Aa.1, Aa.3, C2.7. This cluster was representative of sample with similar abundance of actinobacteria. The second unique cluster identified consisted of samples C1.8, C1.6, Ua1, C1.4, C1.9, C1.5, Ua3, C1.7, C2.4, C2.10. This cluster was unique with the comparatively higher abundance of firmicutes present within all samples. Sample C1.2 was unique as it was separated from all other clusters within the plot with a higher comparative proteobacteria abundance. The X-axis from left to right indicates the dominance of actinobacteria (left) and firmicutes (Right).

At the same time, the Y-axis from bottom to top suggests the presence of proteobacteria as a dominant phylum within the samples.

The PCoA plot representing the unweighted Unifrac metrics assesses the members of the community within samples based on the absence or presence of OTU (Lozupone & Knight, 2005). There were 3 main clusters as illustrated on the PCoA plot (Fig.2.23b). Bacterial communities in samples C1.9, C1.5, C1.2, C1.7, C2.4, C2.6, Ua1 and Aa3, clustered close to each another. The clusters had the most diversity comprising mainly of the most abundant OTUs. The other clusters comprise of samples C2.2, C2.5, C1.8, C2.7, C1.4, C2.9, and C1.6 and was distinct from Aa1 and Ua3. Cluster 2 comprises sample C2.2, C2.5, C1.8, C2.7, C1.4, C2.9, and C1.6 had the least diverse OTUs within the samples. Cluster 3, comprising of Aa1 and Ua3, had a widely diverse OTU. In addition to some of the predominant Phyla, they contained the most minor OTUs. It is also important to note that the diversity of the OTUs is reflected on the X-axis from left to right and the abundance of the Key OTUs is represented on the Y-axis from down to the top of the plot.

2.3.3.5.2 Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis

The UPGMA cluster analysis provides information on the similarity and differences between the cassava sampling points. The UPGMA cluster tree for both weighted and unweighted Unifrac is illustrated in Fig. 2.24 below

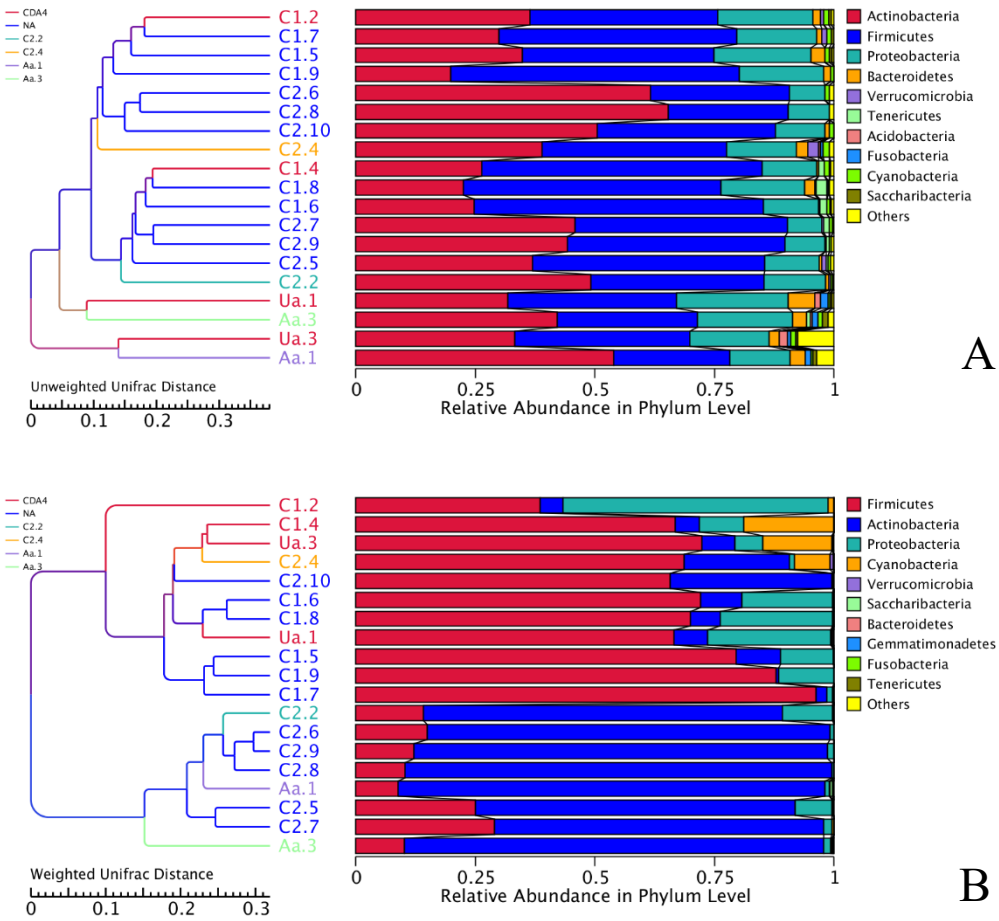


Figure 2.24: UPGMA clustering tree based on (A) Unweighted Unifrac distance (B) Weighted Unifrac distance. The analysis was performed and plotted with the UPGMA tree on the left and the relative abundance on the right. Others represent the culmination of the relative abundances for all other Phyla excluding the ten of the figure

Based on the analysis of Unweighted Unifrac distance matrix Fig. 2.24a if the abundance and size difference of the phyla in each sample is not considered, sample Ua.3 and Aa1 were closest to each other and had the most diversity. These similarities can be associated with the degree of absence or presence of minor OTUs. Sample C2.7 and C2.9 were closest and had similar low diversity within the samples. From samples sequenced based on universal 16S rRNA primers, sample C1.4 and C1.8 were closest and had the least diversity. Within

the actinobacteria 16S rRNA amplicon sequence result, sample Aa1 was farthest from sample C2.7 and C2.9.

As illustrated in the weighted Unifrac distance matrix Fig. 2.24b, there were two main groups within the distance tree with good similarities between parallel samples. Considering the size and abundance of the phyla in each sample, C1.2 and C1.7 were at opposite ends. These samples were least similar based on samples sequenced from universal 16S rRNA primer. From the actinobacteria 16S rRNA amplicon sequence result, samples Aa.3 was farthest away in similarity from sample C2.4.

2.4. Discussion

Over time, the bulk of microbiology studies elucidating the microbial diversity of cassava and its effluent have been reported using various culture-dependent and independent approaches (Elijah *et al.*, 2014; Miambi *et al.*, 2003). However, microbial diversity studies from cassava effluent at different cassava processing stages using culture-dependent and culture-independent approaches are yet to be reported. Therefore, this present study is the first to elucidate the use of culture-dependent and culture-independent approaches to compare the microbial constituents at different stages of cassava processing. This report was also designed to target the potential actinobacteria species that are useful for biofuel production. Understanding the potentials of indigenous actinobacteria within the different cassava processing stages will provide information about the untapped microbial diversity, some of which may present with potential application in biofuel studies.

The pH readings of all effluent samples show that they were acidic. The acidity of cassava effluent has been previously reported (Obueh & Odesiri-Eruteyan,

2016). From all cassava flake effluent stages, based on the result above, pH varied across the different effluent stages but was lowest in the cassava pressing stage after fermentation. It is unclear why pH at the initial washing stage was acidic. However, similar acidity has been reported in the secondary waste (cassava peel) obtained from this initial step (Souza *et al.*, 2013). The second effluent stage in this study involves ongoing fermentation which can cause acidity as reported in the cassava effluent pH result. Finally, the acidity of the third effluent stage (pressing effluent) could be expected as the effluent from this stage comprise of water obtained during dewatering of completely fermented cassava paste. The pH of sampling point 4 (Cassava starch effluent) was also similar to previously published casava effluent pH as earlier stated.

Culture-dependent approach with five media (SCA, HVA, AGS, YMA and GA) targeting actinobacteria was used to isolate the indigenous microorganisms as shown in fig. 2.1. From the colony-forming units observed on isolation media, a unique trend in microbial load was observed. The microbial load was relatively higher in the effluent from the pressing stage and washing stage and lowest in the effluents obtained from the fermentation stage. The initial cassava processing is carried out to remove unwanted organic and inorganic contaminants from cassava (Oyewole, 1992). Hence, after washing step a decrease in microbial load could be expected before a steady increase as a result of the development of fermentation related bacteria. The lower microbial load in the fermentation effluent stage in this study in comparison to pressing stage could be expected. This is because once the grated cassava has been fermented, a hydraulic press is then used to compress the pulp to release water. This stage which is the pressing stage would be expected to contain the effluents from the

whole fermentation process and more. This could account for the higher microbial load observed during the pressing stage. As seen in Table 2.3, AGS media was most suitable to isolate bacteria present within effluents. However, the media was not suitable to isolate the actinobacteria target group. GA and SCA from this study seemed more appropriate to isolate actinobacteria. GA had the most variety of actinobacteria (*Streptomyces, Micrococcus* and *Micromonospora*) but could only detect these in 4 sample points. SCA showed the presence of actinobacteria in 7 of the 10 sampling points. This, however, was predominantly *Micromonospora*. The result of GA and SCA as the most appropriate media contrasts with the hypothesis suggested by Qin *et al.* (2012). The author suggested that media containing amino acids as nitrogen sources were the more suitable media to infer the diversity of actinobacteria.

Streptomyces, Micrococcus, Micromonospora, Bacillus, Lactobacillus, Staphylococcus, Oceanobacillus and *Pseudomonas* were the distinct genera as inferred from 16S rRNA gene sequencing. *Bacillus* was the most dominant isolate across all sample points and media used. The extent of diversity inferred from a specific medium can be affected by the presence of dominant population capable of growing on that media (Ercolini *et al.*, 2003; Ercolini *et al.*, 2001). This can explain the observation of *Bacillus* on most of the isolation plates. Furthermore, the ease by which firmicutes develop on plates compared with actinomycetes can also be a factor for the dominance of *Bacillus, Lactobacillus* and *Oceanobacillus* in the results obtained (Al-Awadhi *et al.*, 2013). This result of *Bacillus* dominance, *Lactobacillus* and *Staphylococcus* tally with the previous report by Elijah *et al.* (2014) on the indigenous microbiota in cassava waste effluent.

The actinobacteria isolates within the culture-dependent analysis have not been previously reported in association with cassava effluents. *Micromonospora* was the most dominant actinobacteria and was observed in 7 out of the 10 sample points. Isolates showed close similarities to type strains of *Micromonospora cremea* (Carro *et al*., 2012), *Micromonospora coriariae* (Trujillo *et al*., 2006) and *Micromonospora endolithica* (Hirsch *et al*., 2004). The latter species have been previously isolated from soil. The initial stage of cassava processing involves the direct washing and peeling of harvested cassava which were uprooted from soil (Jekayinfa & Olajide, 2007; Uchechukwu-Agua *et al.*, 2015). The water used for this washing forms a bulk of cassava waste effluent as unwanted debris, so remnant soil and contaminants have to be removed before downstream processing. It could, therefore, be expected that some soil microorganisms can be found in the effluent. This can possibly explain why *Micromonospora* was observed.

The 16S rRNA gene led phylogenetic analysis of actinobacteria observed from culture method (*Streptomyces, Micromonospora* and *Micrococcus*) inferred uniqueness in sequences of *Streptomyces* and *Micromonospora*. The distinct clustering of both isolates separate from the closest relatives, as seen in Fig. 2.8 and Fig. 2.9, This could suggest potential new *Micromonospora* and *Streptomyces* species.

Sequencing of universal and actinobacterial 16S rRNA amplicons was carried out by amplifying the V3–V4 variable bacterial region. The high-throughput sequencing method utilised suggested the taxonomic dominance of firmicutes, proteobacteria, cyanobacteria, and actinobacteria (Fig. 2.10). These phyla were present in all samples analysed, although the proportions varied based on the

amplicon sequenced (Figs. 2.11 and 2.12). Firmicutes was the most abundant phylum, in which the genus *Lactobacillus* was the most abundant genus (Fig.2.13). *Lactobacillus* dominance in cassava processing effluent is not unexpected. *Lactobacillus* is one such bacteria that mediate the chemical process of fermentation during cassava processing (Colehour *et al.*, 2014). The dominance of *Lactobacillus* within cassava derived product and its waste has been widely reported (Anyogu *et al.*, 2014; Ayodeji *et al.*, 2017; Banwo *et al.*, 2012; Padonou *et al.*, 2009). *Bifidobacterium, Corynebacterium* and *Micromonospora* were found to be the dominant genera within the actinobacteria phylum from using actinobacterial specific primers. *Cellulomonas, Propionibacterium, Dermatophilus, Glutamicibacter* was observed along with *Rothia, Actinomyces, Brachybacterium* and *Microbacterium* in minor proportion. These genera were detected by using both universal primers and also actinobacterial specific primers. The genus *Bifidobacterium* has been recognised as a probiotic strain which can utilise cassava for its growth (Varnam & Awamaria, 2007) suggesting their presence in the cassava effluent. *Corynebacterium* and *Micromonospora* and have been reported to exist as a constituent of soil (Carro *et al.*, 2012; Chen *et al.*, 2004). Their occurrence in cassava effluent can be attributed to cassava being a root tuber crop. Interestingly, although actinobacteria such as *Corynebacterium* and *Bifidobacterium* have been reported within the consortium of indigenous microorganism of cassava and its linked product, there are no studies prior to this report suggesting the occurrence of *Micromonospora* within cassava effluent samples. The occurrence of proteobacteria and cyanobacteria within the consortium of cassava effluent microflora reported in this study correlates with

reports on cassava linked microorganisms (Amao *et al.*, 2019; Ayodeji *et al.*, 2017; Elijah *et al.*, 2014; Gao *et al.*, 2014). From the alpha and beta diversity analysis, it can be inferred that the microbial richness differs between samples. This analysis also postulates that each stage of cassava processing impacts the abundance of bacteria observed.

Results from this experiment clearly confirm the need to combine both culture-dependent and independent methods to generate a more precise understanding to the nature of indigenous actinobacteria within cassava effluent samples. The limitations of culture-dependent approach for microbial study has been widely reported (Amann *et al.*, 1995; Muyzer *et al.*, 1993). These limitations can account for the observation of only *Streptomyces, Micromonospora* and *Micrococcus* in the culture-dependent approach. *Bifidobacterium, Corynebacterium, Cellulomonas Propionibacterium Dermatophilus, Glutamicibacter. Rothia, Actinomyces, Brachybacterium* and *Microbacterium* were observed within the culture-independent analysis but were not isolated from cassava effluent. *Micromonospora, Streptomyces* and *Micrococcus* were the only actinobacteria detected in both approaches used in this diversity studies. Although *Streptomyces* seems in low proportion when compared to *Micromonospora*. There could be biases relating to culture-dependent approach such as of the preferential lysis in which some targets being missed during DNA extraction. Furthermore, primer mismatches or low rate of hybridisation of primers to certain template due to different denaturation temperature could also cause the loss of certain targets (Giraffa & Neviani, 2001; Jany & Barbier, 2008). Holistically, the application of both culture-dependent and independent analysis provided qualitative information on the diversity of actinobacteria within our

samples. Actinobacteria distribution varied based on the companies and sampling points. Variation was also observed within the different stages (washing, fermentation and pressing) and the different cassava processing methods (Cassava flakes and cassava starch). From the combination of data indicated from both approaches used in this study, the washing and pressing stages had the most significant proportion of the actinobacteria diversity observed. This diversity was least in the fermentation stage of processing. This can be attributed to the loss of abundance from the initial washing step before the fermentation stage.

Similar diversity of actinobacteria was observed between the two cassava processing methods (Cassava flakes and cassava starch). Their effluents only differed in the presence of *Microbacterium* and *Brachybacterium*. This was observed in the effluent from the cassava flake processing method.

In conclusion, the two methods employed in investigating actinobacteria diversity within cassava effluents are limited by biases. These methods did provide complementary information on microbial diversity present in the cassava effluent at different processing stages. As such, it is necessary to implement both approaches in tandem to provide an overview of diversity. The findings from this experiment provide a new perspective on the diversity of resourceful actinobacteria within cassava effluent.

2.5. References

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CHAPTER 3: Functional characterisation of actinobacteria isolated from cassava waste effluent

3.1. Introduction

In previous years, various studies have focused on the potential of microorganisms from cassava and its effluent (Avancini *et al.*, 2007; Costa *et al.*, 2010; Elijah *et al.*, 2014; Nitschke & Pastore, 2006). The importance of these studies cannot be understated. Cassava effluent is a prominent waste obtained from cassava processing (Okunade, 2013). With the absence of succinct waste treatment options in countries like Nigeria, this waste contributes to aesthetic and environmental nuisance (Oboh, 2005). Izonfuo *et al.* (2013), (Oghenejoboh, 2015) and Arimoro *et al.* (2008) investigated the negative impact of indiscriminate disposal of cassava effluent. They suggested more investigations into the sustainable use of cassava effluent were needed to provide alternative options in mitigating the deleterious effects of this waste effluent.

Previous work predominantly focused on investigating *Bacillus subtilis* from the indigenous microflora of cassava effluent (Barros *et al.*, 2013; Nitschke & Pastore, 2006). However, there remains a gap in understanding the functional potential of indigenous microorganisms from cassava effluent. The possibility of obtaining industrially significant actinobacteria from cassava effluent is yet to be explored.

In this study, the microbial diversity from cassava effluent at different cassava processing stages was explored initially. Culture-dependent and independent approaches were used to investigate the actinobacterial diversity within effluent samples. Novel members of the genus *Micromonospora* and *Streptomyces* related isolates were investigated further in this chapter. Seven isolates belonging to the genus *Micromonospora* and one isolate belonging to *Streptomyces* were analysed for their functional properties. The physiological

traits and enzymatic activities of CWW isolate were explored further, which can aid in the objective of assessing their suitability in biodiesel production.

3.2. Materials and methods

3.2.1. Microbial isolates

Functional attributes of isolates from cassava effluent belonging to genera *Micromonospora* samples 2CPEGATSA42 (Accession number:MWA26320), CMESCA4TSA11 (Accession number:MWA26316), 3CWEGATSA52 (Accession number:MWA26318), 3CPEGATSA1 (Accession number:MWA26314), 3CPEGATSA1B (Accession number:MWA26315), 2CFEGATSA32 (Accession number:MWA26321), 3CPEGATSA70 (Accession number:MWA26317) and *Streptomyces* isolate 3CPEGATSA66 (Accession number:MWA26323) were investigated. These isolates were previously identified based on 16S rRNA gene and were propagated on Glucose yeast malt extract media (GYM).

3.2.2. Colony and cell morphology

The isolates were grown on Tryptone Soy Agar (TSA) for 5 days at 28^{o}C. Colony characteristics such as; colony size, margins, elevation, and pigmentation were observed and recorded. Spore chain morphology was observed using the scanning electron microscope (SEM). Sample preparation for SEM was followed as described by Hirsch *et al*. (2004). Colonies grown on media were picked and fixed in 2 % paraformaldehyde in phosphate buffer for 30 minutes. These cells were rinsed twice and allowed to rest in phosphate buffer for 10 minutes. Colonies were treated with aqueous 1% Osmium tetroxide (OsO_4) for 1 hour. Samples were further rinsed twice before dehydration occurred. Dehydration of bacterial isolates was carried out in graded series of 2x

2minutes distilled water, 2x10minutes 50% ethanol, 2x 10minutes in 70% ethanol, 2x 10minutes in 90 % ethanol and 3x 10minutes in 100% ethanol. The Leica EM CP300 was used for critical drying. Small pots for critical drying were initially cleaned and loaded with the sample. Alcohol was poured into the sample holder before pots were loaded. Sample holder was re-inserted into the critical dryer. The critical dryer was run at 28ºC, 1.0 bar with a processing time of 1.5 hours following machines protocol book. Following critical point drying, samples were taken out and subjected to sputter coating. The sputter coater Polaron SC7640 was used for gold coating. Morphology was then viewed under the JEOL Jsm-6969LV scanning electron microscope.

3.2.3. Gram staining

Bacterial cells were heat fixed on grease free microscopic slides. The slides were flooded with crystal violet staining reagent for 1 minute and washed off with distilled water. Care was taken to ensure the cell concentration was not too light or heavy. The mordant, Gram's iodine, was added for 1 minute and washed off with distilled water. The decolourising agent was added and incubated for 15 seconds before rinsing off with distilled water. Safranin was added and counterstained for 1 minute. Slides were gently washed under a steady stream of tap water until no colour ran off. Slides were finally blotted and allowed to air dry. The result was observed under oil immersion using a Brightfield microscope (Smith & Hussey, 2005).

3.2.4. Phenotypic characterisation of novel species

Based on 16S rRNA gene analysis, closely related type strains to the novel isolates from CWW (cassava wastewater) were selected for phenotypic characterisation. The type strains *M.coriairiae* (DSM 44875), *M.olivastetospora*

(DSM 43868) *M.cremea* (DSM 45599) and *M.endolithica* (DSM 44398*)* were identified to be closest relatives to novel *Micromonospora* isolates. Similarly, *S.albidoflavus* (DSM 41815) was the closest relative to novel *Streptomyces* isolate from CWW. All media sterilised by autoclaving was done at 15 lbs pressure (121°C) for 15 minutes. This was then allowed to cool to 50°C.

3.2.4.1. Preparation of fresh bacterial suspensions

Standardised bacterial suspension for all isolates was prepared to achieve consistency and reproducibility in results. Isolates were inoculated for 3 days on Tryptone soya agar (TSA) (Oxoid, CM0131). Colonies on agar plate were picked and mixed using ¼ strength Ringers solution (Oxoid) to a suspension of McFarland Standard 3 turbidity (McFarland Standard, bioMerieux UK). This was carried out in sterile bijou bottles.

3.2.4.2. Temperature tolerance test

The ability of isolates to grow at various temperatures was determined on the basal GYM medium as described by Williams *et al.* (1983) which contained 4g Glucose, 4g Yeast extract, 10g Malt extract, 2g $CaCO_3$, 12g Agar dissolved in 1000ml distilled water. The pH was adjusted to 7.2 and autoclaved. Temperature tolerance tests were conducted in replidishes on all isolates over a range of temperatures from 4^{o}C, 15^{o}C, 25^{o}C, 28^{o}C, 37^{o}C, 45^{o}C to 55^{o}C and examined for growth after 7 and 14 days while plates at 4^{o}C, 15^{o}C was measured after 6 weeks.

3.2.4.3. pH tolerance test for CWW isolates

The ability of isolates to grow at various pH range was determined on the GYM basal medium described above. The medium was adjusted to 4.3, 7.3 and 10.3, respectively and autoclaved. Replidishes at the respective pH were incubated at 28^{o}C. Replidishes were examined after 7, 14 and 28 days for growth.

3.2.4.4. Growth in the presence of sodium chloride

The ability of isolates to grow at various salt concentrations was determined on the GYM basal medium (see above). Medium was supplemented with sodium chloride at 1%, 5%, 10%, 20% (w/v) concentration. The pH was adjusted to 7.2, and medium was autoclaved. Replidishes were inoculated with isolates and incubated at 28°C. Replidishes were examined after 7,14 and 28 days for growth.

3.2.4.5. Organic compounds as a sole carbon source

The ability of isolates to use 20 different carbon sources was determined on the basal medium described by Shirling and Gottlieb (1966). Carbon sources used were sodium acetate, ribose, arabinose, arginine, citrate, fructose, fucose, galactose, glucose, histidine, maltose, maltotriose, mannitol, mannose, raffinose, rhamnose, sorbitol, starch, sucrose and xylose. Each carbon source at 1% w/v was added to the basal media, with the exception of citrate and sodium acetate, which were added at 0.1 % (w/v). Basal medium consisted of 2.640 g $(NH_4)_2SO_4$, 2.380g KH_2PO_4 , 5.650g $K_2HPO_4.3H_2O$, 1.000g $MgSO_4.7H2O$, 0.0064g $CuSO_4.5H_2O$, 0.0011 $FeSO_4.7H_2O$, 0.0079g $MnCl_2.7H_2O$, 0.0015g $ZnSO_4.7H_2O$, adjusted to pH 7.0(at 25°C). For each carbon source, 24.83g of the dehydrated basal medium was suspended in 900ml distilled water. The medium was autoclaved before carbon source was added (100ml of 10% filter sterilised carbohydrate solution). The medium was mixed and dispensed into replidishes. Test isolates were added, and replidishes were incubated at 28^{o}C. Growth was observed after 7, 14 and 21 days.

3.2.4.6. Utilisation of nitrogen source for growth

The ability of isolates to use 12 different nitrogen sources was determined on the medium described by Williams *et al.* (1983). Arginine, glutamine, valine,

phenylalanine, threonine, glycine, serine, asparagine, methionine, glutamic acid, histidine, and cysteine were used as nitrogen sources. The basal medium comprised of 1.0% (w/v) D-glucose, 0.1 % (w/v) K_2HP0_4, $FeSO_4$. $7H_20$, 0.001 % (w/v), 0.05% (w/v) NaCl, 0.05% (w/v) $MgSO_4$. $7H_20$, 1.2% (w/v) Bacto-agar (Difco), 1000ml dH_20. The pH was adjusted to 7.0. The medium was heated slightly to ensure all components were completely dissolved before it was autoclaved. Nitrogen sources were filter sterilised at 0.1% w/v into autoclaved medium and dispensed into replidishes. Test isolates were streaked, and replidishes were incubated at 28^{o}C. Growth was observed after 7, 14 and 21 days for all test isolates.

3.2.4.7. Degradation tests

Degradation tests were carried out on Adenine (0.4%, w/v), tyrosine (0.4%, w/v), hypoxanthine (0.4%, w/v), xanthine (0.4%, w/v), xylan (0.4%, w/v), guanine (0.4%, w/v), casein (0.5%, w/v), cellulose (1.0 %, w/v). The basal medium described by Williams *et al.* (1983) was utilised to test for the degradation of each compound. Each compound was filter sterilised respectively into separate GYM agar (See above). Care was taken to ensure insoluble materials were evenly distributed. Replidishes were examined after 7, 14 and 21 days for all test isolates. A clear zone under and around the bacteria growth suggested degradation by test isolate.

3.2.4.8. Allantoin hydrolysis

The degradation of allantoin (a diureide of glyoxylic acid) was determined on the medium described by Gordon *et al.* (1974). 0.33% (w/v) allantoin was added to the medium containing 0.2g $MgSO_4.7H_2O$, 0.2g KC1, 1g $(NH_4)_2HPO_4$ and 15g Agar. The pH of the media was optimised to 7.2 before supplementing with

phenol red as pH indicator (0.04% solution of phenol red, 20 ml; distilled water, 1,000 ml). 0.5 ml of standardised culture was pipetted into wells of replidishes containing basal medium. Replidishes were read after 14 days upon incubation at 28^{o}C. A colour change from orange to pink-red in the medium infers the presence of two hydrolytic enzymes.

3.2.4.9. Urease production

The degradation of urea was determined on the urease medium described by Gordon *et al.* (1974). Urea (1.76 % w/v) was filter sterilised and added to the medium containing 1g yeast extract, 9.5g Na_2HPO_4, 10g KH2PO4, 0.04% solution of phenol red (20 ml in 1000 ml distilled water), pH adjusted to 6.7 and was autoclaved. Inoculated agar plates were examined for colour change after 7 and 14 days. Urea is degraded by urease to release ammonia. The release of ammonia therefore increases the pH of the medium. A colour change from yellow to pink-red indicates a positive urea degradation.

3.2.4.10. Aesculin and arbutin hydrolysis

The hydrolysis of aesculin and arbutin a hydroquinone β glucopyranoside was determined on the basal medium described by Kroppenstedt and Kutzner (1976). Slopes of aesculin were prepared by filter sterilising 0.1 % w/v aesculin into basal medium containing 0.75g Soy peptone,0.5g ferric ammonium citrate, and 3g yeast extract (Oxoid), 15g agar in 1000 ml distilled water. The pH was adjusted to 7.4, and the medium was autoclaved. Isolates were streaked onto the surface of agar slopes. Slopes were examined for blackening after 7 and 14 days following incubation at 28^{o}C. Test control was prepared by inoculating test isolates on slopes without aesculin and arbutin, respectively. Colour change after 7 and 14 days was also observed.

3.2.4.11. Starch hydrolysis

The hydrolysis of starch was carried out to test the presence of extracellular α and β amylases following the protocol described by Gordon *et al.* (1974). An initial 1.5g of potato starch was suspended in 10ml of distilled water and added to 100ml of nutrient agar. Nutrient agar was prepared (w/v): 0.5% peptone, 0.3% yeast extract, 0.5% NaCl, 1.5% agar and autoclaved. Duplicate replidishes were prepared for each isolate to be inoculated. The first set of replidishes were flooded with 95% ethanol after 5 days, and the other sets were flooded after 10 days. Replidishes were allowed to stand for 15 to 30 minutes each time. A clear zone under and around the bacteria growth indicates starch hydrolysis.

3.2.4.12. Degradation of tween 20, 40, 60, and 80

The degradation of water soluble esters of higher fatty acids and polyoxyethylene derivatives of sorbitan mono-laurate (Tweens 20), palmitate (Tweens 40), stearate (Tweens 60) and oleate (Tweens 80) were determined on the basal medium described by Sierra (1957). To prepare the media, 1.0 % v/v of each tween were sterilised separately into 500ml of basal media containing 0.1g CaCl2.H20, 10g Difco Bacto-peptone, 5g NaCl, 15 g Agar, in 1000 ml distilled water. The pH was adjusted to 7.2 and autoclaved. Replidishes were incubated for 14 days at 28^{o}C. Following incubation, replidishes were observed for the precipitation of fatty acid calcium salts produced by the activities of specific esterases. An opaque halo under and around the bacteria growth indicates a positive reaction.

3.2.4.13. Susceptibility and resistance to antibiotics

The ability of isolates to grow in the presence of 10 different antibiotics was measured using the disc diffusion assay described by Jorgensen (1998). Test

bacterial suspensions were initially adjusted to a turbidity equivalent of 0.5 McFarland standard. This was followed by the inoculation of 0.1 ml of isolates on GYM medium. The medium pH was adjusted to 7.2 and autoclaved before dispensing into plates. Discs previously soaked in Oxytetracycline (Oxoid; 30µg ml^{-1}), ampicillin (Oxoid; 25µg ml^{-1}), tetracycline (Oxoid; 30µg ml^{-1}), polymyxin B (Oxoid; 300 µg ml^{-l}), penicillin (Oxoid; 10 µgml^{-l}), Amoxycillin/clavulanic acid (Oxoid; 3µg ml^{-1}), oxacillin (Oxoid; 1µg ml^{-1}), vancomycin (Oxoid; 5µg ml^{-1}), Ciprofloxacin (Oxoid; 10µg ml^{-1}) and Neomycin (Oxoid; 10µg ml^{-1}) were systematically placed on the surface of plates containing the test isolates. Plates were incubated at $28^{O}C$ for 7 days. A clear zone around the bacteria growth suggests susceptibility to the antibiotics. The diameter of inhibition zone (mm) was measured for each antibiotic.

3.2.4.14. Growth in the presence of lysozyme

The ability for isolates to grow in the presence of lysozyme was determined on the basal medium described by Gordon *et al.* (1974). 0.005% (w/v) lysozyme was added to glycerol broth containing 70 ml glycerol, 3 g beef extract, 5 g peptone, in 1000 ml distilled water; pH 7.0. The medium was autoclaved. Three millilitres of media was aseptically dispensed into the wells of sterile replidishes after which, 5µl of test isolate was used to seed each replidish before incubating at $28^{o}C$ for 2 weeks. Replidishes were observed and scored based on the growth in relation to the positive and negative controls. Positive results are isolates which were resistant to lysozyme.

3.2.4.15. Nitrate reduction

Test bacterial suspensions were initially adjusted to a turbidity equivalent of 0.3 McFarland standard using ¼ strength Ringer's solution. For all test isolates, 5µl

was inoculated in nitrate broth containing: 5.0g Peptic digest of animal tissue, 3.0g Meat extract, 1.0g Potassium nitrate, 30.0g Sodium chloride dissolved in 1000 ml distilled water and autoclaved. Tests were incubated for 14 days at 28^{O}C. A few drops of Nitrate A reagent (Microgen 10ml Ref MID61a) and Nitrate B (Microgen 10ml Ref MID61b) was added. The formation of a red colour following the addition of nitrate B indicates the presence of nitrite and was positive for nitrate reduction and negative for nitrite reduction.

3.2.4.16. Enzyme detection

The ability of isolates to produce the following 20 enzymes was determined using the API ZYM Kits (Biomerieux): Alkaline phosphatase, C-4 esterase, C-8 esterase lipase, C-14 lipase, Leucine aminopeptidase, Valine aminopeptidase, Cystine aminopeptidase, Trypsin, α-Chymotrypsin, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, α-Galactosidase, β-Galactosidase, β-Glucuronidase, α-Glucosidase, β-Glucosidase, N-Acetyl-P-glucosaminidase, α-Mannosidase, α-Fucosidase. Bacteria test isolates were initially grown on TSA agar plates. Colonies were picked and mixed using ¼ strength Ringers solution (Oxoid) to make a suspension equivalent to McFarland Standard 5 turbidity. The API ZYM incubation box was prepared by adding 5ml of distilled water into the honeycombed wells. The test strip was removed from the package and was placed in the incubation box. 65µl of standardised test isolate was added to each cupule. Strip was covered and incubated at 28^{o}C in humidified trays overnight. Same conditions were ensured for all test isolates. Following incubation, a drop of ZYM A and ZYM B reagent was added to each cupule. ZYM A contained Tris-HCL and sodium lauryl sulphate in water while ZYM B contained diazonium salt Fast Blue BB in 2-methoxyethanol. API strips for each test

isolate were allowed to stand in bright light for 5minutes to allow the development of colour. A colour change revealed the activities of enzymes present in each cupule. Each cupule was scored based on the intensity of the colour produced. Scoring was from zero representing an absence of enzyme activity to 5, indicating very strong enzyme activity. Scores from 0 to 2 were classified as a negative reaction, while 3 to 5 was scored as a positive reaction.

3.2.4.17. Hydrogen sulphide production

The ability of isolates to produce Hydrogen sulphide (H_2S) was determined by the method of Williams *et al.* (1983). Isolates were inoculated in a nitrate medium containing,5.0g Peptic digest of animal tissue, 3.0g Meat extract, 1.0g Potassium nitrate, 30.0g Sodium chloride dissolved in 1000ml distilled water. Isolates were inoculated into the broth and incubated for 14 days at $28^{O}C$. Hydrogen sulphide (H_2S) formation was determined after 14 days by inserting lead acetate filter paper on the neck of the nitrate medium. Blackening of sterile lead acetate filter paper indicates positive result.

3.2.4.18. Catalase activity

Catalase activity was detected on a microscopic slide as described by Williams *et al.* (1983). Few drops of 20% (v/v) hydrogen peroxide was added to a loop full of 7-day old biomass grown on Tryptone soya agar plates. The evolution of oxygen bubbles on the surface of the mixture within 5 minutes of addition, suggests a positive catalase activity.

3.2.4.19. Acid fastness

Acid fastness was deduced for all test isolates as described by Gordon *et al.* (1974). Bacterial smear of representative isolates was heat fixed on grease free microscopic slides. The slides were flooded with carbol fuschin stain (saturated

ethanol solution of basic fuchsin, 10 ml; 5% aqueous phenol solution, 90 ml) and heated for 5 minutes to steaming temperature. The slide was rinsed off with water. The smears were decolourised using acid alcohol (95% ethanol, 97 ml; concentrated HC1, 3 ml). Slides were washed gently in running sterile distilled water. Next, slides were counterstained with 0.1% methylene blue for 1-2 minutes. Slides were finally air-dried for 30 minutes and examined under the microscope.

3.2.5. Fatty acid analysis

Fatty acid analysis was carried out on the freeze-dried biomass of all isolates. Isolates were grown for 48 hours in GYM broth medium and harvested. Harvested cells were frozen overnight at -80^{O}c. The Edwards freeze dryer (3983) was used to obtain the freeze-dried biomass. The type strains *M.coriairiae* (DSM 44875), *M.cremea* (DSM 45599), *M.endolithica* (DSM 44398*)* and *M.olivastetospora* (DSM 43868) were compared with *Micromonospora* isolates 2CPEGATSA42, 3CWEGATSA52, CMESCA4TSA11, 3CPEGATSA1, 3CPEGATSA1B, 2CFEGATSA32, 3CPEGATSA70 while *S.albidoflavus* (DSM 41815) was compared with 3CPEGATSA66 from genus *Streptomyces*.

The extraction of cellular fatty acids was carried out, as described by Sasser (2006). 40 mg of each freeze-dried sample was added to a clean, dry Teflon-lined screw cap test tube. 1 ml of reagent 1 (45g sodium hydroxide, 150ml methanol, 150ml deionised distilled water) was added into the Teflon-lined screw cap, tightly sealed and vortexed for 5-10 seconds. Each sample was placed on a rack and then boiled for 5 minutes in a water bath. Samples were vortexed for 5 seconds checked for leakage and boiled for another 25 minutes. Tubes were cooled at room temperature in a water bath following boiling. Two ml of reagent

2 (195ml of 12N Hydrochloric acid, 275ml methanol, 130ml of deionised distilled water) was added to each tube. Tubes were tightly sealed and vortexed for 10 seconds then heated in a water bath for 10 minutes at 80°C. Tubes were cooled quickly by transferring into a water bath set at room temperature. Following cooling, each tube was uncapped, and 1.25ml of reagent 3 (200ml Hexane, 200ml Methyl-tert-butyl-ether) was added to the tube. Tubes were tightly sealed and placed in a rotator mixed end-over for 10 minutes. Tubes were uncapped in batch, and the lower aqueous phase was discarded. 3 ml of reagent 4 (10.8g sodium hydroxide, 900ml deionised distilled water) was added to each tube. Teflon-lined tubes were screwed tightly and rotated end–over for 5 minutes. Tubes were centrifuged for 3 minutes at 2000 rpm (1920 xg). Two-thirds of the upper solvent phase following centrifugation was removed and placed in a GC sample bottle for fatty acid profiling.

The Trace 1300 series Gas Chromatograph coupled with a single quadrupole mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to profile fatty acids extracted from bacteria cells. All FAME extracts were incubated at 40°C for 2 minutes with intermittent shaking. A FAME mix C8-C24 (Sigma Aldrich, USA) was used as the standard for this GC analysis. A 50/30 μm (Supelco, Sigma Aldrich, UK) solid-phase microextraction (SPME) fibre was used for 40 minutes extraction of samples then desorbed for 1min. The separation was carried with a ZB-WAX capillary gas chromatography column (length 30 m, internal diameter 1 mm, 1.00 μm film thickness). The injector temperature was set at 200°C in splitless mode with a constant carrier pressure of 18psi. The column temperature was held initially at 40°C for 2min, increased

by 8°C/minute to reach 240°C and held for 1 min. Full scan mode was used to detect fatty acids (mass range from m/z 35 to 200).

3.2.6. Growth of novel isolates on cassava waste effluent

3.2.6.1. Culture on agar

The ability of all actinobacterial isolates to grow on CWW effluent was tested. Representatives from each genus previously isolated were incubated on CWW agar. Isolates used were *Micromonospora* (3CPEGATSA70), *Streptomyces* (3CPEGATSA66) *Micrococcus* (CMEYMATSA12B) including *Rhodococcus opacus* type strain (DSMZ 43205). Cassava waste effluent from which these isolates were originally isolated from were used in the preparation of the agar media. For *Micromonospora* (3CPEGATSA70) and *Streptomyces* (3CPEGATSA66) isolates, cassava pressing effluent from company 3 (Reputable food and allied) was used. *Micrococcus* (CMEYMATSA12B), cassava starch effluent from company 4 (Matna foods) was used (Table 3.1 summarises the sample details). For *Rhodococcus opacus* type strain (DSMZ 43205), the ability to grow on cassava effluent was investigated equally. Cassava pressing effluent from Reputable food and allied company was used to investigate this potential. This was because this sampling point had a high diversity of actinobacteria when investigated during the culture-dependent analysis.

For each isolate, 100 ml of CWW agar was prepared by adding 50 ml CWW into 50 ml autoclaved agar supplemented with Nystatin (25 µg/ml). This agar was initially prepared by adding 1.5g agar into 50ml of distilled water. Potassium phosphate buffer was used in the preparation of CWW agar to

maintain the pH 7.0. Potassium Phosphate buffer (0.1M concentration) was prepared by adding 9.3g K2HPO4, and 6.3g KH2PO4, in 1000ml distilled water. *Micromonospora*, *Streptomyces, Micrococcus* were the isolates obtained from CWW effluent to be incubated on CWW agar. *Rhodococcus opacus* was also incubated on CWW agar to observe their ability to grow on waste effluent. A loopful of cells from *Micromonospora* (3CPEGATSA70), *Streptomyces* (3CPEGATSA66) *Micrococcus* (CMEYMATSA12B) and *Rhodococcus* (DSMZ 43205) initially grown on TSA were inoculated in TSB for 3 days at 28^{o}C. Test isolates were washed twice in deionised water before standardisation to Mcfarland standard 3 (McFarland Standard, bioMerieux UK) using ¼ strength Ringer's solution (Oxoid). One ml of standardised inocula for all isolates was agitated to produce an even suspension before pipetting into the wells of sterile replidishes containing CWW agar. Replidishes were incubated at 28^{o}C and was examined after 7 and 14 days for growth.

3.2.6.2. Confirmation of isolates grown on waste effluent agar

To confirm the identity of isolates grown on CWW agar, Repetitive Extragenic Palindromic (REP) PCR was used to compare the REP fingerprint profiles between isolates retrieved on CWW replidishes and the original inoculated cultures. DNA extraction was carried out by following the protocol of Legras and Karst (2003) (see section 2.2.2.2). REP PCR was carried out using REP primers RepIRI: iiiCGIGCICATCIGGC and Rep2I: iCGiCGTATCiGGCCTAC (De Bruijn, 1992). Protocol for REP PCR was carried out as earlier described (see section 2.2.2.3.1)

3.3. Results

3.3.1. Microbial isolates

The eight isolates used were previously obtained from cassava waste effluent at different sampling points. These isolates were chosen based on their unique REP fingerprint pattern which delineated them from other species within the consortium observed. They represent the isolated diversity within cassava effluent which suggested potential novelty when compared with published type strains. Table 3.1 shows the origin and details of isolates

Table 3.1: Actinobacteria isolates in this study obtained from CWW

Isolates	**Isolate code**	**Genus**	**Origin of isolation**
1	2CPEGATSA42 (Accession number:MWA26320)	*Micromonospora*	Pressing stage (Bilatrade food)
2	3CWEGATSA52 (Accession number:MWA26318)	*Micromonospora*	Washing stage (Oxford Foods),
3	CMESCA4TSA11 (Accession number:MWA26316)	*Micromonospora*	Cassava starch effluent (Matna foods)
4	3CPEGATSA1 (Accession number:MWA26314)	*Micromonospora*	Pressing stage (Reputable food)
5	3CPEGATSA1B (Accession number:MWA26315)	*Micromonospora*	Pressing stage (Reputable food)
6	2CFEGATSA32 (Accession number:MWA26321)	*Micromonospora*	Fermentation stage (Bilatrade food)
7	3CPEGATSA70 (Accession number:MWA26317)	*Micromonospora*	Pressing stage (Reputable food)
8	3CPEGATSA66 (Accession number:MWA26323)	*Streptomyces*	Pressing stage (Reputable food)

3.3.2. Morphology

The morphology of all unique actinomycetes isolated from CWW was observed under a microscope. All tested isolates, as seen in Table 3.1, grew well on both GYM and TSA. All closely related isolates to *Micromonospora* (isolate 1-7) were gram-positive and filamentous in morphology. These isolates varied in colour from cream to intense orange. Colonies were raised and folded. They changed to dark colour after 4 to 10 days of growth. Fig 3.1 shows the morphology of isolates cultured on GYM agar. SEM image (Fig 3.2) after 5 days of development on GYM agar, show single non-motile spores on both short and long tip of the hyphae for *Micromonospora*.

Isolate 8 (*Streptomyces)* was gram-positive, filamentous bacteria with a smooth, raised morphology and was white in colour. They turned into bright white colonies after 4 days of growth with complex substrate mycelium and stuck tightly on the surface of agar plates (Fig. 3.3)

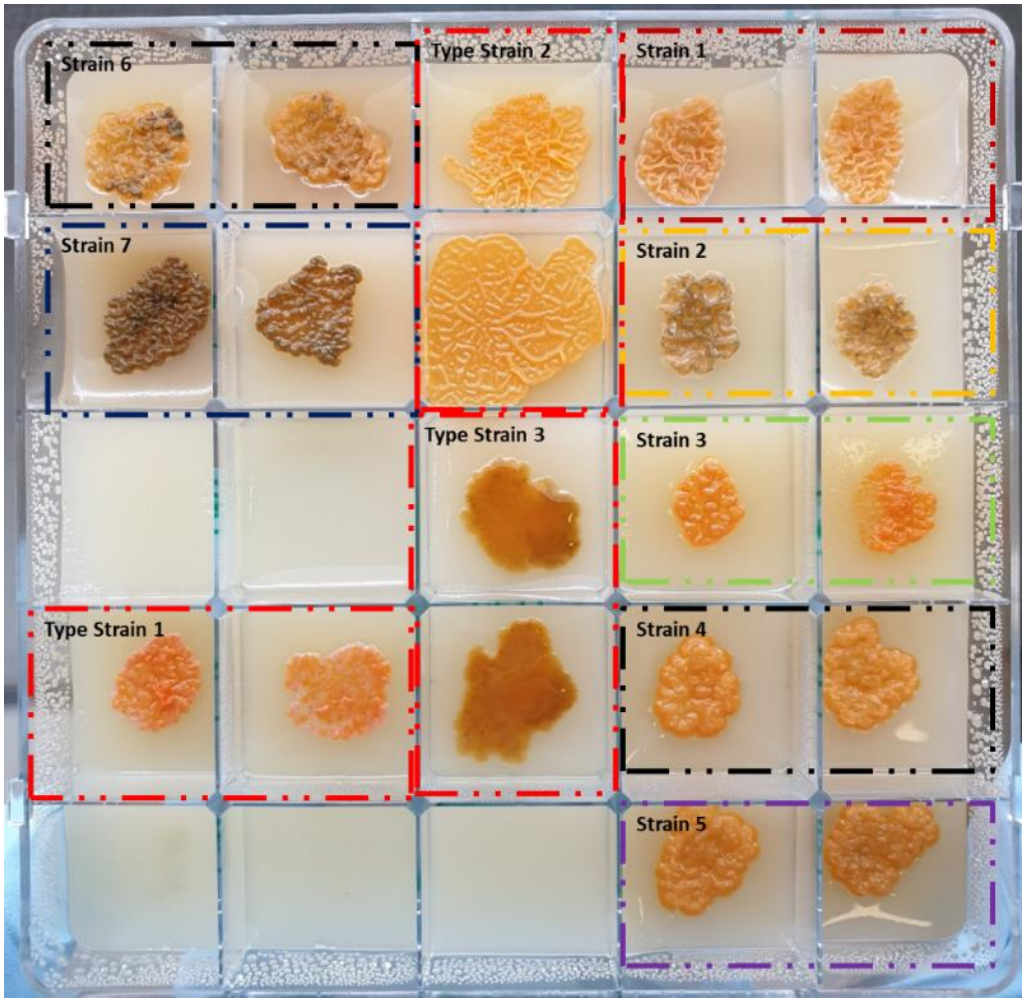


Figure 3.1: Morphology of all *Micromonospora* isolates cultured on GYM agar. Strain 1(2CPEGATSA42), Strain 2 (3CWEGATSA52), Strain 3 (CMESCA4TSA11), Strain 4 (3CPEGATSA1), Strain 5 (3CPEGATSA1B), Strain 6 (2CFEGATSA32), Strain 7, 3CPEGATSA70), Strain 8 (3CPEGATSA66) type strain 1= *Micromonospora coriariae.* type strain 2= *Micromonospora cremea.* type strain 3= *Micromonospora endolithica*

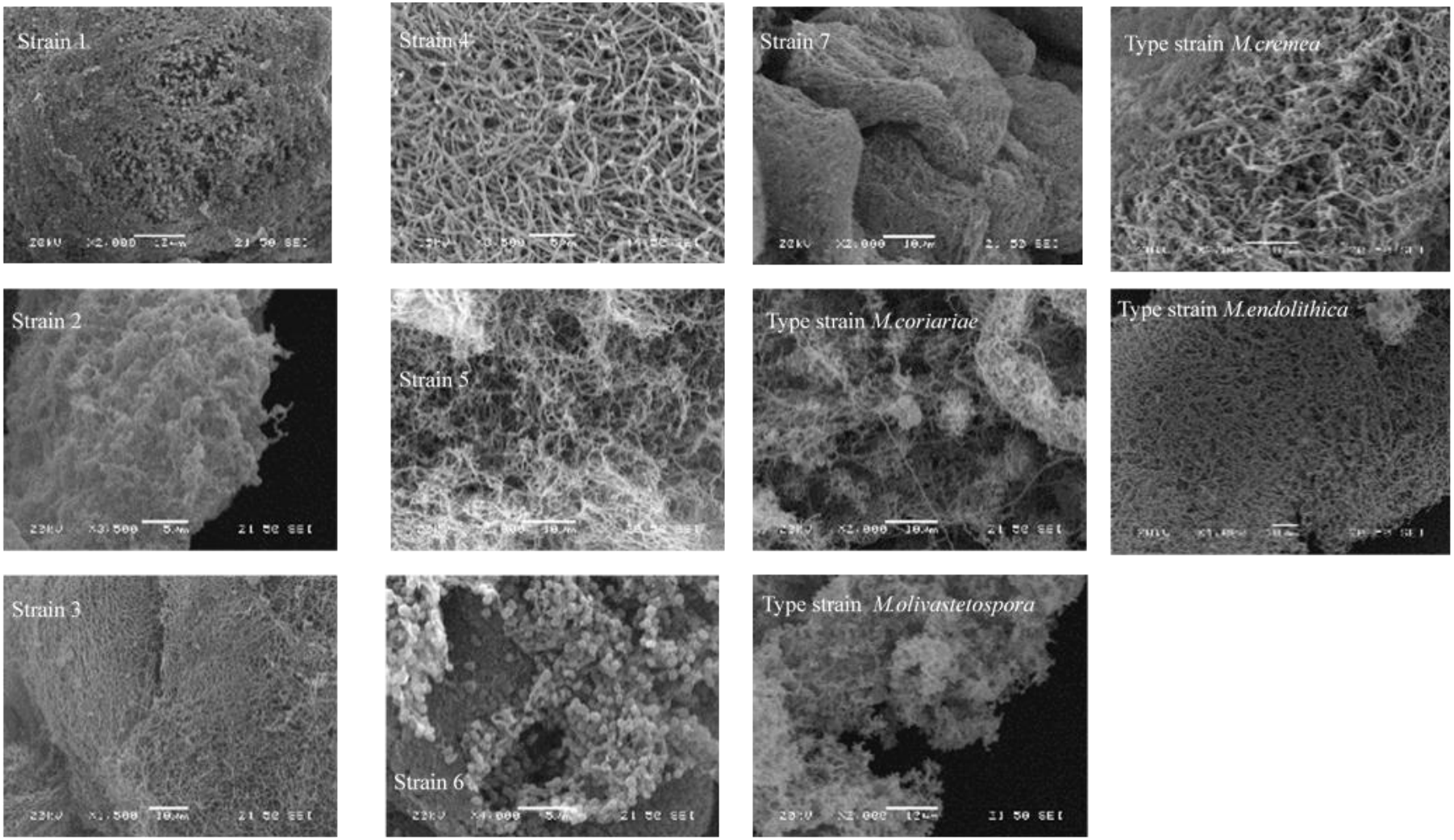


Figure 3.2 : Scanning electron micrographs of culture showing spores on both short and long tip of the hyphae. *Micromonospora* isolates 1-7 and type strain *M.cremea, M.coriariae, M.endolithica* and *M.olivastetospora.* The micron (µm) scale bars in the respective image legend is used to determine average spore sizes

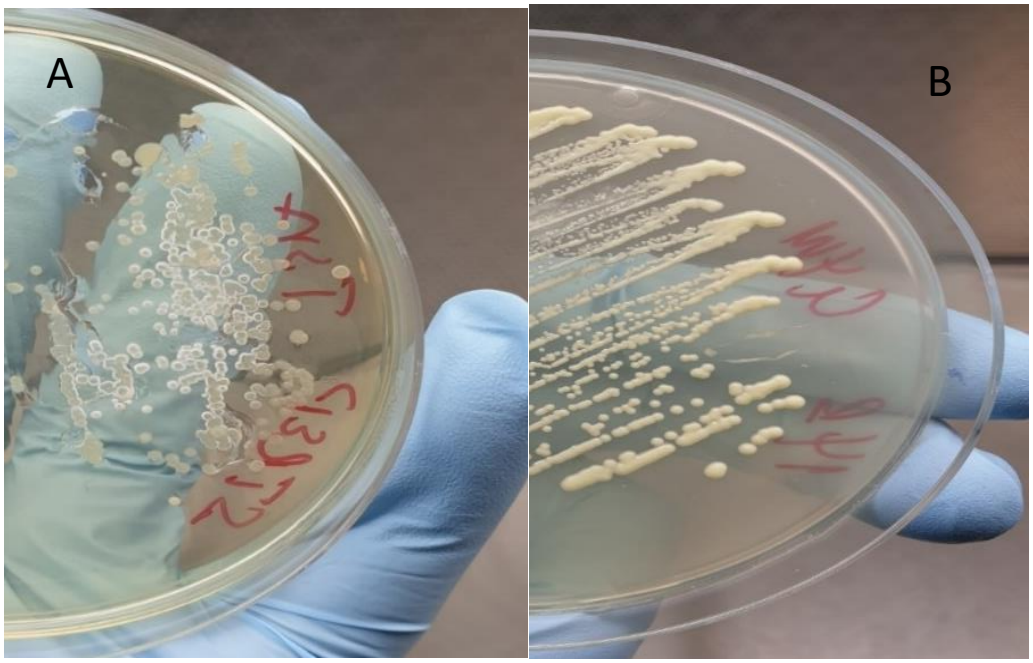


Figure 3.3: Morphology of *Streptomyces* isolates and closely related type strain. (a) Isolate 8=3CPEGATSA66 (*Streptomyces*) (b) type strain *Streptomyces albidoflavus*

3.3.3. Phenotypic characterisation of novel species

3.3.3.1. Growth test

The optimal pH, temperature and NaCl concentrations for optimum growth were investigated for all isolates. As shown in Table 3.2 below, all *Micromonospora* isolates 1-7 grew best between 25^{o}C and 37^{o}C, albeit at some temperatures they grow well at different pH levels. *Streptomyces* isolate 8 grew best at temperatures of 15 ^{o}C to 37 ^{o}C. No growth detected on all plates incubated at temperatures of 4^{o}C and 55^{o}C. Sparing growth was observed in isolates 1, 4 and 5 at 45^{o}C. As seen in Table 3.2, pH results suggest the rate at which isolates are affected by a change in pH levels. All *Micromonospora* isolates grew well at pH of 4.3, 7.3 and 10.3. Isolate 8 was unable to grow properly at a pH of 4.3 but grew well at pH 7.3 and 10.3.

The ability of all isolates to withstand salt stress was also evaluated by culturing with NaCl concentrations of 1%, 5%, 10% and 20%. From the result, sodium

chloride tolerance for all *Micromonospora* isolates was only at 1%. Isolate 8 was able to develop at concentrations of 1-5% NaCl concentrations only.

Table 3.2: Effect of temperature, pH and sodium chloride on the growth of actinomycete isolates obtained from cassava waste effluent

TESTS		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	***S.albidoflavus***	***M.coriariae***	***M.cremea***	***M.endolithica***	***M.olivastetospora***
Temperature 4°C	pH 4.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	pH 7.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	pH 10.3	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature 15°C	pH 4.3	-	-	-	+	+	-	-	-	-	-	-	-	-
	pH 7.3	+	-	+	+	+	-	+	+	+	+	+	+	+
	pH 10.3	-	-	-	-	-	-	-	-	+	-	-	-	-
Temperature 25°C	pH 4.3	+	+	+	+	+	+	+	-	-	+	+	-	-
	pH 7.3	+	+	+	+	+	+	+	+	+	+	+	+	+
	pH 10.3	+	+	-	+	+	-	+	+	+	-	-	-	-
Temperature 28°C	pH 4.3	+	+	+	+	+	+	+	-	-	+	+	+	-
	pH 7.3	+	+	+	+	+	+	+	+	+	+	+	+	+
	pH 10.3	+	+	-	+	+	-	+	+	+	-	+	-	-
Temperature 37°C	pH 4.3	-	+	+	+	+	+	+	-	-	+	+	-	-
	pH 7.3	-	+	+	+	+	+	+	+	+	+	+	+	+
	pH 10.3	+	+	-	+	+	-	+	+	+	-	-	-	-
Temperature 45°C	pH 4.3	+	-	-	-	+	-	-	-	-	-	-	-	-
	pH 7.3	+	-	-	+	+	-	-	-	-	-	-	-	-
	pH 10.3	+	-	-	+	-	-	-	-	-	-	-	-	-
Temperature 55°C	pH 4.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	pH 7.3	-	-	-	-	-	-	-	-	-	-	-	-	-
	pH 10.3	-	-	-	-	-	-	-	-	-	-	-	-	
NaCl Tolerance	1%	+	+	+	+	+	+	+	+	+	+	+	+	+
	5%	-	-	-	-	-	-	-	+	+	-	-	-	-
	10%	-	-	-	-	-	-	-	-	-	-	-	-	-
	20%	-	-	-	-	-	-	-	-	-	-	-	-	-

(−) negative, (+) positive.

3.3.3.2. Catalase activity

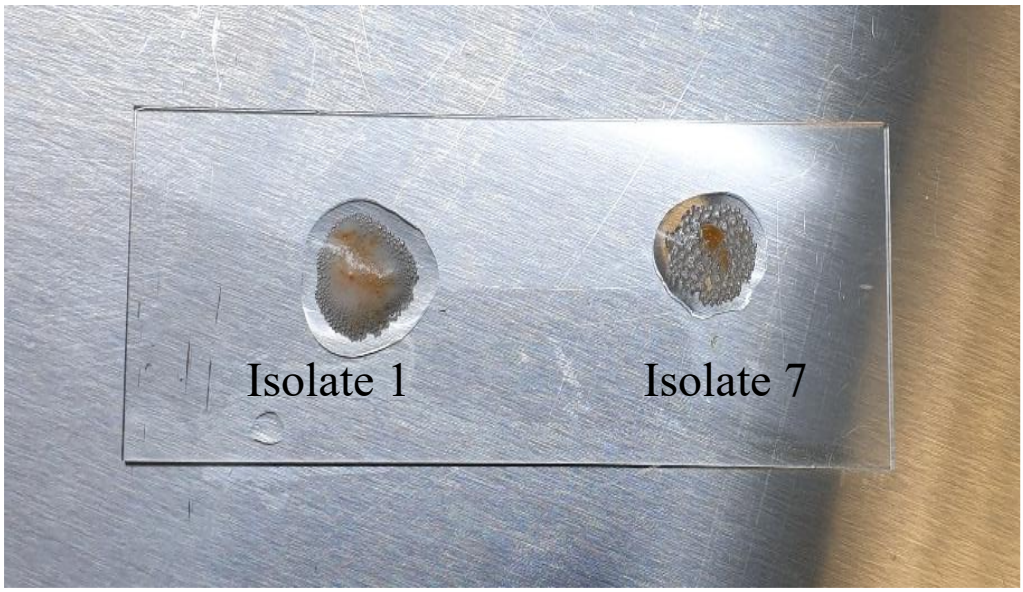


Figure 3.4: Catalase activity indicated by the formation of oxygen bubbles following the addition of H_2O_2. Isolate 1= 2CPEGATSA42, Isolate 7= 3CPEGATSA70.

The ability to secrete enzymes involved in catalysing oxygen release from hydrogen peroxide (H_2O_2) was determined for test isolates from 1-8. All isolates tested positive for catalase activity due to the formation of oxygen bubble on the microscopic slide's surface. Fig. 3.4 illustrates a positive catalase activity indicated by the appearance of oxygen bubbles following the addition of H_2O_2.

3.3.3.3. Acid fastness

Isolates 1-8 were tested for mycolic acids to differentiate isolates into acid-fast or non-acid fast groups. Following the addition of carbol-fushin and decolourising with acid alcohol, all isolates 1-8 retained the counterstain methylene blue colour.

3.3.3.4. Hydrogen sulphide production

H_2S test was carried out on all isolates 1-8. Cell growth was observed in the nitrate broth for all samples, indicating suitability to utilise the medium for growth after 14 days. However, there was no colour change or blackening of the lead acetate filter paper suggesting that H_2S was not produced from all tested isolates.

3.3.3.5. Carbon source utilisation

In this study, 20 different carbons sources were used to evaluate the isolates for growth and assimilation on each source. Incubation was carried out on replidishes containing the different carbon sources, as seen in Table 3.3. Isolates (1-7) could be differentiated from each other based on the utilisation pattern. From all isolates, 1 and 2 had similarities between the carbon sources they assimilated. Isolate 8 showed similarities to type strain *S.albidoflavus* in 18 of the 20 carbon sources tested for. All other isolates suggested a slight difference from each other based on their carbon utilisation. These isolates also showed differences in comparison with the type strains. From the result, amongst the 20 carbon sources, only citrate was not used by any of the isolates for growth.

Table 3.3: Carbon source utilisation for CWW isolate and respective type strains

TESTS	1	2	3	4	5	6	7	8	*S.albidoflavus*	*M.coriariae*	*M.cremea*	*M.endolithica*	*M.olivastetospora*
carbon source													
Sodium acetate	+	+	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	-	-	-	-	+	-	-	-	-	-	-
Arabinose	+	+	+	-	-	-	-	+	+	-	+	-	-
Arginine	+	+	+	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	-	-	-	-	+	+	-	+	-	+
Fucose	+	+	+	-	-	-	-	+	+	+	+	-	+
Galactose	+	+	+	-	-	-	-	+	+	-	+	-	+
Glucose	+	+	+	+	+	+	-	+	+	+	+	-	-
Histidine	+	+	-	-	-	-	-	+	+	-	+	-	-
Maltose	+	+	+	-	-	-	-	+	+	-	+	-	+
Maltotriose	+	+	+	-	-	-	-	+	+	+	+	-	+
Mannitol	+	+	+	-	+	-	-	+	+	+	+	-	-
Mannose	+	+	+	-	-	-	-	+	+	-	+	-	+
Raffinose	+	+	+	+	-	-	-	+	+	-	-	-	-
Rhamnose	+	+	-	-	-	-	-	-	+	+	-	-	+
Sorbitol	+	+	+	-	-	-	-	+	+	-	+	-	+
Starch	+	+	+	-	-	-	-	+	+	+	+	-	-
sucrose	+	+	+	-	-	-	-	-	+	+	+	-	+
Xylose	+	+	+	-	-	-	-	+	+	+	+	-	+

(−) negative, (+) positive

3.3.3.6. Utilisation of nitrogen

The ability to utilise 12 different nitrogen sources was determined for all novel isolates. Isolates were able to assimilate nitrogen sources, as described in Table 3.4 below. Isolates 1-7 showed differences in the assimilation of tested nitrogen sources. This test distinguishes each isolate between themselves and the type strains providing information on the nutrient requirements affecting growth. Isolate 8, however, was similar to the closest type strain *S.albidoflavus* for all tested nitrogen sources.

Table 3.4: Nitrogen source utilisation for CWW isolate and respective type strains

TESTS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	***S.albidoflavus***	***M.coriariae***	***M.cremea***	***M.endolithica***	***M.olivastetospora***
Utilisation of Nitrogen													
Arginine	+	-	+	-	-	-	-	-	-	-	-	-	-
Glutamine	+	+	+	-	-	+	-	+	+	-	+	-	-
Valine	+	+	+	+	+	+	+	+	+	+	+	-	-
PhenylAlanine	-	-	-	-	-	-	-	+	+	-	-	-	+
Threonine	-	+	+	-	+	-	-	+	+	-	+	-	+
Glycine	-	-	-	-	-	-	-	+	+	-	-	-	-
Serine	-	-	-	-	-	-	-	+	+	-	+	-	-
Asparagine	-	-	+	-	-	-	+	+	+	-	-	+	-
Methionine	+	-	+	+	+	+	+	+	+	-	+	-	+
Glutamic acid	+	-	-	-	-	-	-	+	+	-	-	-	+
Histidine	-	-	-	-	-	-	-	+	+	-	-	-	-
Cysteine	-	-	-	-	-	-	-	+	+	-	-	-	-

(−) negative, (+) positive.

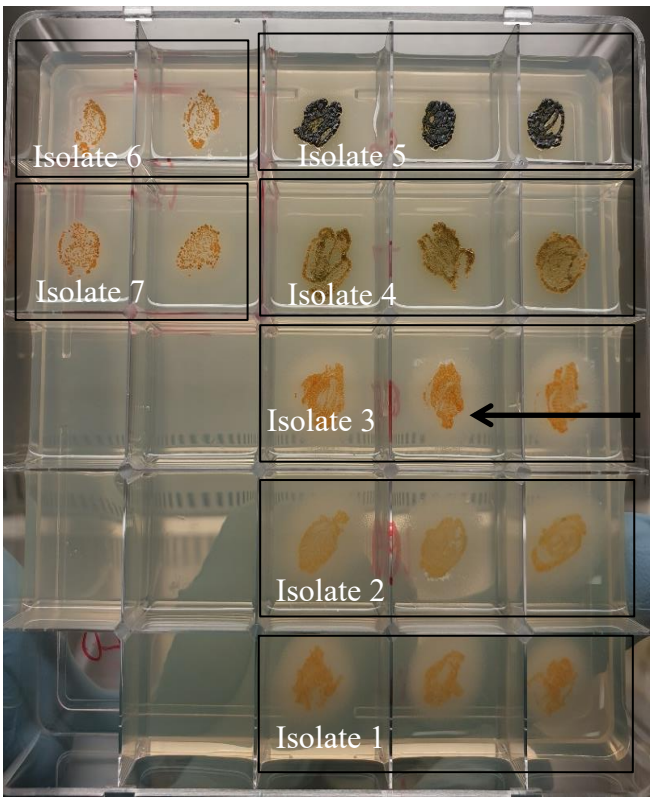
3.3.3.7. Degradation test

The ability for all isolates to degrade a variety of compounds was determined. The clearing of the insoluble compound around and under the growth areas indicated a positive reaction for these tests. From Table 3.5 below, esculin, casein, and starch were hydrolysed by the majority of isolates. There was no esculin hydrolysis observed on plates inoculated with isolates 2 and 8. Casein was not hydrolysed by isolate 5. There were negative results on isolate 1,2, and 7 tested on Tween 40 and isolate 7 tested on Tween 80. Isolate 5 was able to hydrolyse both urea and tyrosine in comparison with other tested isolates. Isolate 8 was also able to hydrolyse tyrosine. Figs. 3.5,3.6,3.7,3.8 and 3.9 illustrate the reactions occurring in tween, esculin, arbutin, urea and starch tests, respectively.

Table 3.5: Degradation test carried out on CWW isolates and respective type strains

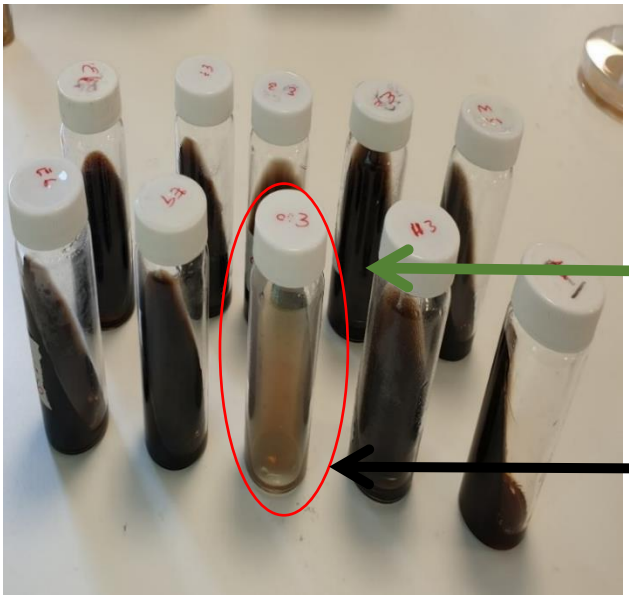
TESTS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	***S.albidoflavus***	***M.coriariae***	***M.cremea***	***M.endolithica***	***M.olivastetospora***
Degradation													
Arbutin	-	-	-	-	-	-	-	-	-	-	+	-	-
Allantoin	-	-	-	-	-	-	-	+	+	-	-	-	-
Urease	-	-	-	-	+	-	-	-	-	-	-	-	-
Aesculin	+	-	+	+	+	+	+	-	-	+	+	+	-
Adenine	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	-	-	+	-	-	+	+	-	-	-	+
Hypoxanthine	-	-	-	-	-	-	-	-	-	-	-	-	-
xanthine	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylan	-	-	-	-	-	-	-	-	-	-	-	-	-
Guanine	-	-	-	-	-	-	-	-	-	-	-	-	-
Casein	+	+	+	-	+	+	+	+	+	+	+	+	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-	-	-	-	-	-
Tweens													
20	+	+	+	+	+	+	+	+	+	+	+	+	+
40	-	-	+	+	+	+	-	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	-	+	+	+	+	+	+

(−) negative, (+) positive.



zone of calcium precipitates around the colonies indicating hydrolysis of Tween

Figure 3.5: Hydrolysis of Tweens by Isolate 1-7 showing zones of precipitation around colonies. Isolate 1= 2CPEGATSA42, Isolate 2= 3CWEGATSA52, Isolate 3= CMESCA4TSA11, Isolate 4= 3CPEGATSA1, Isolate 5= 3CPEGATSA1B, Isolate 6= 2CFEGATSA32, Isolate 7= 3CPEGATSA70



Darkening of agar slope

No color change on strain 2 following incubation on esculin agar slope

Figure 3.6: Hydrolysis of esculin showing darkened agar (positive result) and absence of colour change (negative result)

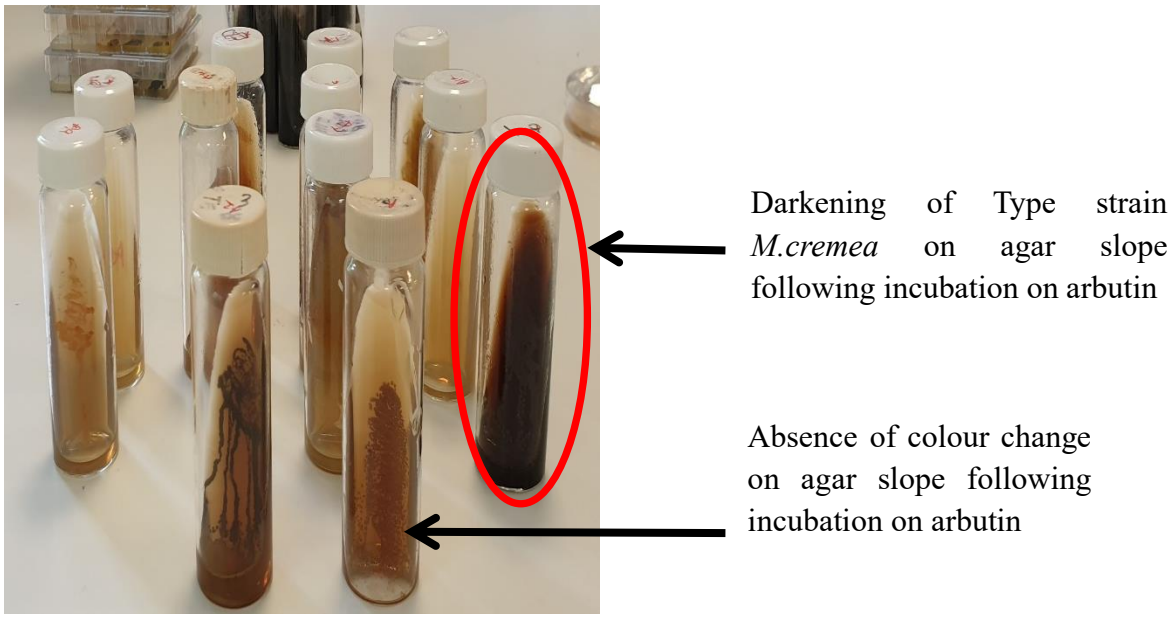


Figure 3.7: Hydrolysis of arbutin showing growth of isolates on agar slopes.

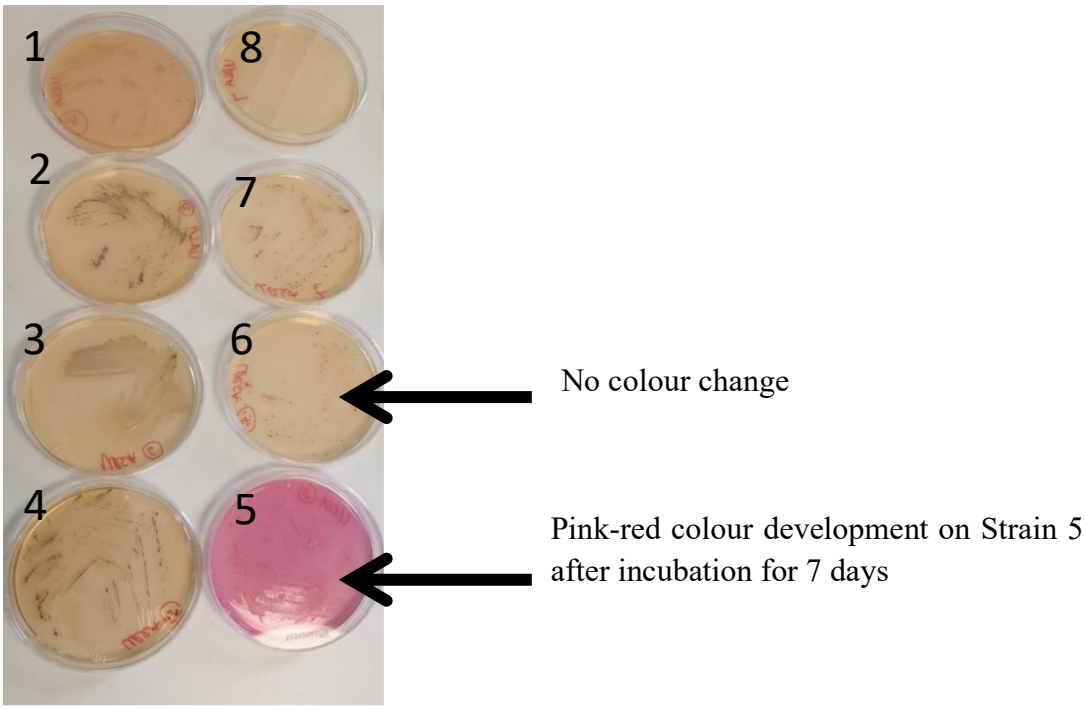


Figure 3.8: Hydrolysis of urea showing positive reaction. Isolate 1= 2CPEGATSA42, Isolate 2= 3CWEGATSA52, Isolate 3= CMESCA4TSA11, Isolate 4= 3CPEGATSA1, Isolate 5= 3CPEGATSA1B, Isolate 6= 2CFEGATSA32, Isolate 7= 3CPEGATSA70 Isolate 8= 3CPEGATSA66

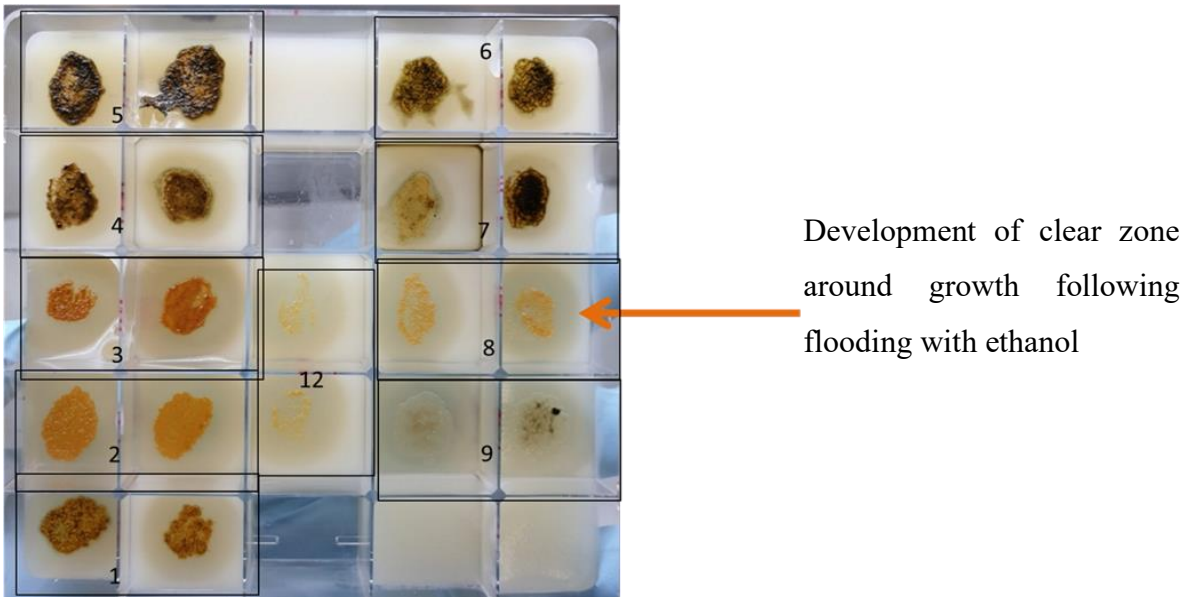


Figure 3.9: Hydrolysis of starch showing region of clearing around growth area. 1= 2CPEGATSA42, 2= 3CWEGATSA52, 3= CMESCA4TSA11, 4= 3CPEGATSA1, 5= 3CPEGATSA1B, 6= 2CFEGATSA32, 7= 3CPEGATSA70. 8= type strain *M.coriairiae* (DSM 44875), 9= 3CPEGATSA66 12=*M.endolithica* (DSM 44398*)*

3.3.3.8. Antibiotic test

Isolates were tested for resistance and susceptibility to different antibiotics. The patterns of resistance of isolates (isolate 1-8) and comparative type strains can be seen in Table 3.6 below. Isolate 2 and 5 were resistant to all tested antibiotics. Isolate 4 and 6 showed similarities in the pattern of resistance and susceptibility to the different antibiotics. In comparison with the types strain, Isolate 7 showed a similar pattern of susceptibility to *M.coriariae* and *M.endolithica* for all antibiotics tested. Isolate 8 showed similar response with type strain *S.albidoflavus* for the antibiotics tested with exception to Neomycin in which isolate 8 was susceptible, and *S.albidoflavus* was resistant. Fig. 3.10 illustrate zone of inhibition patterns and the effect of disc antibiotic on the isolates tested.



Figure 3.10: Antibiotics test plates (Isolate 7= 3CPEGATSA70) showing antagonistic effect of disc antibiotics. (A) Vancomycin (B) Penicillin (C) Tetracycline (D) Oxacillin (E) Ampicillin (F) Neomycin (G) Oxytetracycline (H) Amoxylin (I) Polymyxin (J) Ciprofloxacin

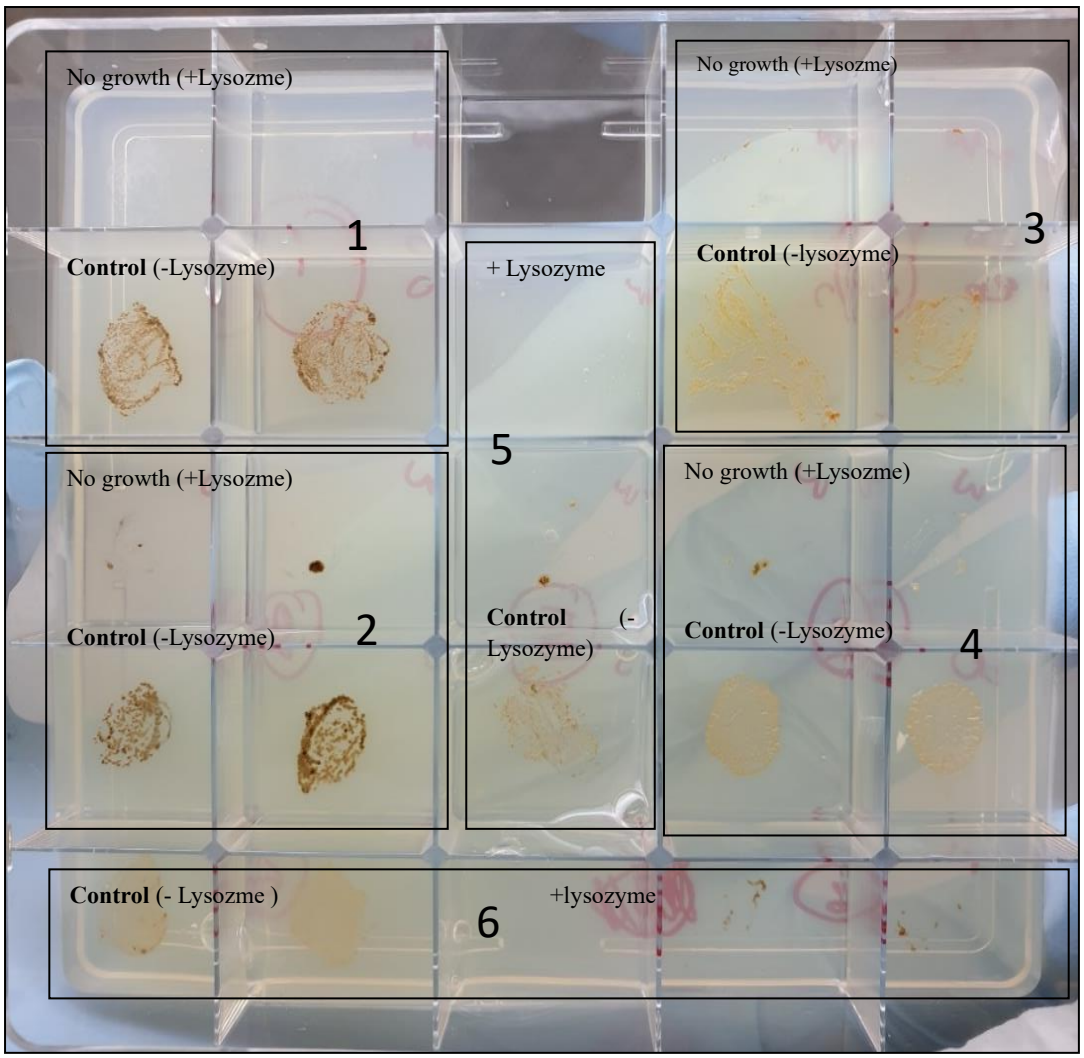
Table 3.6: Effect of antibiotics on CWW isolates

Antimicrobial agent (Disk content)	Diameter of inhibition zone (mm)			Isolates								Type strain				
	Resistance (R)	Intermediate (I)	Susceptible (S)	1	2	3	4	5	6	7	8	*S.albidoflavus*	*M.coriariae*	*M.cremea*	*M.endolithica*	*M.olivastetospora*
Tetracycline (30µg)	≤ 18	N/A	≥ 19	S	R	S	R	R	R	S	R	R	R	ND	ND	S
Penicillin (10 µg)	≤ 14	N/A	≥ 15	S	R	S	R	R	R	S	R	R	S	ND	S	R
Vancomycin (5µg)	≤14	15-16	≥17	R	R	S	R	R	R	S	R	R	S	ND	S	R
Ampicillin (25µg)	≤11	12-14	≥15	S	R	S	R	R	R	S	R	R	S	ND	S	R
Oxacillin (1µg)	≤10	11-12	≥13	S	R	S	R	R	R	R	R	R	R	S	R	R
Amoxycillin (3µg)	≤13	14-17	≥18	S	R	S	R	R	R	S	R	R	S	ND	S	R
Oxytetracycline (30µg)	≤ 18	N/A	≥ 19	R	R	R	R	R	R	S	R	R	S	ND	ND	R
Neomycin (10µg)	≤13	14-15	≥16	S	R	S	S	R	S	S	S	R	S	ND	S	R
Ciprofloxacin (10µg)	≤15	16-20	≥21	S	R	S	S	R	S	S	S	S	R	ND	ND	S
Polymyxin B (300 µg)	≤11	N/A	≥12	S	R	R	R	R	R	S	S	R	S	S	S	S

(S) Susceptible, (I) Intermediate, (R) Resistant (ND) Not determined.

3.3.3.9. Growth in the presence of lysozyme

The ability to grow in the presence of lysozyme was determined. For all *Micromonospora* isolates, no growth was observed across all tested isolates in relation to the control (Fig. 3.11). Isolate 8 developed in the presence of lysozyme. This was in contrast to the type strain *S.albidoflavus* which was susceptible to lysozyme.



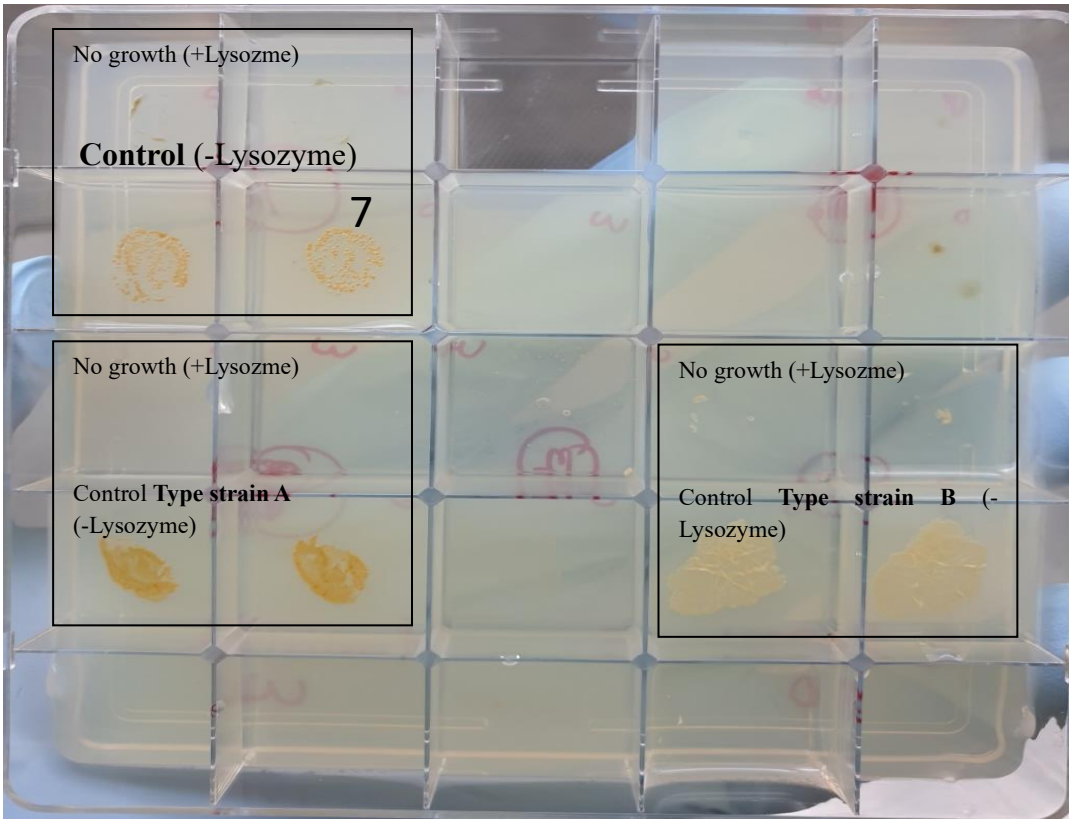


Figure 3.11: Plates showing effect of lysozyme on growth of Isolates. Isolate 1= 2CPEGATSA42, Isolate 2= 3CWEGATSA52, Isolate 3= CMESCA4TSA11, Isolate 4= 3CPEGATSA1, Isolate 5= 3CPEGATSA1B, Isolate 6= 2CFEGATSA32, Isolate 7= 3CPEGATSA70 type strains A –*Micromonospora cremea* and type strain B- *Micromonospora endolithica*

3.3.3.10. Nitrate reduction

The ability for isolates to reduce nitrate to nitrite was tested across all isolates. A colour change detected the reduction of nitrate to nitrite by test isolate following the addition of Nitrate A and B reagent as seen in Fig. 3.12. All test isolates 1-7 showed a significant colour change in contrast to the type strains tested against isolates. There was no colour change in isolate 8 upon the addition of Nitrate A and B. This result was also observed on the type strain *S.albidoflavus.*

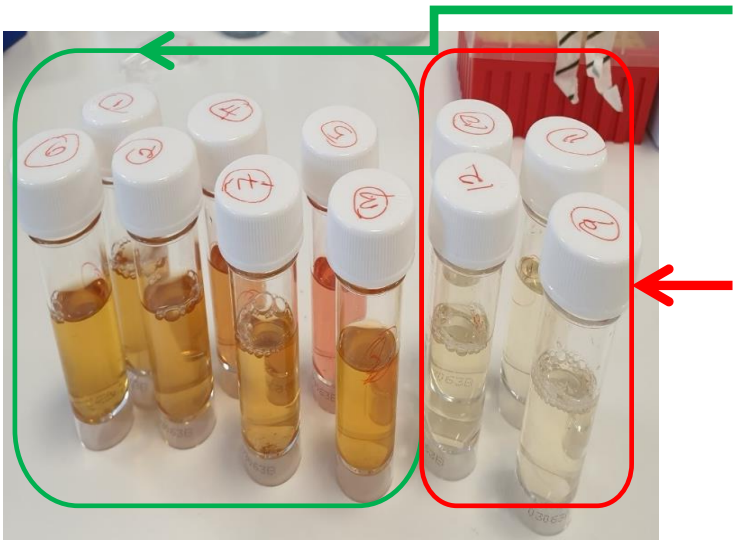


Figure 3.12: Reduction of nitrate by isolates 1-7.

3.3.3.11. Enzyme detection

The enzymatic profiles of isolates 1-8 were rapidly determined using the API ZYM kit (Biomerieux). In reference to the API ZYM expected colour intensity, reactions were graded negative (grade 0-2), positive (grade 3 to 5). Enzymatic activity for tested isolates 1-8 as inferred from API ZYM system is summarised in Table 3.7 below. Isolates showed differences between each other based on their enzyme activities across 19 substrates. All isolates tested positive for Naphthol-AS-BI-phosphohydrolase activity. These isolates, with the exception of isolate 8, also indicated α-Chymotrypsin activity. Isolate 4 showed positive enzyme activity to 12 out of the 20 enzymes tested. α-Fucosidase, α-Mannosidase, β-Glucosidase and N-Acetyl-β-glucosaminidase activities were not observed in all tested isolates. Equally, β-Glucuronidase was not produced by all isolates. Fig. 3.13 illustrates the enzymatic activity of CWW isolates occurring on the API ZYM assay kit.

Table 3.7: Enzymatic activity tested using the API system for CWW isolates.

TESTS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	***S.albidoflavus***	***M.coriariae***	***M.cremea***	***M.endolithica***	***M.olivastetospora***
Enzymes													
Alkaline phosphatase	-	-	-	+	-	-	-	+	+	-	+	-	+
C-4 esterase	+	-	+	+	-	+	-	+	+	+	+	+	+
C-8 esterase lipase	+	-	+	+	-	-	+	+	+	+	-	+	+
C-14 lipase	-	-	+	+	-	-	-	+	+	-	-	-	-
Leucine arylamidase	-	-	+	+	-	-	-	+	+	+	+	-	-
Valine arylamidase	-	-	-	+	-	-	-	+	-	-	+	-	-
Cystine arylamidase	-	-	-	+	-	-	-	+	-	+	+	-	-
Trypsin	-	-	-	+	+	-	-	-	-	-	-	-	-
α-Chymotrypsin	+	+	+	+	+	+	+	-	-	-	-	-	-
Acid phosphatase	+	-	-	+	-	-	-	-	-	+	-	-	-
Naphtol-AS-BI-phosphohyrdrolase	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	+	-	-	-	-	-	-	-	-	-	-	-	-
β-Galactosidase	-	-	+	-	-	-	-	-	-	-	-	-	-
β-Glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Glucosidase	+	-	-	+	+	-	-	-	-	-	+	+	+
β-Glucosidase	-	-	-	-	-	-	-	-	+	-	-	-	-
N-Acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-	+	-	+	-	-
α-Mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-

(−) negative, (+) positive.

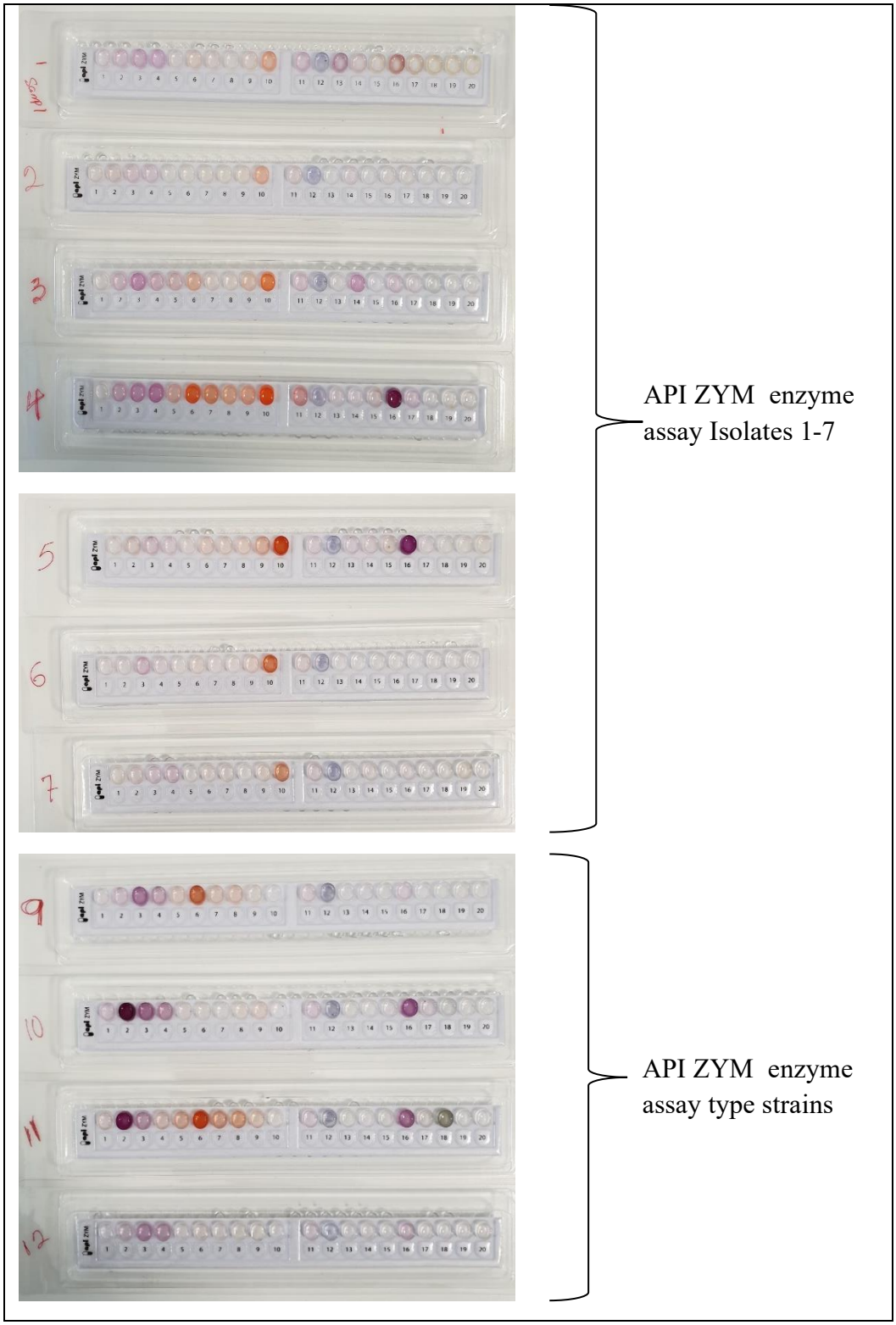
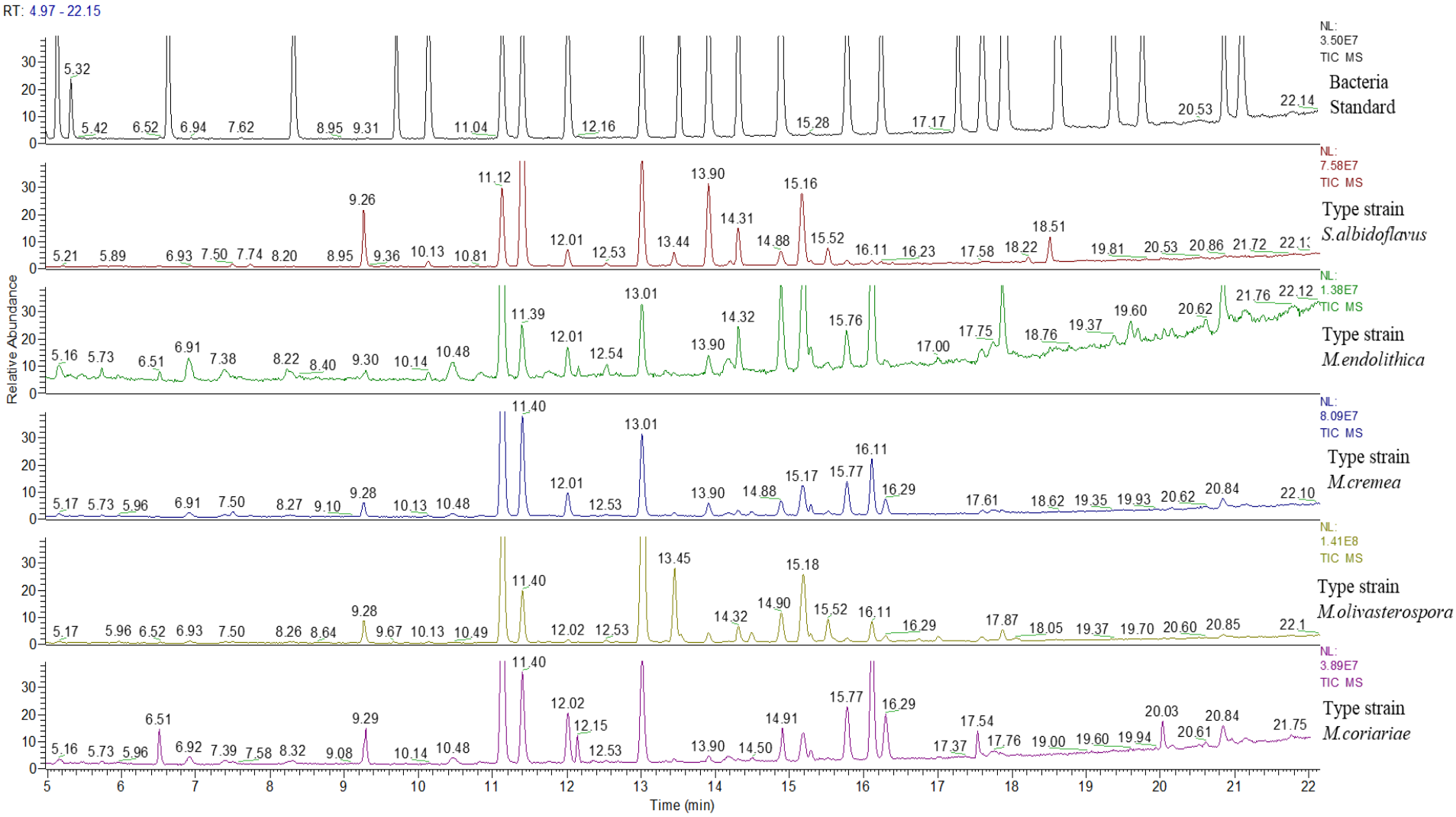


Figure 3.13: API ZYM assay kit representing enzymatic activity of CWW isolates. 1= 2CPEGATSA42, 2= 3CWEGATSA52, 3= CMESCA4TSA11, 4= 3CPEGATSA1, 5= 3CPEGATSA1B, 6= 2CFEGATSA32, 7= 3CPEGATSA70 9= type strains= *M.coriairiae* 10 =*M.olivastetospora* 11=*M.cremea* and 12= *M.endolithica*

3.3.4. Fatty acid analysis

The chromatographic profile of fatty acid methyl ester (FAME) composition for all isolates is represented in Fig. 3.14. The result from the fatty acid profile suggests close similarities amongst all isolates from the same genus. The common fatty acids between *Micromonospora* isolates were 13-methyltetradecanoic acid $C_{i15:0}$, 12-methyltetradecanoic acid $C_{a15:0}$, 14-methylpentadecanoic acid $C_{i16:0}$, 15-methylhexadecanoate $C_{i17:0}$, 14-methyl hexadecanoic acid $C_{a17:0}$ and Cis-9, 10 methylenehexadecanoate $C_{17:0\wedge}$. The proportion of each fatty acids varied between isolates within same genus, as seen in Table 3.8. *Micromonospora* isolates 1, 2, 3and 7 were mainly comprised of $C_{a17:0,}$ although they showed varying abundance of other fatty acids. Isolate 4,5,6 were similar in the higher percentage of $C_{i15:0}$. These isolates showed FAME profile with the closest similarity to type strains *M.Coriariae, M.cremea* and *M endolithica*. *Streptomyces* isolate 8 had similar high proportion of $C_{a15:0}$ with type strain *S.albidoflavus*. Isolates varied based on the percentage of other minor FAME profile as observed from the analysis.


RT: 4.97 - 22.15
NL:
3.50E7
TIC MS
Bacteria
Standard
NL:
7.58E7
TIC MS
Type strain
S.albidoflavus
NL:
1.38E7
TIC MS
Type strain
M.endolithica
NL:
8.09E7
TIC MS
Type strain
M.cremea
NL:
1.41E8
TIC MS
Type strain
M.olivasterospora
NL:
3.89E7
TIC MS
Type strain
M.coriariae
Relative Abundance
Time (min)

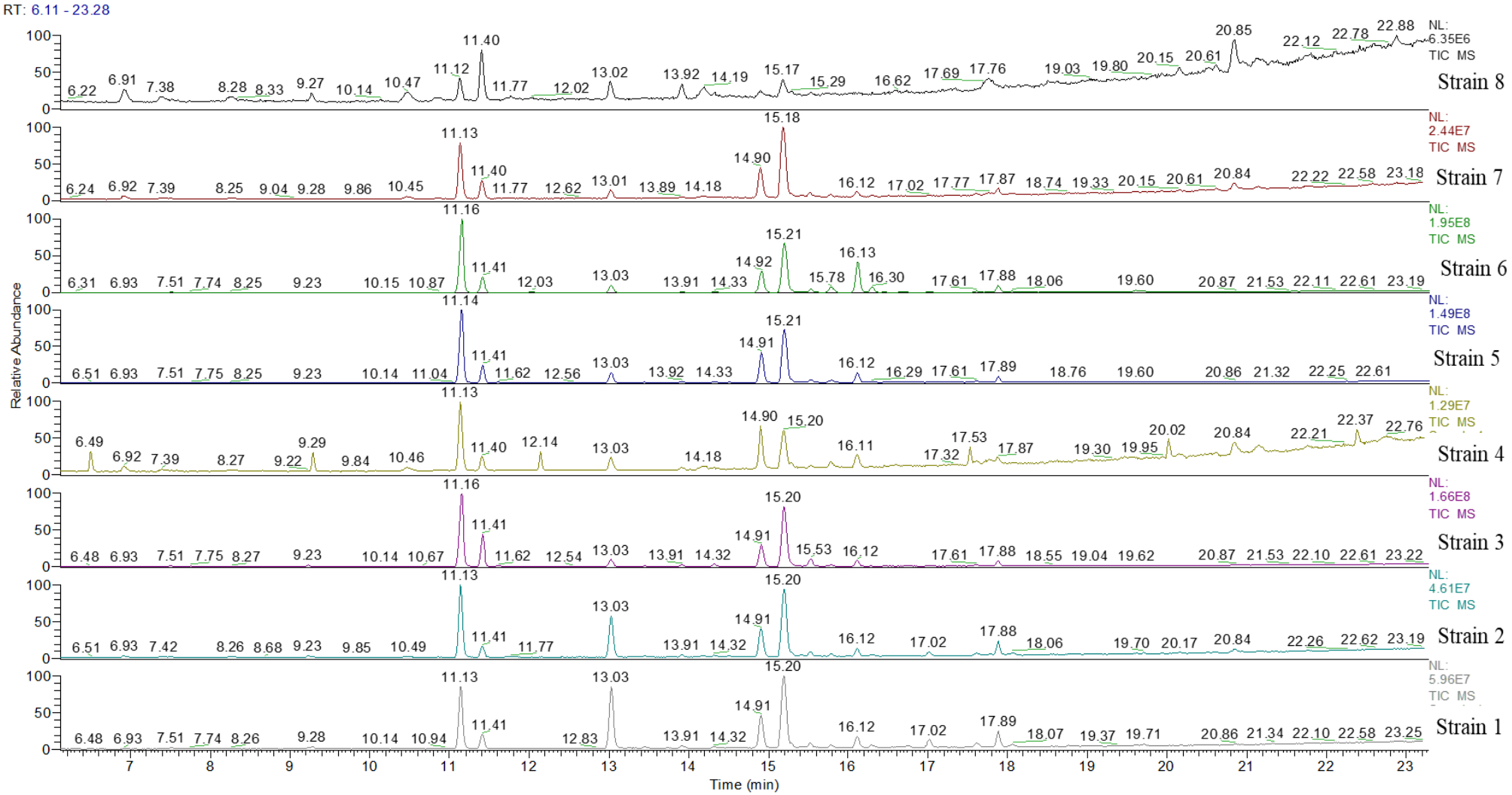


Figure 3.14: GC/ MS chromatograph of FAME profiles for CWW isolates.

Table 3.8: Percentage composition of fatty acid methyl esters within CWW isolates.

% Composition of principal fatty acid in samples															
Sample no	**i14:0**	**i15:0**	**a15:0**	**15:0**	**i16:0**	**16:1_i**	**16:cis9**	**i17:0**	**a17:0**	**17:1br_a**	**17:0**	**17:0^**	**18:1_i**	**18:0**	**18:1_9**
Isolate 1	-	18.2	4.6	-	19.4	-	-	10.9	28.8	1.5	-	3.4	2.4	1.6	4.9
Isolate 2	-	23.7	4.1	-	14.8	-	-	10.9	30.1	1.5	-	2.6	1.7	1.2	4.8
Isolate 3	-	30.7	12.7	-	3.2	-	-	9.9	32.0	3.2	-	2.4	-	-	2.4
Isolate 4	5.1	28.2	6.7	-	6.0	-	-	17.1	23.0	-	3.3	5.3	-	1.5	2.1
Isolate 5	-	31.3	7.3	-	4.9	-	-	13.9	29.8	1.4	1.4	4.3	-	1.1	2.7
Isolate 6	-	30.4	6.3	-	3.3	-	-	10.2	27.5	1.2	2.4	12.9	-	-	3.0
Isolate 7	-	23.1	7.7	-	4.0	-	-	13.9	39.0	1.6	1.2	2.8	-	1.3	2.7
Type strain *M.coriariae*	3.8	30.3	10.6	5.8	13.3	-	-	3.3	4.3	-	6.9	16.6	-	2.5	1.0
Typestrain *M.olivasterospora*	2.6	22.8	5.9	-	34.4	8.1	1.8	3.9	10.1	3.0	-	2.2	-	-	1.3
Type strain *M.cremea*	2.0	40.7	15.4	3.7	13.1	-	-	2.6	6.0	0.5	5.5	7.8	-	-	-
Type strain *M.endolithica*	1.2	23.7	5.3	2.7	7.5	-	3.5	9.9	14.3	0.8	3.9	18.2	-	1.6	6.2
Isolate 8	4.9	14.4	31.8	3.6	13.0	1.0	-	6.6	12.4	2.1	-	-	1.2	-	8.9
Type strain *S.albidoflavus*	6.7	11.2	37.3	2.4	16.6	1.8	4.7	2.6	11.7	2.4	-	-	-	1.1	-

3.3.5. Growth of *Rhodococcus opacus*, novel isolates and type strains on cassava waste effluent

3.3.5.1. Culture on agar

Result of isolates grown on replidishes can be seen in Fig. 3.15. *Rhodococcus opacus* has been previously reported to produce a significant proportion of useful lipids which can be employed in biodiesel generation (Kurosawa *et al.*, 2010). Hence, it was important to investigate this isolate's potential to grow along with indigenous isolates on cassava effluent. Growth was only observed in wells buffered CWW media for isolates belonging to *Micromononspora, Streptomyces* and *R.opacus*. There was no growth of *Micrococcus* in all growth conditions employed.

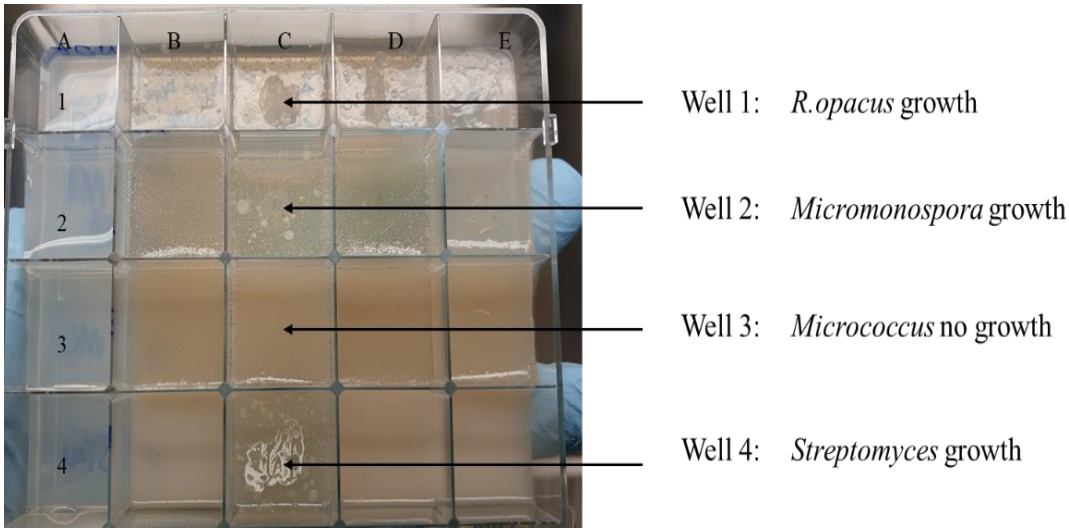


Figure 3.15: Replidish illustrating growth of isolates on CWW agar. A) Well A1-A4 represents agar alone without isolates B) Well B1-B4 Isolates grown on buffered CWW agar C) Well C1-C4 Isolates grown on unbuffered agar D) Well D1-D4 Isolates grown on autoclaved CWW agar

3.3.5.2. Confirmation of Isolates Grown on Waste Effluent Agar

The REP fingerprint pattern of CWW agar inoculum and observed isolates were compared as seen in Fig.3.16. From the 1.5% gel image, similar patterns were observed between all CWW agar isolates and their inoculums respectively.

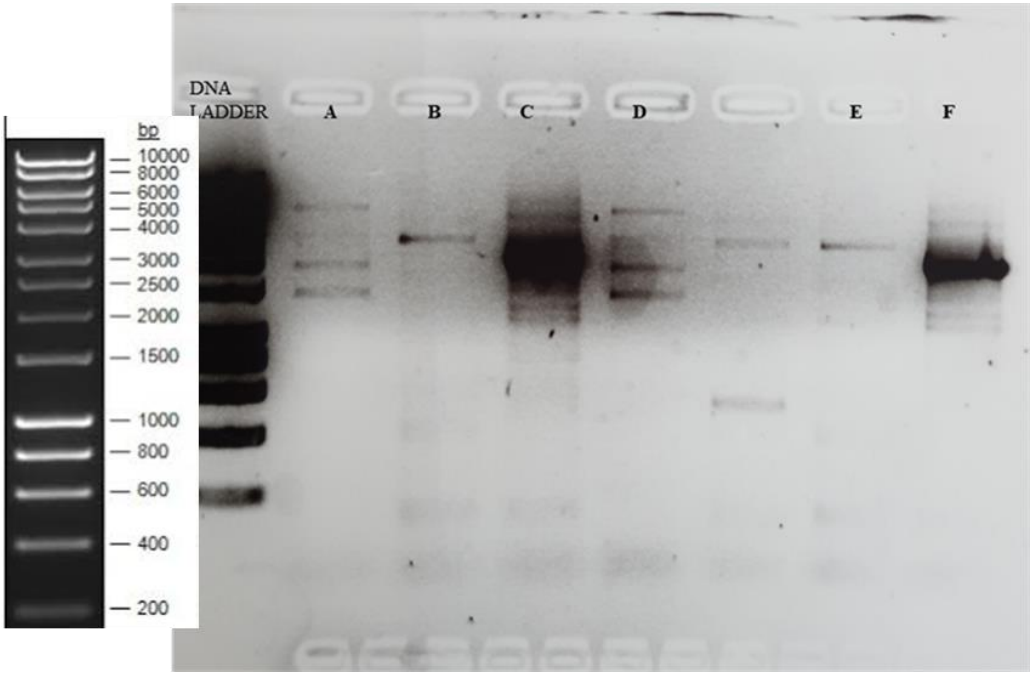


Figure 3.16: 1.5% Gel image showing REP PCR fingerprint patterns obtained from CWW agar isolates and inoculated strains. A) CWW agar *R.opacus* REP pattern B) CWW agar *Micromonospora* REP pattern C) CWW agar *Streptomyces* REP pattern D) *R.opacus* (DSMZ 43205) inoculum REP pattern E) *Micromonospora* inoculum (3CPEGATSA70) REP pattern F) *Streptomyces* (3CPEGATSA66) inoculum REP pattern

3.4. Discussion

This is the first report regarding the description of actinobacteria presence within cassava waste effluent to the best of our knowledge. The characterisation of these isolates ensures their suitability in many contexts for various industrial processes, notably in biodiesel production. Understanding of the properties of

effluent and isolate which can be improved can aid in significantly increasing isolates productivity for commercial use. Meier *et al.* (2020) justified the importance of effluent fortification based on the knowledge on ability of strains to utilise glycerol for development. In their report, authors used residual glycerol as an addictive to cassava wastewater in an anaerobic digestion to produce biohydrogen. The fortification of CWW with glycerol resulted in increased productivity. This suggesting the importance of understanding the conditions that can be amended to improve productivity of strains cultured on CWW for commercial purposes.

Isolates indicated similar morphological characteristics and growth pattern to previously described species of *Micromonospora* (Isolate 1-7) (Hirsch *et al.,* 2004; Trujillo *et al.,* 2006) and *Streptomyces* (Isolate 8) (Rong *et al.,* 2009; Shepherd *et al.,* 2010). Furthermore, isolates were positive for catalase enzymes, indicating their potential to nullify the harmful effect of H_2O_2 produced as a by-product to various metabolic processes (Reiner, 2010). This property is a characteristic of most actinomycetes and has been widely reported positive in similar description reports on *Streptomyces* (Khan *et al.,* 2010) and *Micromonospora* species (Barka *et al.,* 2016; Garcia *et al.,* 2010; Trujillo *et al.,* 2007; Zhang *et al.,* 2014). Isolates were unable to produce mycolic acid which coincides with previous reports on related *Micromonospora* species (Gao *et al.,* 2014; Garcia *et al.,* 2010; Kim *et al.,* 2002) and *Streptomyces* (Taddei *et al.,* 2006). The inability to release H_2S was also determined. Results could suggest that these isolates do not secrete enzymes capable of releasing H_2S from sulphur-containing amino acids (Xia *et al.,* 2017). This variation, particularly in the

Micromonospora species, has been observed (Fang *et al.,* 2015; Huang *et al.,* 2008; Zhang *et al.,* 2014).

Understanding the factors that promote the growth of isolates can aid in their optimisation for biodiesel production from cassava effluent. Growth in different temperature ranges (Table 3.2) suggests that both *Micromonospora* and *Streptomyces* isolates are mainly mesophiles (Brock, 1985). The ability of all isolates to grow within moderate temperature, as seen in Table 3.2 supports their potential cultivation on cassava effluent for biodiesel production. This is because the processing of cassava usually occurs at 30- 35^{o}C. Hence, the effluents from the processing of cassava vary within this temperature range (Oyewole, 1992).

The pH results shown in Table 3.2 infer the rate at which isolates are affected by a pH change. All *Micromonospora* isolates (1-7) grew well at pH tested within this study. This would suggest that isolates can tolerate a wide range of pH in the environment for their growth. *Streptomyces* isolate 8 (3CPEGATSA66) was more selective in the pH in which it can grow. Isolate grew best in neutral to alkaline pH. A similar pattern of pH growth condition was also observed on the type strain *Streptomyces albidoflavus*. The ability of *Micromonospora* to grow in a wide range of pH explains the abundance of *Micromonospora* within different stages of cassava effluent as inferred from culture-dependent and independent study.

All CWW isolates exhibited variation in esculin hydrolysis and lipase activity. Lipase activity was defined by the ability of isolates to release fatty acids which can bind the calcium present in the medium to form insoluble crystals around the point of inoculation (Ramnath *et al.,* 2017). The hydrolysis of esculin a hydroquinone-β-glucopyranoside, casein and starch is a common characteristic

of many actinobacteria. These compounds have been hydrolysed by closely related isolates of *Micromonospora* (Trujillo *et al.*, 2007; Trujillo *et al.*, 2006) and isolates of *Streptomyces* (Lambert & Loria, 1989). The hydrolysis of urea by isolate 5 (3CPEGATSA1B) would suggest this isolate's ability to produce a hydrolytic enzyme capable of attacking the amide linkage in urea to liberate ammonia (Hemraj *et al.*, 2013). Isolate 8 (3CPEGATSA66) was also able to hydrolyse tyrosine, which has been reported as a common characteristic within the species of *Streptomyces* (Goodfellow *et al.*, 2017). Variation in tyrosine hydrolysis as observed in results from this study also coincides with previous report (Trujillo *et al.*, 2006) on closely related *Micromonospora* species. From the hydrolysis results, biochemical properties varied between isolates and the type strain of *Micromonospora* and *Streptomyces*. The degradation test results provide information relating to isolates' suitability to extract viable nutrients from various complex compounds that can contribute to their growth. This is particularly important if these strains are to be applied to other low-cost waste in biofuel production. One such low cost and available waste as an alternative to cassava effluent is the cassava peel. The ability of isolates to degrade starch would suggest the peels of cassava can be optimised to grow these isolates if the cost benefits are higher than cassava effluent.

The enzymatic profiles of isolates were rapidly determined using the API ZYM kit. This system has been previously used to study and understand actinomycetes' enzymatic profiles (Garcia *et al.*, 2010; Kilian, 1978; Trujillo *et al.*, 2007). All isolates tested positive for Naphthol-AS-BI-phosphohydrolase activity. These isolates, with the exception of isolate 8 (3CPEGATSA66), also indicated α-Chymotrypsin activity. Chymotrypsin cleaves amides, esters and

peptides. This cleavage is preferentially carried out at the carboxyl groups of the hydrophobic amino acids (Kilian, 1978). Interestingly this serine protease is usually considered an animal enzyme but has been found to be present in actinomycetes of the genera *Streptomyces* (Khan *et al.*, 2010; Kilian, 1978) and *Micromonospora* (Trujillo *et al.,* 2007). Naphthol-AS-BI-phosphohydrolase production has also been previously reported in previously described species of *Micromonospora* (Garcia *et al.,* 2010). Isolate 4 (3CPEGATSA1) showed positive enzyme activity to 12 of the 20 enzymes tested. Amongst which the observation of isolates positivity to cysteine, valine and leucine arylamidase indicates the proteolytic potential of isolate compared to other isolates (Acosta-Martínez & Tabatabai, 2001; Kilian, 1978). α-Fucosidase, α-Mannosidase, β-Glucosidase and N-Acetyl-β-glucosaminidase activities were not observed in all tested isolates. Reports on *Micromonospora* have indicated the presence of some of these enzymes (Garcia *et al.,* 2010; Trujillo *et al.,* 2007). Equally, β-Glucuronidase was not produced by all isolates. This enzyme has been observed as a character exclusive to *Escherichia coli* and some *Shigella* species within *Enterobacteriaceae* (Kilian, 1978).

Isolates were tested for resistance and susceptibility to different ranges of antibiotics. The formation of a circular uniform zone of inhibition around the test antibiotics indicated resistance to test isolates (Reller *et al.*, 2009). This information is particularly useful to control other unwanted bacteria which may compete with isolates for nutrients during biodiesel production. Isolate indicated variation in response to different antibiotics. From the result, Neomycin and Ciprofloxacin were the most effective against the majority of isolates tested. Isolate 2 (3CWEGATSA52) and 5 (3CPEGATSA1B) did, however, indicate

resistance to all antibiotics tested. Their resistance to the antibiotics, as seen in Table 3.6 could be due to the complex internal mechanisms within the isolates. Isolates could limit these antibiotics' uptake, modify antibiotics target or inactivate the specific antibiotic (Reygaert, 2018).

The lytic action of lysozyme on gram-positive bacteria has been described (Salton, 1958). Lysozyme breaks down the cell wall of some aerobic actinomycetes. The sensitivity of these actinomycetes to lysozyme was reported (Helmke & Weyland, 1984; Muricy *et al.*, 2014). For all *Micromonospora* isolates, no growth was observed when lysozyme was added in media (Fig.3.11). There are few reports on the action of lysozyme on *Micromonospora.* Horan and Brodshy (1986) inferred the sensitivity of *Micromonospora rosaria* to lysozyme. Isolate 8 (3CPEGATSA66), however, developed in the presence of lysozyme. This was in contrast with its type strain *S.albidoflavus* which was susceptible to lysozyme. The resistance of some *Streptomyces* to lysozyme has been previously reported (Sohler *et al.*, 1958). In their report, it was inferred that the hydrolysed hexosamine and low amino sugar were responsible for the different responses to lysozyme. Lysozyme sensitive isolates in their report contained acetylated hexosamine within their cell walls, whereas this was not observed in resistant isolates. Interestingly, our CWW isolate 8 (3CPEGATSA66) when tested with the API ZYM suggested the absence of N-Acetyl-β-glucosaminidase activity. The resistance to lysozyme based on the presence of N-Acetyl-β-glucosaminidase in this report is not conclusive. This is because the variation in this activity had no effect on the responsiveness of *Micromonospora* isolates to lysozyme.

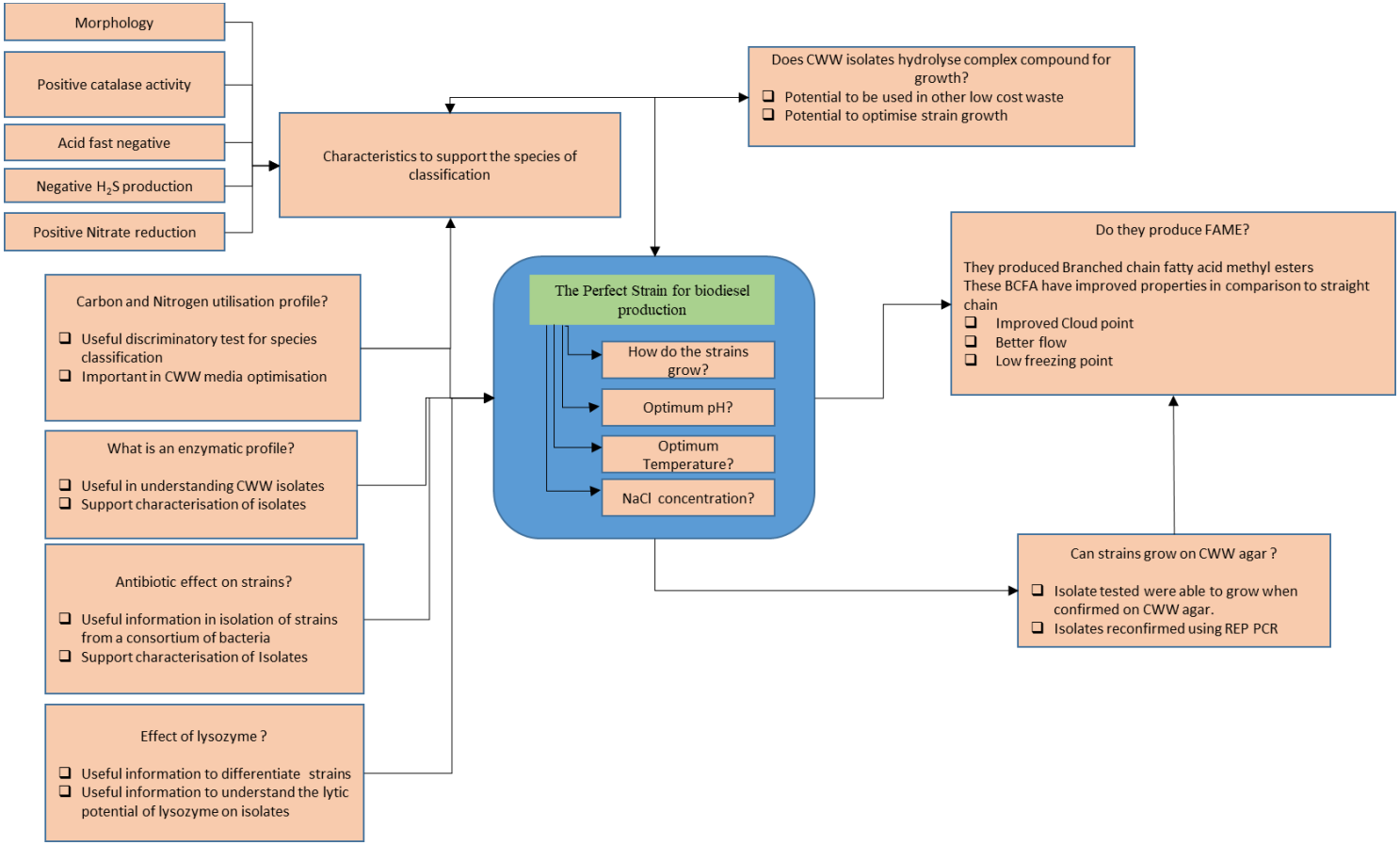
The FAME profile of all isolates was analysed to deduce the potential of fatty acid esters. The properties of individual fatty acid esters (Gopinath *et al.*, 2015) such as the branching of the chains, degree of unsaturation, and chain length attribute these isolates' biodiesel potential (Gopinath *et al.*, 2015). From Table 3.8, it is evident that all CWW isolates of *Micromonospora* and *Streptomyces,* which were examined, are characterised by a significant percentage of branched-chain fatty acids (BCFAs). This property has also been observed in isolates belonging to the actinobacteria phylum (Zelles, 1999). All isolates had a significant percentage of their fatty esters composed of saturated fatty acyl chain with the methyl group branched at the iso or anteiso position. The biodiesel properties of BCFAs that make them vital to biofuels' practical use have been extensively discussed (Knothe, 2005, 2009; Knothe *et al.*, 2005). These fuels have improved properties including lower cloud point, better cold flow and lower freezing point than biofuels comprising straight-chain fatty acids. This, therefore, suggests the potential of these isolates to produce viable biodiesel.

Based on the growth result and REP confirmation of fingerprint pattern, the ability of isolates from *Micromonospora, Streptomyces* and *R.opacus* to grow on cassava effluent was confirmed in this study. This would suggest that cassava effluent contains nutrient requirements that can support the growth of these isolates. Interesting although *Micrococcus* was previously isolated from this effluent, there was no growth on reconfirmation on CWW Agar. The previous result from our culture-dependent and independent analysis suggests the presence of this genus within the consortium of cassava effluent. Hence, it is more likely that the biases associated with culturing of isolates could give rise to the negative result obtained (Salmonová & Bunešová, 2017). Furthermore,

the ability of isolate to grow might slow down based on the available nutrient present within the effluent media. The pH or temperature of incubation could also impact on the ability of isolates to grow (Stewart, 2012; Vartoukian *et al.*, 2010).

From the assessment of the properties of isolates within this study, it can be concluded that *Micromonospora* and *Streptomyces* isolate previously obtained within cassava effluent can play a role in biodiesel production from this effluent. The dynamics of their cultivation and the analysis of their FAME profiles provide more evidence to this claim. The alteration of carbon and nitrogen ratio within effluent is an aspect that can be further explored to improve the lipid productivity of isolates (Kurosawa *et al.*, 2010). Interestingly, the ability of *R.opacus* to grow on this effluent infers the suitability of effluent to support the growth of non-indigenous actinomycetes equally. *R.opacus* has been reported to be quality lipids producers and have been suggested as useful actinomycetes in biodiesel production (Holder *et al.*, 2011; Kurosawa *et al.*, 2010). This creates a similar perspective, which can also be exploited in the utilisation of cassava effluent for biofuel production. The utilisation of cassava effluent for growth of these actinomycetes in biodiesel production can therefore reduce the negative environmental impacts of its disposal.

3.5. Flowchart summarising physiological characteristics



3.6. References

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CHAPTER 4: Analysis of TAG biosynthesis potential from novel cassava wastewater isolates

4.1. Introduction

Bacteria utilise different mechanisms to thrive in fluctuating environmental conditions. Accumulation of storage lipids such as triacylglycerol (TAG) is a key mechanism utilised by some prokaryotes to adapt and tolerate the main stresses in an environment. This characteristic was initially considered a defining eukaryotic feature, since then a few prokaryotes, mostly actinomycetes, have been identified as capable of making TAG – including some, but not all, *Streptomyces* (Alvarez & Steinbüchel, 2002). These triglycerides are water-insoluble triesters of glycerol bound with three fatty acid molecules (Shields-Menard *et al.*, 2018). TAGs are excellent energy stores due to higher energy yields when oxidised compared to other storage compounds such as polyhydroxyalkanoates (PHA) and carbohydrates (Alvarez & Steinbüchel, 2002). The oxidation of these triglycerides in desiccation conditions can also provide cells with a source of metabolic water.

Further to their excellent energy reserve, their highly hydrophobic properties allow large accumulation of TAGs in cells without a change in the cytoplasm's osmolarity. TAGs also play other important roles within the cells. They are effective precursors for the synthesis of cell envelopes and membranes. They are also crucial in the maintenance of redox state of the cell and serve as a form of detoxification of unusual fatty acids or removal of excess free fatty acids (Alvarez *et al.*, 2017).

The accumulation of TAGs occurs after phospholipids synthesis during the stationary phase of growth or in response to external stress factors. This accumulation of TAGs can be related to the presence of an excessive amount of carbon source with a limited nitrogen source within a growth environment

(Alvarez & Steinbüchel, 2002; Alvarez *et al.*, 1997; Packter & Olukoshi, 1995; Ratledge, 2004). Therefore, the amount of TAG synthesised depends on the condition of strains and the nutrient sources for growth.

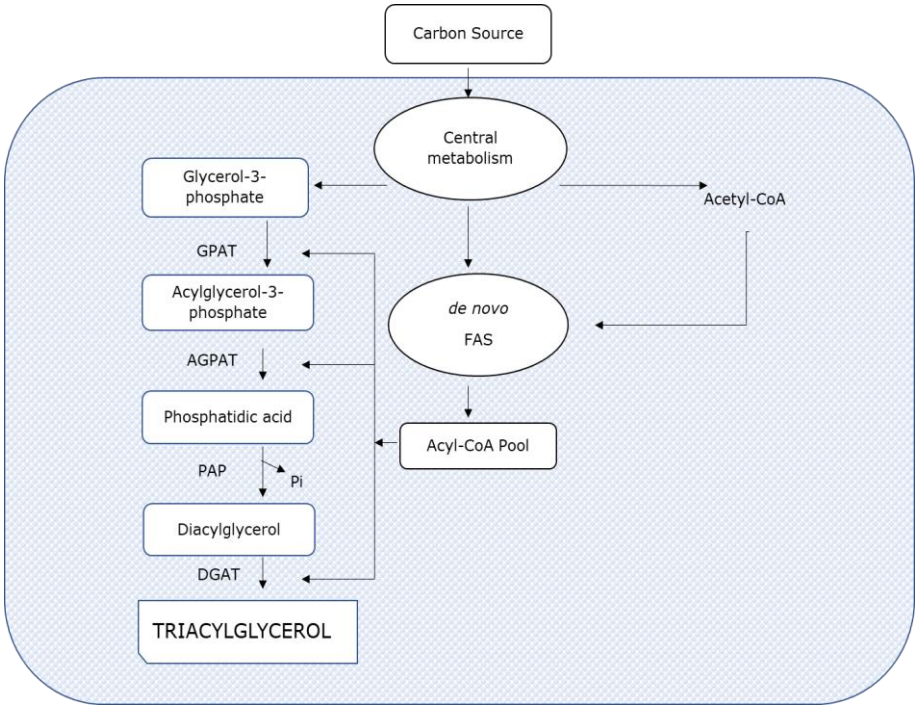


Figure 4.1: Schematic TAG metabolic pathway in bacteria. FAS (Fatty acid synthesis. DGAT (diacylglycerol acyltransferase), GPAT (glycerol-3-phosphate O-acyltransferase), AGPAT (acyl-glycerol-3-phosphate acyltransferase), PAP (phosphatidic acid phosphatase). Adapted from (Alvarez *et al.*, 2017)

The synthesis of TAG occurs via the Kennedy pathway (Fig. 4.1). The fatty acid esterified to the glycerol backbone is obtained from the *de novo* Fatty acid synthesis (FAS) as described by (White *et al.*, 2005). Usually, there are two basic types of FAS in nature. These FAS based on protein architecture are classed as the FAS type 1 and FAS type 2. The FAS1 utilises a single gene to produce a polypeptide that possesses all the necessary reaction centres involved in synthesising fatty acid. FAS 2, more commonly found in oleaginous bacteria

involves a dissociation system in which different genes encode each FAS component (Gago *et al.*, 2011). Fig 4.2 illustrates the bacterial FAS pathway.

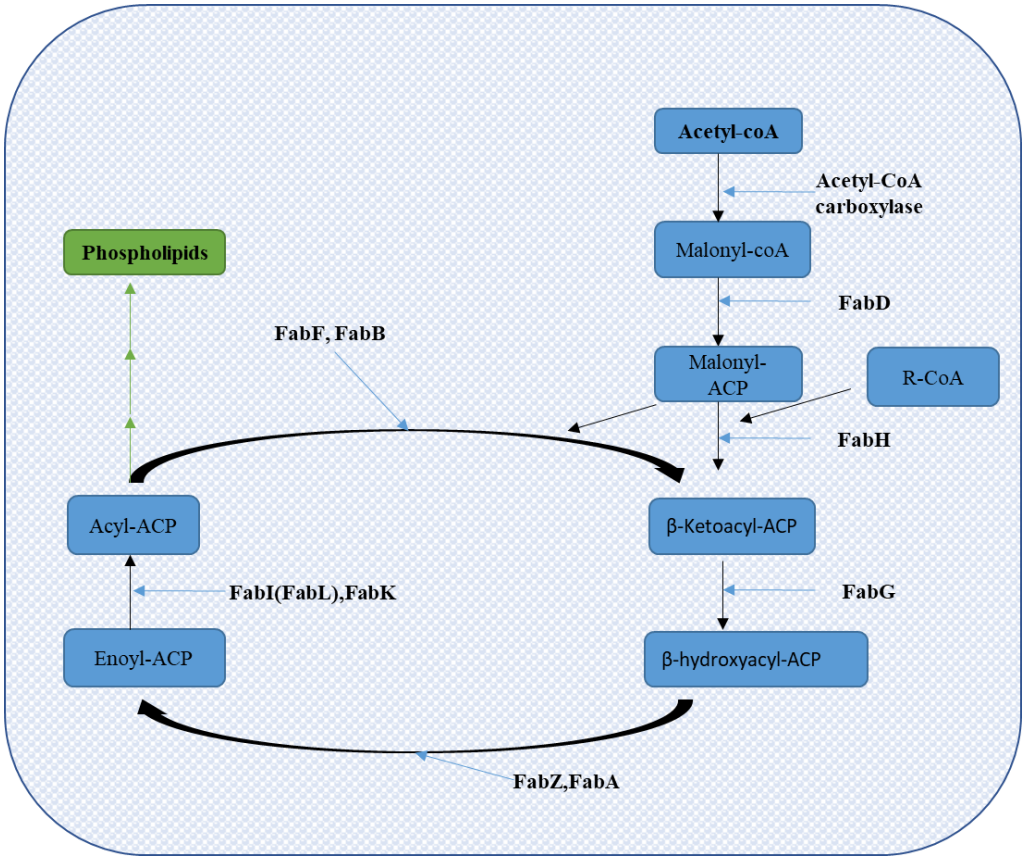


Figure 4.2: Schematic FAS type 2 metabolic pathway in bacteria. Abbreviations: malonyl-CoA:ACP transacylase (FabD), β-Ketoacyl-ACP synthase III (FabH), β-Ketoacyl-ACP reductase (FabG), enoyl-ACP reductase (FabI), β-hydroxyacyl-ACP dehydratases (FabZ) β-Ketoacyl-acyl carrier protein synthase (FabF). Adapted from (Freiberg *et al.*, 2004)

Within the FAS2 architecture, acetyl coenzyme A (CoA) carboxylase (ACC) initiates fatty acid synthesis. This enzyme catalyses the conversion of acetyl-CoA to malonyl-CoA. This step is the first committed step within the fatty acid pathway. Following this step, the malonyl group of malonyl-CoA is transferred to ACP by malonyl-CoA: ACP transacylase (FabD). β-Ketoacyl-ACP synthase III (FabH) catalyses the first condensation step in the pathway. This reaction

utilises malonyl-ACP as the donor and acetyl-CoA as the primer to form acetoacetyl-ACP. The elongation cycle contains four core enzymatic processes that progressively attach two carbon to units to the elongating acyl chain attached to an ACP. Acetoacetyl-ACP is converted by β-ketoacyl-ACP reductase (FabG) into β-hydroxyacylACP. The resulting β-hydroxyacylACP intermediate is further dehydrated by the β-hydroxyacyl-ACP dehydratases (FabZ). Finally, an NADH dependent enoyl-ACP reductase (FabI) reduces the enoyl-ACP produced to complete the cycle. The FabF or FabB condensing enzyme initiates subsequent elongation rounds. This enzyme is responsible for extending fatty acid chain by two carbons via the condensation of the growing acyl-ACP with malonyl-ACP (Revill *et al.*, 2001; Singh & Reynolds, 2015; White *et al.*, 2005).

Streptomyces is an example of an oleaginous bacteria that shows significant potential by utilising the FAS 2 system to synthesise fatty acid for TAG production (Gago *et al.*, 2011; Revill *et al.*, 2001). *Streptomyces*' FAS type 2 metabolic pathway results in the production of branched-chain fatty acids with only a minor proportion of straight-chain fatty acid (Gago *et al.*, 2011). FabH (β-ketoacyl synthase III) catalyses the first step in the pathway. This initial step involves the carboxylative condensation of malonyl-ACP with an acyl-CoA primer to produce 3-ketoacyl-ACP. The acyl CoA primer required for the synthesis of the branched-chain or straight-chain fatty acid differ. The synthesis of straight-chain fatty acid commonly involves propionyl-CoA and acetyl-CoA primers (for odd and even C chain length) while methyl butyryl-CoA or an isobutyryl-CoA primer is essential in branched-chain fatty acids. The next step involves an NADPH-dependent FabG of 3-ketoacyl-ACP to produce 3-

hydroxyacyl-ACP. FabA then dehydrates the 3-hydroxyacyl-ACP to form an enoyl-ACP. The cycle is completed by the action of NADPH dependent FabI, which catalyses a reduction yielding the corresponding acyl-ACP. Subsequently in the chain elongation, FabF rather than FabH catalyses the condensation step with the acyl-ACP. Malonyl-CoA provides the malonyl-ACP required for each elongation step through the actions of a malonyl CoA:ACP transacylase (encoded by fabD) (Singh & Reynolds, 2015). To complete *Streptomyces* TAG synthesis, acyl-CoA generated from the FAS2 architecture becomes incorporated with glycerol to produce TAG (Fig. 4.1) using the Kennedy pathway (Arabolaza *et al.*, 2008).

The different genes and enzymes responsible for producing TAG have been extensively studied, resulting in several DGAT enzymes characterised in actinobacteria. The presence of this DGAT enzyme has been used to distinguish bacteria strains capable of producing TAG. Since the first report by Kalscheuer and Steinbüchel (2003) of WS/DGAT in *Acinetobacter baylyi* ADP1, which resulted in wax esters and TAG production, various other DGATs have been identified and characterised. The presence of these DGAT/ATF enzymes have been reported in TAG-synthesising bacteria, such as *Rhodococcus, Streptomyces, Micromonospora, Dietzia, Gordonia,* and *Marinobacter* (Alvarez *et al.*, 2008; Alvarez *et al.*, 2017; Arabolaza *et al.*, 2008; Indest *et al.*, 2015; White *et al.*, 2005)

DGAT/ATF enzymes interestingly differ in gene sequence to eukaryotic acyltransferases (Kalscheuer & Steinbüchel, 2003; Wältermann *et al.*, 2007). This class of TAG -synthesising enzymes is abundant equally as isoenzymes within TAG-accumulating actinobacteria (Alvarez *et al.*, 2017). These

isoenzymes are species-dependent, and this confers various regulatory and physiological flexibility to the synthesis of lipids within the bacteria. The abundance of DGAT/ATF isoenzymes can induce these genera's ability to synthesise TAG from a host of intermediates from the cell network under different environmental conditions. In addition to the presence of DGAT/ATF enzymes, oleaginous bacteria's ability to accumulate TAG also depends on other important factors. The accumulation of this lipid depends on the presence of special metabolic and regulatory frameworks. The frameworks encompass various genes that encode for enzymes involved in central metabolism, the synthesis of fatty acid and the generation of reducing equivalents. The synthesis also depends on enzymes pertinent to assembly, stabilisation, and structure of lipid inclusion bodies. MacEachran *et al.* (2010) identified and characterized the TadA protein in *Rhodococcus opacus* PD630, which plays a key structural role in lipid body formation.

Asides from the Kennedy pathway, other routes of TAG synthesis in some bacteria may be available. Phospholipid: diacylglycerol acyltransferase (PDAT) catalyses the transfer of fatty acids from the sn-2 position of phospholipid to DAG at the sn-3 position. This transfer yields sn-1-lysophospholipid and TAG. Although this pathway has been documented widely in higher plants and yeast, the presence in bacteria of (PDAT) like enzymes has only been suggested to be present in *Streptomyces* (Arabolaza *et al.*, 2008).

Different procedures are available to separate the different component of lipids to identify TAG. Thin layer chromatography (TLC) is one such method that has been used to segregate the components of lipids for their identification. TLC involves a quick, inexpensive and simple procedure that can serve as an

alternative to more sophisticated instruments to identify and quantify lipids in bacteria (Arabolaza *et al.*, 2008). Another such procedure for TAG detection involves the direct screening of bacteria for accumulation of TAG using the lipophilic dye (Nile red). This procedure was previously utilised in the staining of colonies and differentiated between TAG accumulating sand non-accumulating strains (Spiekermann *et al.*, 1999).

In this chapter, TAG synthesis and accumulation novel isolates from cassava wastewater (CWW) was investigated. Furthermore, the genes involved in the accumulation of this lipid were explored. Thin layer chromatography, the viable colony staining method based on Nile red to detect lipid inclusions and a PCR approach to amplify PAP and ATF gene based on conserved consensus sequences within strains was adopted. These methods have been used previously to investigate storage lipids in different oleaginous strains of bacteria (Christie, 2011; Hernández *et al.*, 2013; Minnikin *et al.*, 1977; Spiekermann *et al.*, 1999). The report in this chapter is the first report exploring the potential of TAG accumulation from CWW isolates. Furthermore, this report also suggests the presence of PAP enzymes, *lppα* (SCO1102) and *lppβ* (SCO1753) genes in *Streptomyces albidoflavus* and a *Streptomyces* CWW isolate, for TAG accumulation. The potential of alternative TAG pathways within the metabolic network of *Streptomyces* and *Micromonospora* within this study was also inferred.

4.2. Materials and methods

4.2.1. Bacterial strains used in this study

Isolates from cassava effluent belonging to the genera *Micromonospora*; isolate 2CPEGATSA4 (Sample 1), 3CPEGATSA1B (Sample 5) and *Streptomyces*;

isolate 3CPEGATSA66 (Sample 8) were explored for their TAG accumulating potential. Representative type strains of *Micromonospora* (*Micromonospora endolithica* DSM 44398 represented as Sample 12) and *Streptomyces* (*Streptomyces albidoflavus* DSM 41815 sample 13) was used in addition to the CWW strains as controls. These isolates were chosen as a representative of the genera *Micromonospora* and *Streptomyces* being investigated for their potential to accumulate TAG. All strains were reactivated on Glucose yeast malt broth (GYM) for 5 days at 28ºC.

4.2.2. TAG pathway mapping using KEGG databases

In this study, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to perform the pathway analysis (http://www.genome.jp/kegg/). Herein, the KEGG database was explored for the biochemical pathway diagrams involved in the synthesis of TAG within *Micromonospora* and *Streptomyces*' networks. *Streptomyces albidoflavus* and *Micromonospora aurantiaca* within KEGG were used as reference species due to their closeness to CWW isolates.

4.2.3. DNA extraction of CWW isolate and investigation of ATF gene sequence.

DNA was extracted from three-day-old biomass of purified single colonies grown in TSA broth from all study isolates and type strains. Briefly, DNA extraction was carried out by following the protocol of (Legras & Karst, 2003) (see section 2.2.2.2). Following DNA extraction from CWW, three oligonucleotide primers were designed from the consensus sequence obtained from the aligned DGAT/ATF gene of *Streptomyces* (MEGA 7) available on the NCBI website (http://www.ncbi.nlm.nih.gov). These primers were designed to

flank the conserved HHxxxDG motif using primer 3 software (https://primer3.ut.ee/) (See appendix). Table 4.1 shows the primers designed.

Table 4.1: Primers for DGAT/Atf-related genes in *Streptomyces albidoflavus*

Forward primers	Reverse primers	Expected band size
Atf_1F 5'-GGTTTGATCTGCTGGTGACC-3'	Atf_1R 5'-GTTTCCACTTCTTCCGCCAC-3'	226
Atf_2F 5'-GGGGAGTTGATGGAGAGGC-3'	Atf_2R 5'AAATATCCCCGCCCCTATCG-3'	177
TRP F: 5'-CTCATCGGTGACCTCTGCAT-3'	TRP R: 5'-TGTAGCTGGCGGTCTTGTAC-3'	214

PCR amplification was carried out in a Techne TC-512 Thermal cycler on DNA samples of strain 1, 5, 8, 12 and 13. In all tubes, a 50 µl reaction mixture containing 5 µl of 10X PCR Buffer (containing 15mM MgCl2), 1 µl of 200 µM dNTP, 2 µl each from 20 µM of both primers, 1 µl of DNA template, 38.75 µl of nuclease-free water and 0.25 µl of 2.5unit *Taq* DNA polymerase. The polymerase chain reaction was performed with initial denaturation for 5 minutes at 95^{o}C followed by 30 cycles of 45s denaturation at 95^{o}C, 60s annealing at 55^{o}C and 60s extension at 72^{o}C. A final elongation step was carried out at 72^{o}C for 5 minutes. Amplified PCR product was separated on a 1.5% agarose gel electrophoresis containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1x TAE buffer. The gel was scanned in a Gel Doc 1000 apparatus (UVP, Gel Doc-it imaging system) Amplified PCR product was purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

4.2.4. PCR amplification of phosphatidic acid phosphatase (PAP) gene, Lppα (Sco1102) and Lppβ (Sco1753) in CWW isolate

Two oligonucleotide primer pairs were designed from the Blast of Sco1102 against the *Streptomyces albidoflavus* taxid in Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Alignment and visualisation of these hits were used to identify diverse proteins as PAP2-related and multiple enzymes present in *S.albidoflavus*. Alignment of base sequences obtained was used to design primer, as seen in Table 4.2 below.

Table 4.2: Primers for Sco1102-related genes in *Streptomyces albidoflavus* whole genomes

Forward primers	Reverse primers	Expected band size
3F 5'GACCATCGTGACCATGGCA-3'	242 R 5'-CACACCCAGTCGCTGAACA-3'	239
2F 5'TGACCATCGTGACCATGGC-3'	241R 5'-ACACCCAGTCGCTGAACAC-3'	239

A blast of Sco1753 against *Streptomyces albidoflavus* was used to design four oligonucleotide primer pair. The nucleotide sequences corresponding to the proteins in the Sco1753 cluster were identified in Identical proteins, downloaded and aligned. Primers were designed with Primer 3 software as seen in table 4.3 below.

Table 4.3: Primers for Sco1753-related genes in *Streptomyces albidoflavus* whole genomes.

Forward primers	Reverse primers	Expected band size
189F 5'GGACTGGCAGCTCATGTTCT-3'	483R 5' CGGAAATATATCGCCGCCCT-3'	294
189F5'GGACTGGCAGCTCATGTTCT-3'	629R5'-CAGTGCGTACCGAGGTAGAC-3'	440
189F 5'GGACTGGCAGCTCATGTTCT-3'	706R 5'-TGATCATCGGCTCGAACCAG-3'	517
464F 5'AGGGCGGCGATATATTTCCG-3'	706R 5'-TGATCATCGGCTCGAACCAG-3'	242

PCR amplification was carried out in a Techne TC-512 Thermal cycler on DNA sample of *Streptomyces* CWW isolate 8 and *Streptomyces albidoflavus*. In all tubes, a 50 µl reaction mixture containing 5 µl of 10X PCR Buffer (containing 15mM MgCl2), 1 µl of 200 µM dNTP, 2 µl each from 20 µM of both primers, 1 µl of DNA template, 38.75 µl of nuclease-free water and 0.25 µl of 2.5unit *Taq* DNA polymerase. A gradient polymerase chain reaction was performed with initial denaturation for 5 minutes at 95^{o}C followed by 30 cycles of 60s denaturation at 95^{o}C. A gradient annealing temperature was set at 55^{o}C,57^{o}C,60 ^{o}C,62^{o}C and 65^{o}C for 60s annealing and 60s extension at 72^{o}C. A final elongation step was carried out at 72^{o}C for 10 minutes. Amplified PCR product was checked via gel electrophoresis before purification of the amplicon.

4.2.5. Lipid analysis using TLC for TAG accumulation

The protocol used for the TLC analysis to determine the presence of TAG was developed and optimised from Henderson and Tocher (1992), Santiago and Strobel (2013) Touchstone (1995) and Amidzhin and Nikolova-Damyanova (1988). Lipid extraction was carried out from the freeze-dried biomass of all

strains. Strains were initially grown on the basal medium GYM described by Williams *et al.* (1983) which contained 4g Glucose, 4g Yeast extract, 10g Malt extract, 2g $CaCO_3$, and 1000ml distilled water. The pH was adjusted to 7.2 and the media was autoclaved. Strains were harvested after 3 days, frozen and freeze-dried before the extraction of lipids. Lipid from 3 mg of each sample was extracted with 0.3ml of chloroform: methanol (2:1) overnight at room temperature. The supernatants were separated and pipetted off into clean vials. Lipids were also extracted twice with 0.5ml chloroform: methanol (2:1) from 10 mg of biomass for 4 hours with gentle shaking at regular intervals of 10 minutes. Lipids were redissolved in 30 µl solvent (chloroform-methanol (2:1)). 5 µl of redissolved lipid was spotted, using a pipette tip, onto a silica plate preloaded with a solution of 10% $(NH_4)2SO_4$ before detection by charring at 200^oC for 20 minutes (At 200^oC $(NH_4)2SO_4$ decomposes to release H_2SO_4). The TLC loading standards, T1, T2, T3, T4 and T5 were prepared from olive oil (which is primarily triglyceride) at volumes 5, 10, 20, 50 and 100 µg/µl respectively. Aliquots of 1 µl from 5 µl preparation of each sample from the 10 mg extraction (redissolved in 30 µl chloroform: methanol (2:1)), were spotted onto a silica plate. Silica plate was pre-soaked in 10% $(NH_4)2SO_4$ and dried together with standard triglyceride, and this was run in hexane: diethyl ether: acetic acid. Additional 10 µl of the sample was spotted to run in dual solvents chloroform: methanol: water (60:30:5) followed by hexane: diethyl ether: acetic acid (80:20:1) using silica TLC plates without preloading with 10% $(NH_4)2SO_4$ as this seemed to interfere with running in this system when tested with standard triglyceride. The relatively polar pigments, which remained at the origin in hexane: diethyl ether: acetic acid ran in chloroform: methanol: water. The plates

were run to 5 cm (halfway) in chloroform: methanol: water and after thorough drying the plate was run, in the same direction, in hexane: diethyl ether: acetic acid and dried. After drying the plate was then sprayed to saturation with 10% $(NH_4)2SO_4$ and the excess allowed to run off. The plate was then dried initially at room temperature and then at 50^oC before charring for 10 minutes at 200^oC. The remaining sample was dried and redissolved in 20 µl hexane: diethyl ether: acetic acid (80:20:5) and 10 µl spotted onto a silica plate and run in hexane: diethyl ether: acetic acid.

4.2.6. Viable colony staining using Nile red for direct screening of bacteria

Direct screening of strains for their ability to accumulate TAG was performed via a viable colony staining method involving the use of Nile red. The ability of isolates to accumulate TAG was inferred based on cell fluorescence when cultivated in the presence of Nile red. *Micromonospora*; isolate 2CPEGATSA4 (Sample1), and *Streptomyces*; isolate 3CPEGATSA66 (Sample 8) previously isolated from CWW were investigated along with representative type strains of *Micromonospora endolithica* (Sample 12) and *Streptomyces albidoflavus* (sample 13). The protocol was optimised as described by Spiekermann *et al.* (1999). The analysis was carried out by adding 0.002 volume of a 0.25mg solution of Nile red (Sigma, St. Louis, Mo. USA) per ml dimethylsulfoxide (DMSO) to a sterilised MSM media to give a final concentration of 0.5 µg dye/ml media. MSM media was prepared by dissolving $Na_2HPO_4.12H_2O$- 9g , KH_2PO4 –1.5g, $MgSO_4.7\ H_2O$ -0.2g, NH_4Cl- 0.25 g, $CaCl_2.2\ H_2O$ -0.02g, Fe(III)NH_4-citrate - 0.0012g, Agar 15ml in 1000ml dH_20. The medium was autoclaved before trace element solution, 0.5% (w/v) carbon source and 0.5 µg dye/ml Nile red was added. Carbon sources used were sucrose, maltose and

fructose. Trace element solution contained per litre dH_20; $ZnSO_4.7H_2O$- 100 mg, $MnCl_2.4H_2O$ - 30 mg, H_3BO_3 - 300 mg, $CoCl_2.6H_2O$ -200 mg, $CuCl_2.2H_2O$ - 10 mg, $NiCl_2.6H_2O$ - 20 mg, $Na_2MoO_4.2H_2O$ 900 mg and N_2SeO_3 20 mg. Strains were streaked onto agar plates and incubated for 5 days at 28°C. Following the growth and development of colonies, agar plates were exposed to ultraviolet light to detect TAG accumulation (Spiekermann *et al.*, 1999). *Rhodococcus opacus* was used as the positive control as these strains accumulate a large amount of triacylglycerol (Spiekermann *et al.*, 1999).

4.3. Result

4.3.1. TAG pathway mapping using KEGG databases

Micromonospora aurantica and *Streptomyces albidoflavus* were used as the representative strains in the preliminary search of the KEGG database for the TAG metabolic pathway. The KEGG pathway indicated the absence of key enzyme glycerol-3-phosphate O-acyltransferase [EC:2.3.1.15], phosphatidate phosphatase [EC:3.1.3.4], diacylglycerol diphosphate phosphatase/phosphatidate phosphatase [EC:3.1.3.81 3.1.3.4],phospholipid:diacylglycerol acyltransferase [EC:2.3.1.158] and diacylglycerol O-acyltransferase 1 [EC:2.3.1.20 2.3.1.75 2.3.1.76] within the pathways of the representative species. Fig.4.3 and Fig.4.4 shows respectively the TAG pathway for *Streptomyces* and *Micromonospora aurantiaca* obtained from the KEGG database.

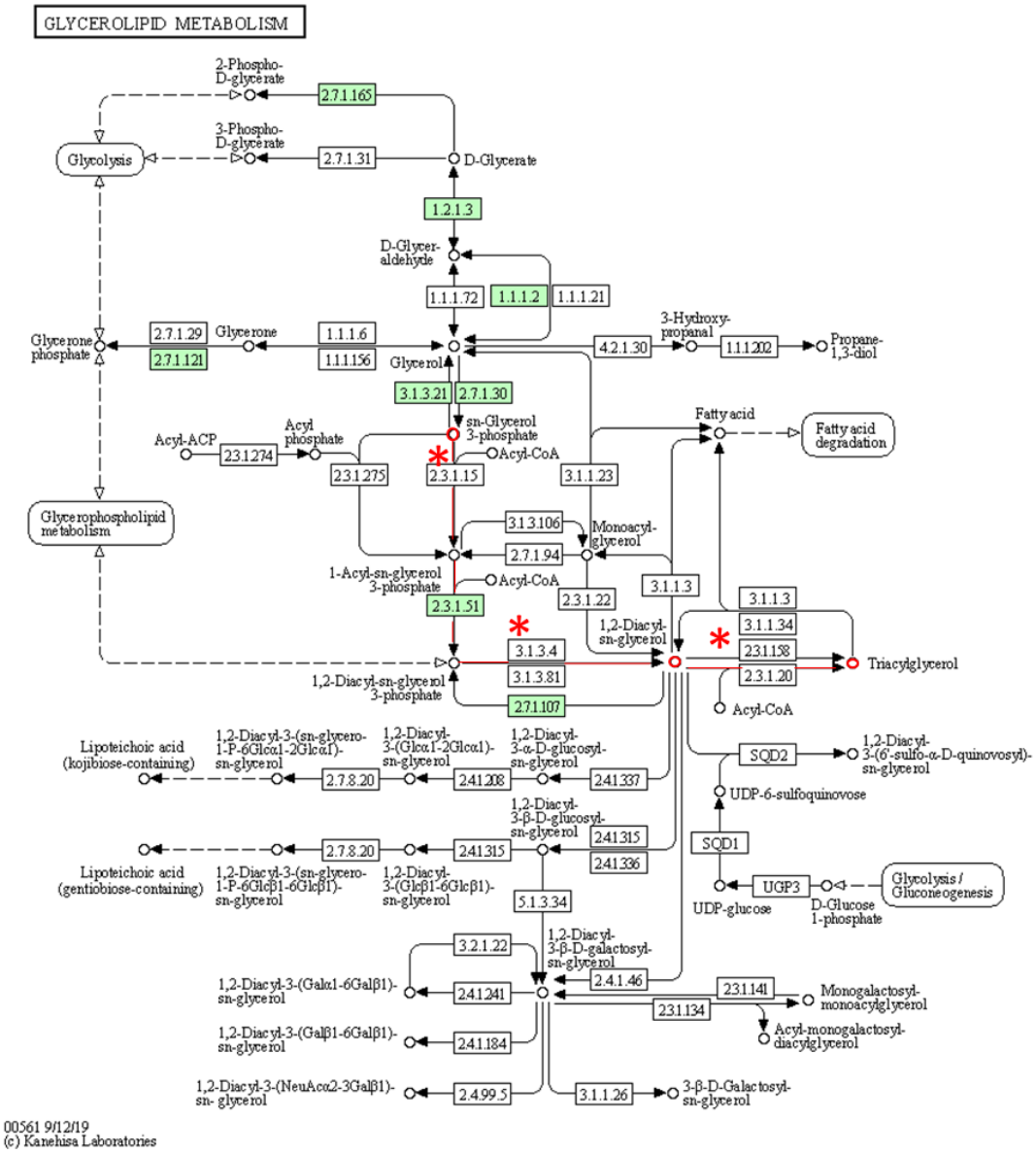


Figure 4.3: Representative glycolipid pathway for *Streptomyces albidoflavus* explored from Kyoto Encyclopedia of Genes and Genomes (KEGG). Key pathway highlighted in Red colour. *Missing enzymes responsible for TAG synthesis. glycerol-3-phosphate O-acyltransferase 1/2 [EC:2.3.1.15] phosphatidate phosphatase [EC:3.1.3.4], diacylglycerol diphosphate phosphatase / phosphatidate phosphatase [EC:3.1.3.81 3.1.3.4] phospholipid:diacylglycerol acyltransferase [EC:2.3.1.158], diacylglycerol O-acyltransferase 1 [EC:2.3.1.20 2.3.1.75 2.3.1.76]. Colored green boxes suggest` available information is present for the gene

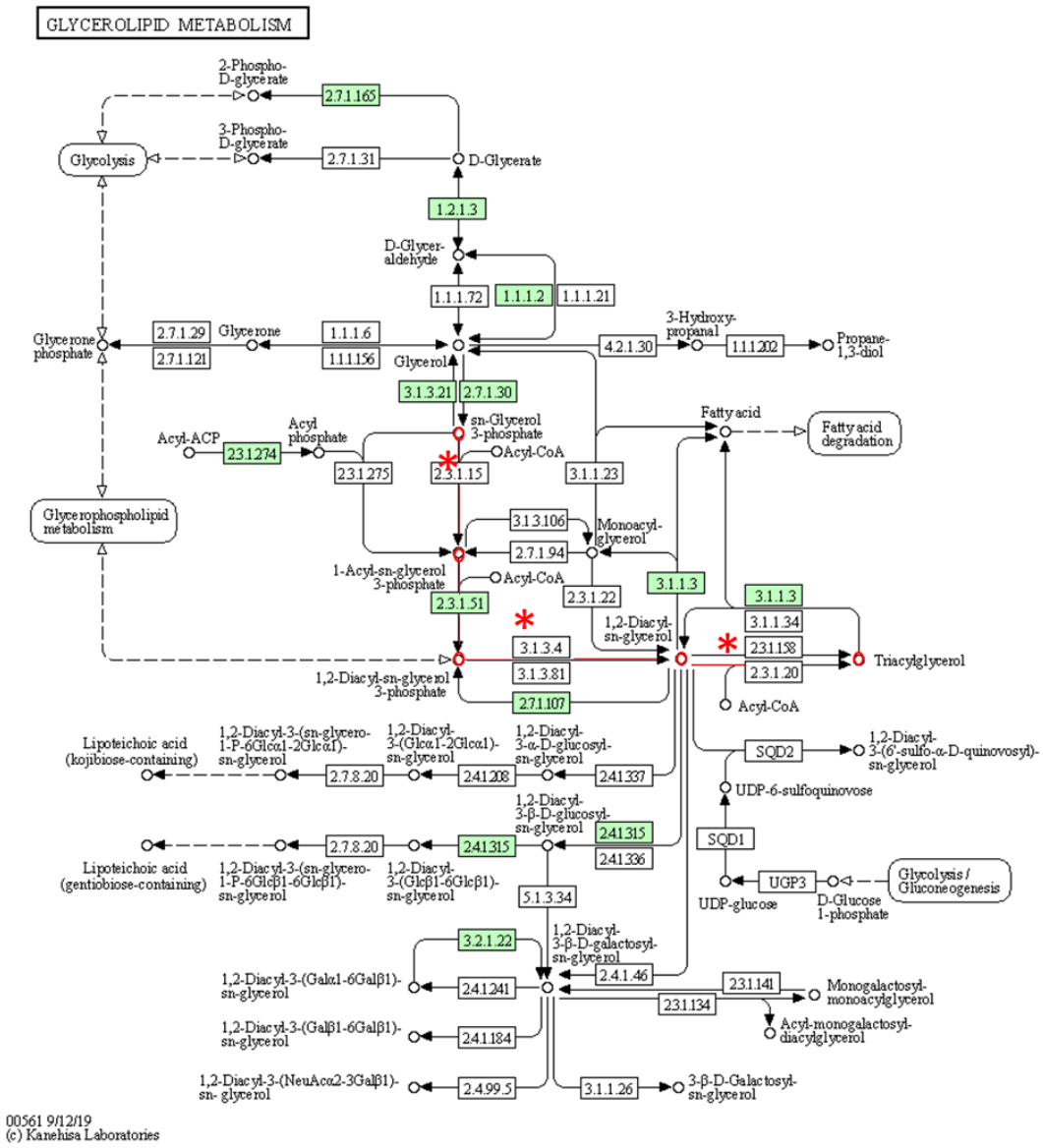


Figure 4.4: Representative glycolipid pathway for *Micromonospora aurantiaca* explored from Kyoto Encyclopedia of Genes and Genomes (KEGG). Key pathway highlighted in red colour. *Missing enzymes responsible for TAG synthesis. glycerol-3-phosphate O-acyltransferase 1/2 [EC:2.3.1.15] phosphatidate phosphatase [EC:3.1.3.4], diacylglycerol diphosphate phosphatase / phosphatidate phosphatase [EC:3.1.3.81 3.1.3.4] phospholipid:diacylglycerol acyltransferase [EC:2.3.1.158], diacylglycerol O-acyltransferase 1 [EC:2.3.1.20 2.3.1.75 2.3.1.76]. Colored green boxes suggest available information is present for the gene

4.3.2. Investigation of ATF gene in CWW isolate

Extracted DNA of *Micromonospora*; strain 2CPEGATSA4 (Sample 1), 3CPEGATSA1B (Sample 5) *Streptomyces*; strain 3CPEGATSA66 (Sample 8) and representative type strains of *Micromonospora* (*Micromonospora endolithica* represented as Sample 12) and *Streptomyces* (*Streptomyces albidoflavus* represented as sample 13) were amplified with DGAT/ATF designed primers. There were no amplicons for all reactions as observed on the gel image (Fig. 4.5).

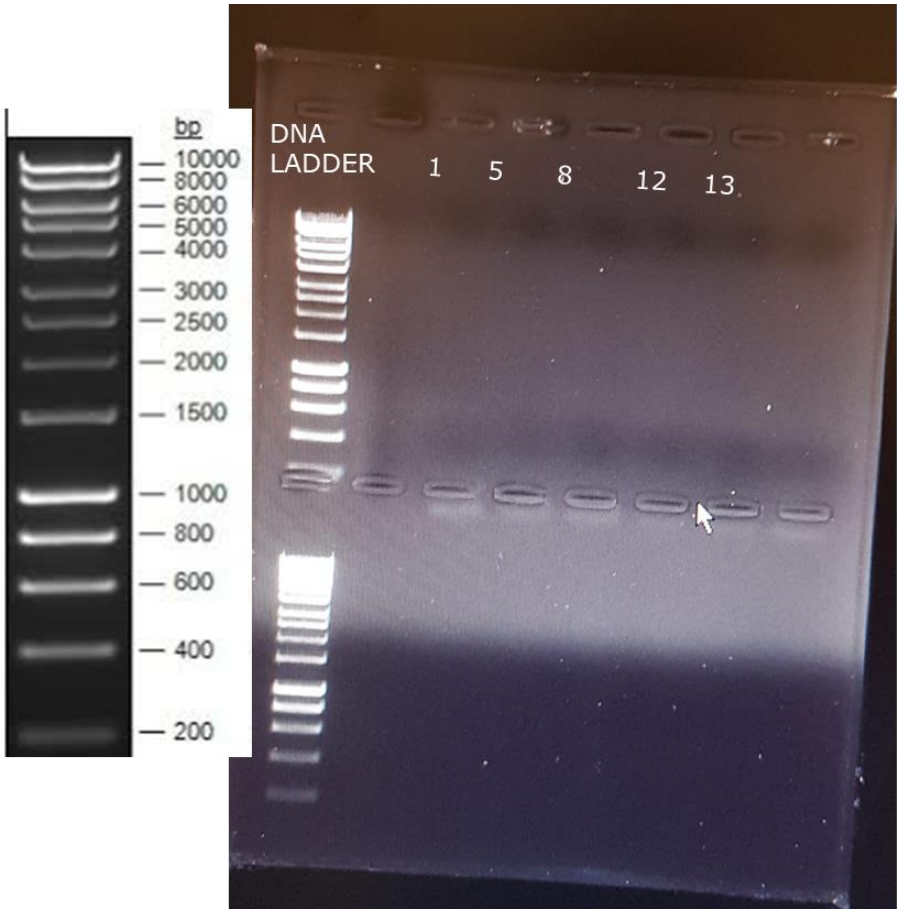


Figure 4.5: 1.5% gel image showing failed amplification from DGAT primers. 2CPEGATSA4 (Sample 1), 3CPEGATSA1B (Sample 5) *Streptomyces*; strain 3CPEGATSA66 (Sample 8) *Micromonospora endolithica* (Sample 12) and *Streptomyces albidoflavus* (sample 13)

4.3.3. PCR amplification of phosphatidic acid phosphatase (PAP) gene, Lppα (Sco1102) and Lppβ (Sco1753) in CWW isolate

The Blast of Sco1102 against the *Streptomyces albidoflavus* taxid in Genbank returned 94 sequence hits closest to Sco1102 from *S.coelicolor* A3(2). Alignment and visualization of these hits show that diverse clusters of proteins identify as PAP2-related and multiple enzymes present in *S.albidoflavus*. Fig.4.6 shows the phylogenetic tree of the PAP2-related cluster of *S.albidoflavus* most closely related to Sco1102 from *S. coelicolor* A3(2). Alignment of Sco1102 related proteins in *Streptomyces albidoflavus* strains was used to design the primer. Fig.4.7 and Fig 4.8 illustrates the alignment of Sco1102 related proteins in *Streptomyces albidoflavus* strains

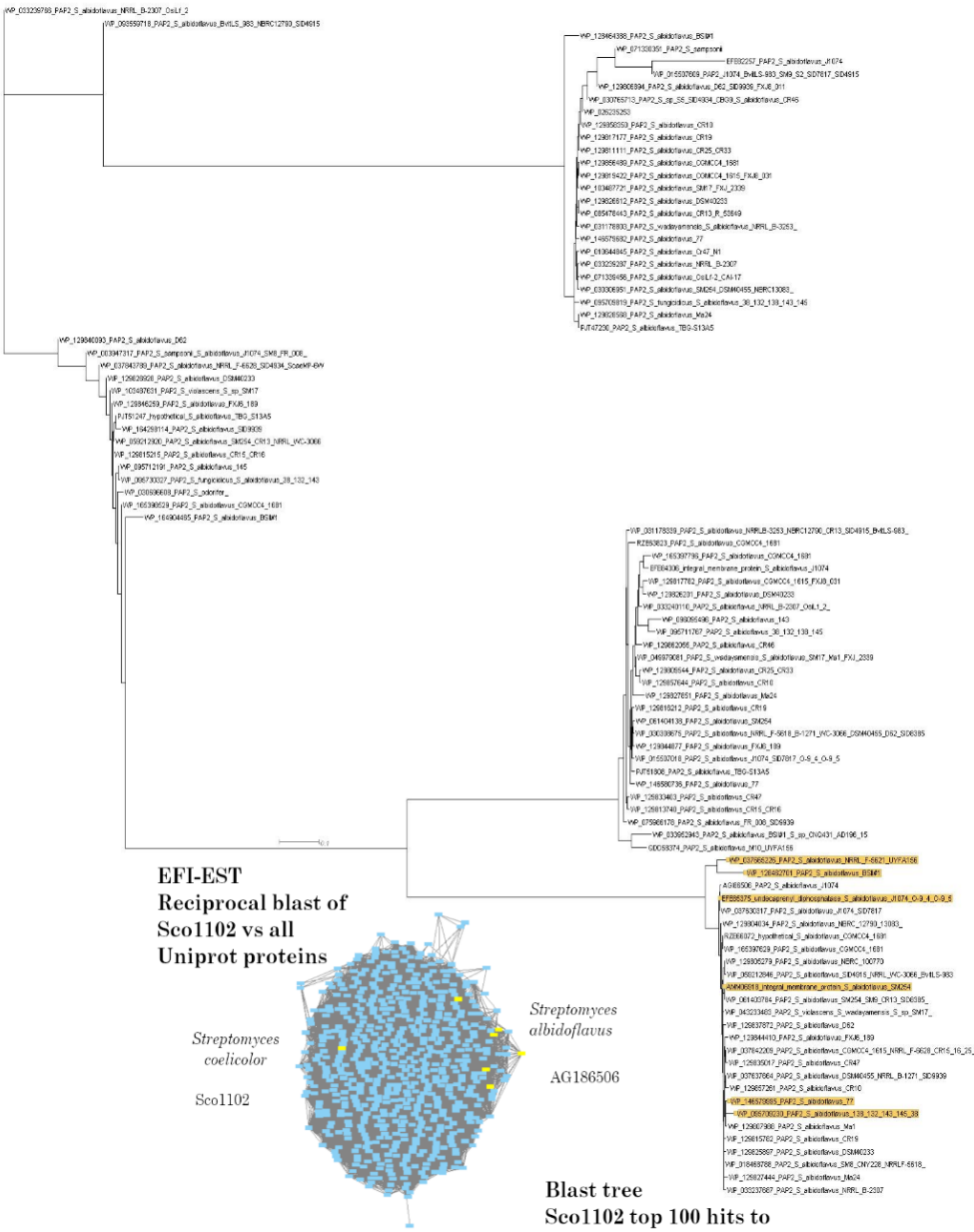


Figure 4.6: Identification of the PAP2-related cluster of *S.albidoflavus* most closely related to Sco1102 from *S. coelicolor* A3(2)

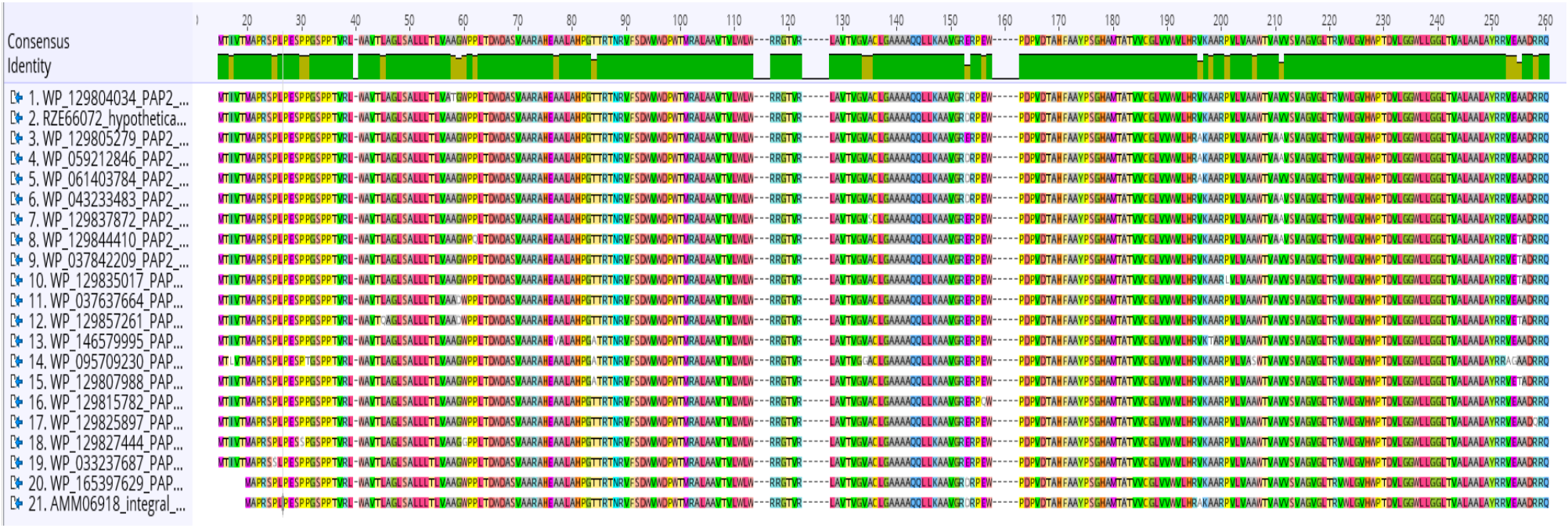


Figure 4.7: Alignment of Sco1102 related proteins in *Streptomyces albidoflavus* strains

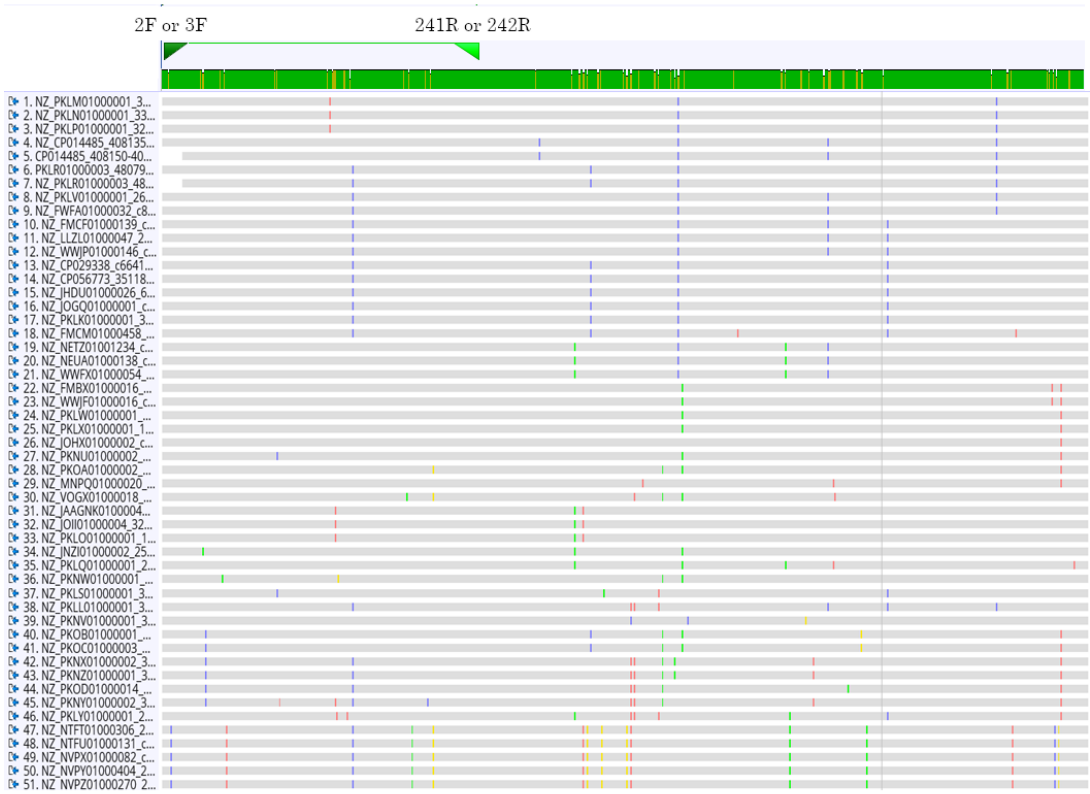


Figure 4.8: Alignment of homologous gene sequences to Sco1102 for *Streptomyces albidoflavus* strain.

The protein Blast of Sco1753 against *Streptomyces albidoflavus* can be seen in Fig. 4.9

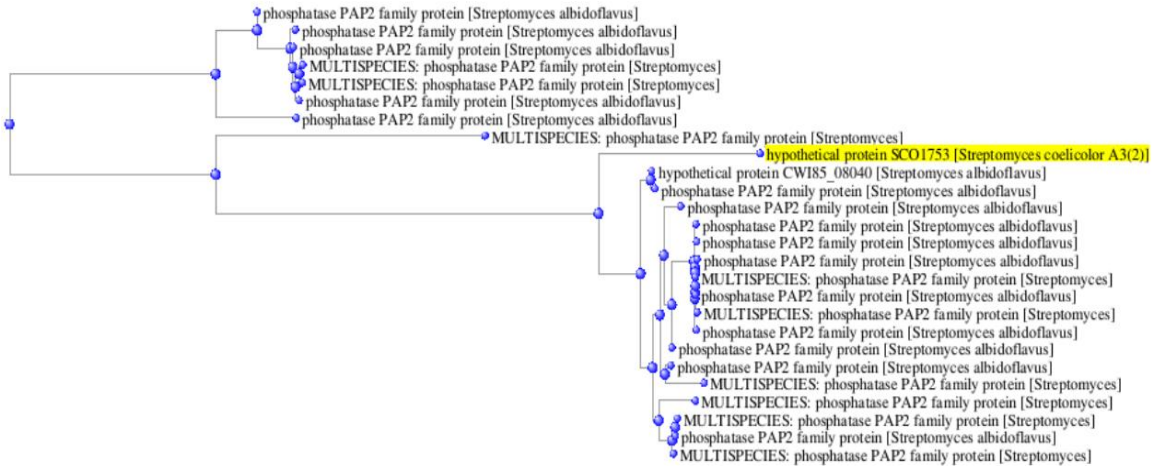


Figure 4.9: Identification of the PAP2-related cluster of *S.albidoflavus* most closely related to Sco1753 from *S. coelicolor* A3(2)

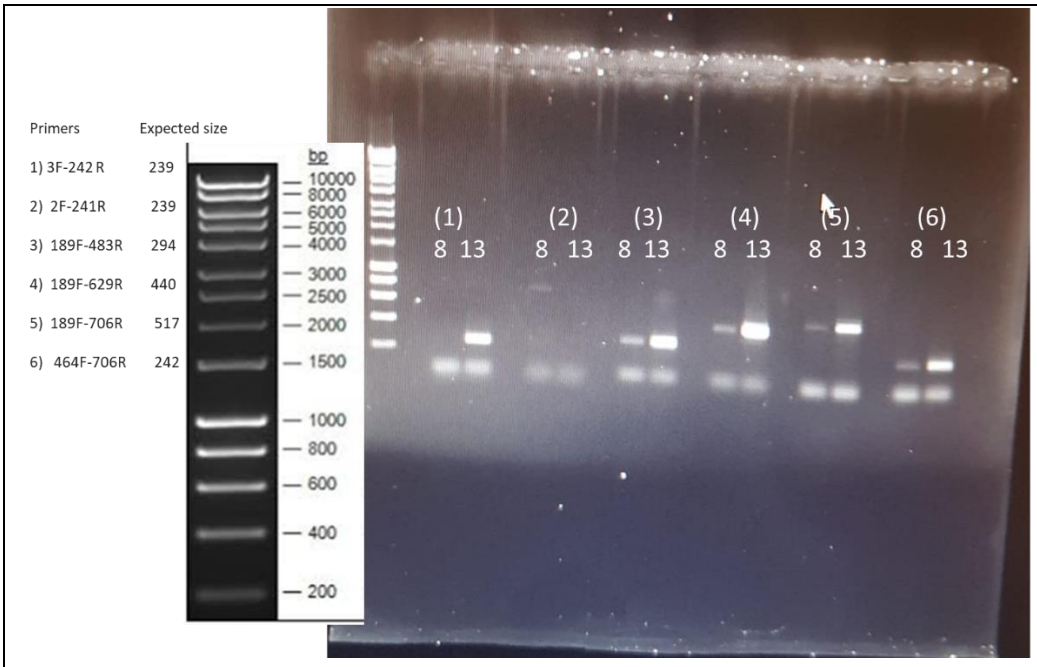
The nucleotide sequences corresponding to the proteins in the Sco1753 cluster were identified in Identical proteins, downloaded and aligned as seen in Fig.10 below. Primers were designed with Primer 3 software.



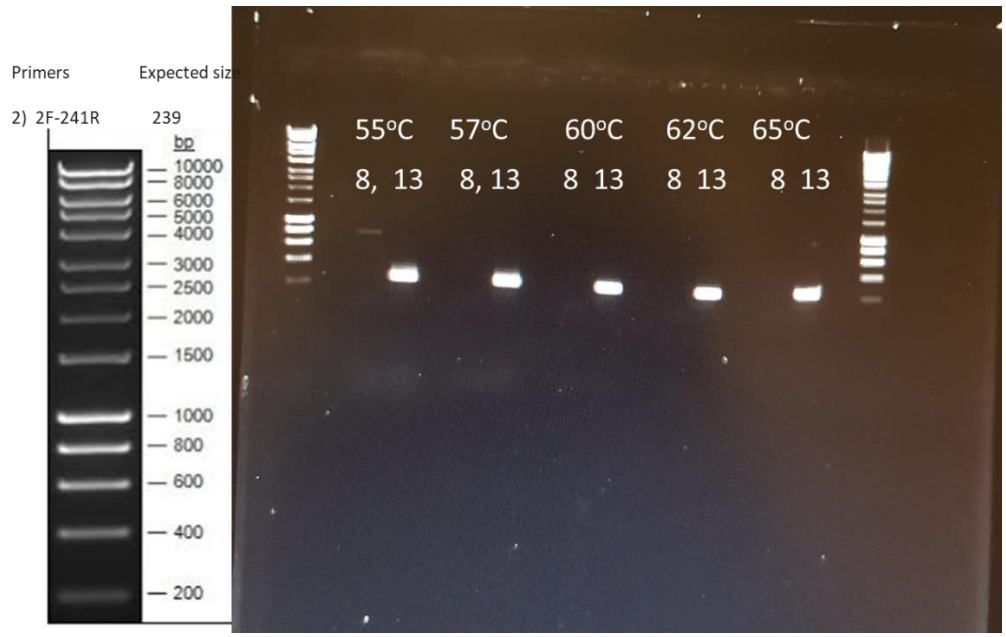
Figure 4.10: Alignment of Sco1753 related proteins in *Streptomyces albidoflavus* strains

4.3.3.1. Amplification of Streptomyces Isolate and Streptomyces albidoflavus with PAP primers

Streptomyces isolate strain 8 and the representative *Streptomyces albidoflavus* type strain were amplified with PAP Sco1102 and Sco1753 designed primers (Fig.4.11). There was no amplification for strain 8 for both 3F-242 R and 2F-241R designed from Sco1102. A gradient PCR (Fig. 4.11B) was used in the amplification of 2F-241R following the failure of both strains to amplify with initial PCR protocol (Fig.4.11A). All amplicons were observed at the expected band size as seen below



A



B

Figure 4.11: 1.5% Gel image showing amplified PCR product with (PAP Sco1102 and Sco1753) primers. A) Amplification with primers 3F-242 R, 2F-241R, 189F-483R, 189F-629R, 189F-706R and 464F-706R (B) Gradient PCR with 2F-241R to amplify PAP gene

4.3.4. Thin layer chromatography lipid analysis

Thin layer chromatography was used to determine the presence of TAG in samples of *Micromonospora* and *Streptomyces* isolated from cassava effluent. A significant red/orange pigment(s) were extracted with the lipids from sample 1 and 5. Samples 12 and 13 although not showing as pigmented biomass did give slightly pigmented extracts, whereas sample 8 only revealed a faint grey extract. Image of extracted lipid for both 3mg freeze-dried biomass and 10mg biomass is shown in Fig.4.12 below

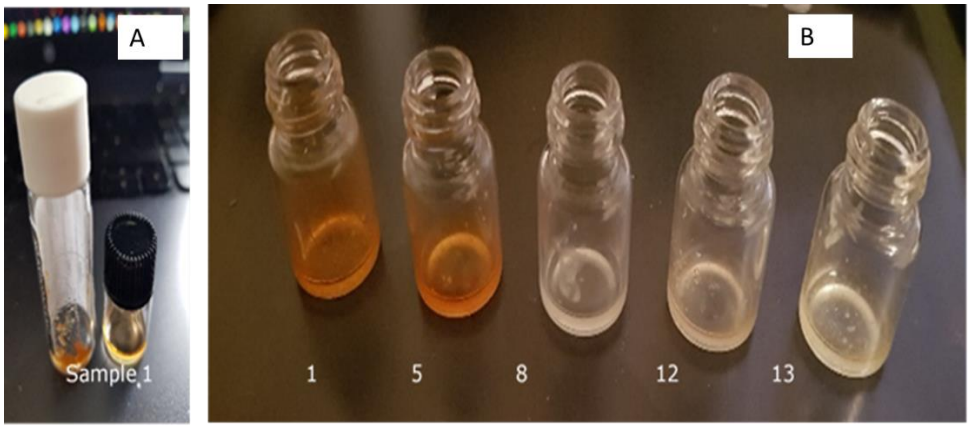


Figure 4.12: Image showing extracted lipids from samples. (A) lipid was initially extracted from 3mg freeze dried biomass (B) sample extracted from 10 mg biomass. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus*

Fig. 4.13 below shows the image of a 5 µl aliquot of each lipid extract from samples 1, 5, 8, 12 and 13 and a 5 µl aliquot of standards T2, T3 and T5 spotted, as a single 5 µl application, onto a silica gel plate and then charred for 20 minutes at 200^{o}C. From the image, lipids from 3 mg biomass extraction were not detected. So biomass was increased to 10 mg, and the biomass was extracted twice with 0.5 ml chloroform: methanol (2:1) for 4 hours with gentle shaking at

regular intervals of 10 minutes. Standard triglyceride was detected as seen from Fig.4.14 and 4.15

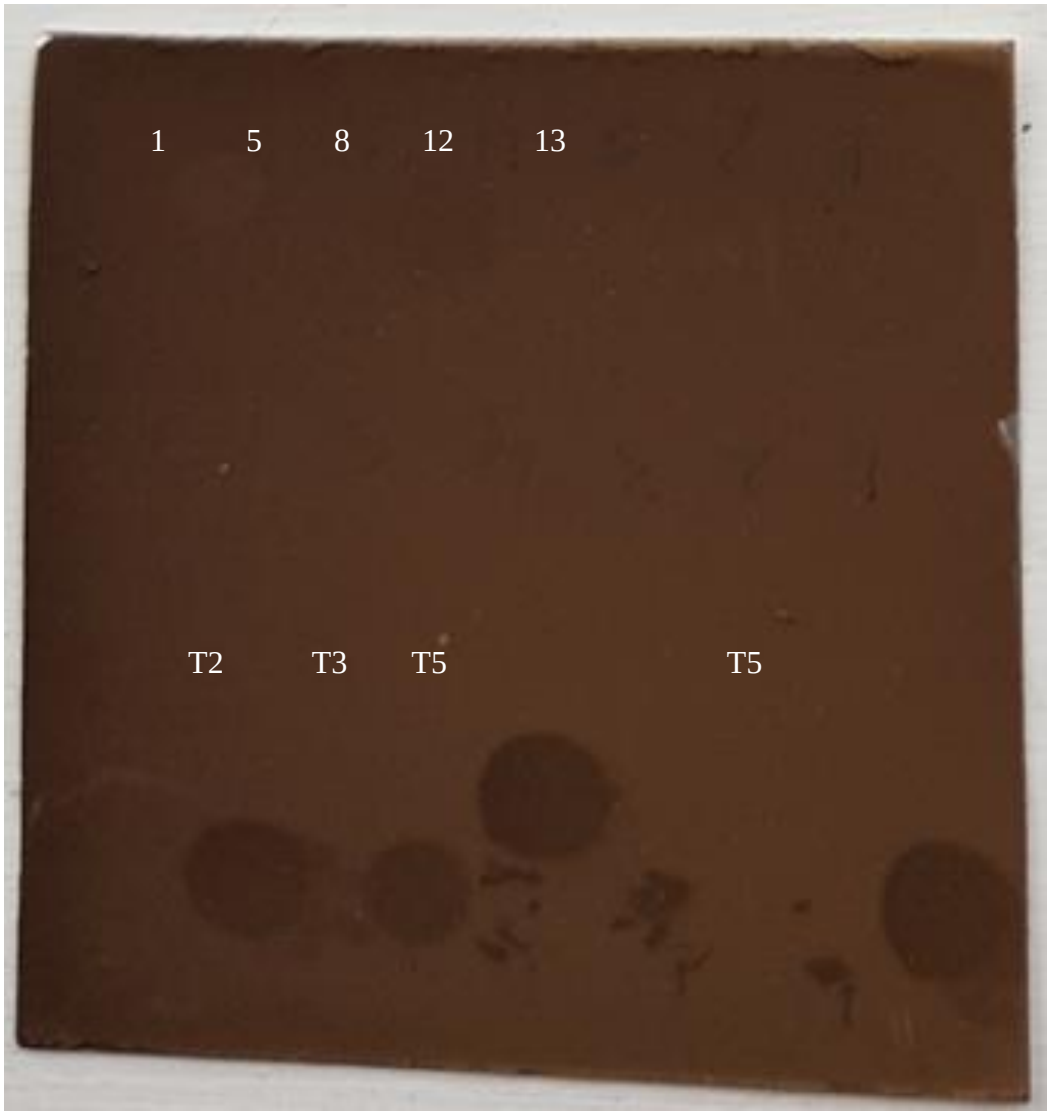


Figure 4.13: Detection of 3mg extracted samples and standards by charring (20 min) on silica gel with $(NH_4)_2SO_4$. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica,* Sample 13 = *Streptomyces albidoflavus.* T2,T3, T5= control

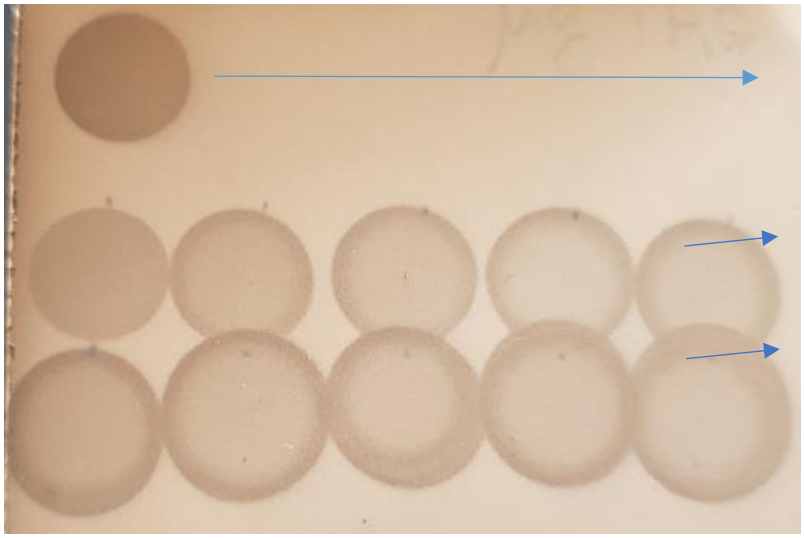


Figure 4.14: Detection of standards by charring (10 min) on silica gel with $(NH_4)_2SO_4$

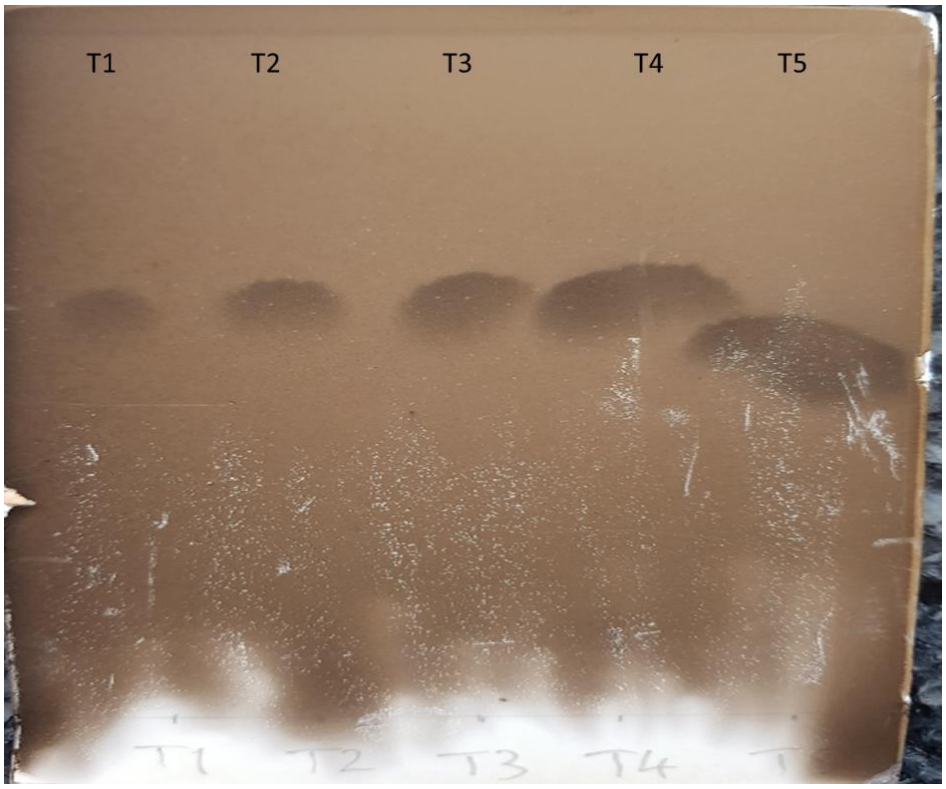


Figure 4.15: Detection of standard triglyceride (olive oil). Standards T1, T2, T3, T4 and T5 were spotted (5 µl) onto silica plate, pre-saturated with 10% $(NH_4)_2SO_4$ and dried, then run in hexane: diethyl ether: acetic acid and charred 10 minutes at 200^oC.

4.3.4.1. Detection of triglyceride in samples

For the TLC run with each sample, 1 µl of the 5 µl aliquot of samples and T3 standard was spotted on silica plate pre-soaked in 10% $(NH_4)_2SO_4$ and dried. Sample aliquot was obtained from the 10 mg extraction (redissolved in 30 µl chloroform: methanol (2:1)). Fig. 4.16 and 4.17 shows the spotted samples for run in hexane: diethyl ether: acetic acid.

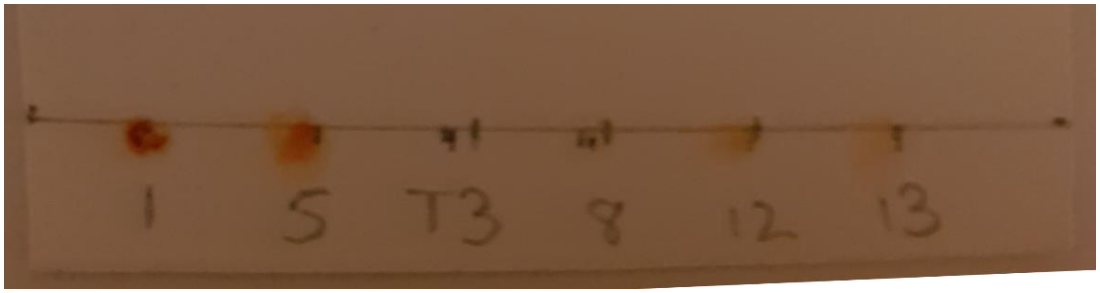


Figure 4.16: Samples spotted for run in hexane: diethyl ether: acetic acid. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus* T3= Control



Figure 4.17: Samples run in hexane: diethyl ether: acetic acid. No TAG was detected in this run. All the lipid seems to be trapped at the origin. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus* T3= Control

Additional sample 10 µl was spotted to run in dual solvents chloroform:methanol: water (60:30:5) followed by hexane: diethyl ether: acetic acid (80:20:1) using silica TLC plates without preloading with 10% $(NH_4)_2SO_4$ as this seemed to interfere with running in this system when tested with standard triglyceride. Fig.4.18 shows the sample spotting in the dual solvent system. Fig.4.19 shows the sample run in chloroform:methanol: water. The relatively polar pigments, which remained at the origin in hexane: diethyl ether: acetic acid ran in chloroform:methanol: water. The plates were run to 5 cm (halfway) in chloroform:methanol: water. After thorough drying, the plate was run in the same direction, in hexane: diethyl ether: acetic acid. Fig.4.20 and Fig.4.21 shows the Samples run in chloroform:methanol: water to separate polar lipids, then hexane: diethyl ether: acetic acid, to separate simple lipids like triglyceride

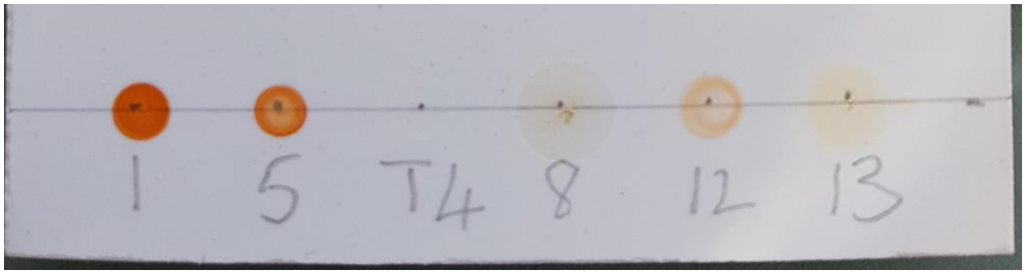


Figure 4.18: Samples spotted for a run in dual solvent systems chloroform:methanol: water and hexane: diethyl ether: acetic acid. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica,* Sample 13 = *Streptomyces albidoflavus* T4= Control

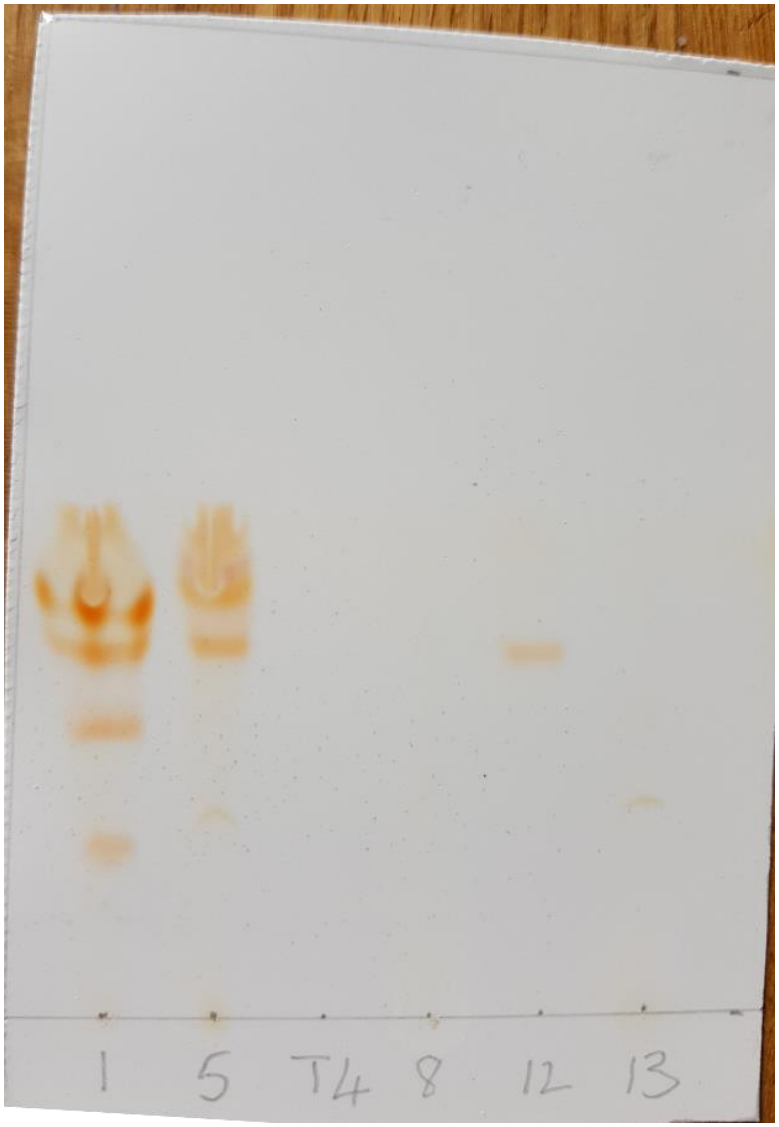


Figure 4.19: Samples run halfway in chloroform:methanol: water to separate polar lipids. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus* T4= Control

The pigments were overloaded in samples 1 and 5 hence the intensity of bands. One of the major pigments detected in 1 and 5 is also seen in sample 12. There are no visible pigments in 8 and only a faint pigment band in 13. Sample was run further in hexane: diethyl ether: acetic acid, to separate simple lipids like triglycerides as seen in Fig. 4.20



Figure 4.20: Samples run in chloroform:methanol: water to separate polar lipids, then hexane: diethyl ether: acetic acid, to separate simple lipids like triglyceride. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus* T4= Control

The remaining sample was dried and redissolved in 20 µl hexane: diethyl ether: acetic acid (80:20:5) and 10 µl spotted onto a silica plate and run in hexane: diethyl ether: acetic acid. With this concentration of non-polar lipids from the remaining sample, a spot corresponding to triglyceride is just visible in all the samples (Fig 4.21)

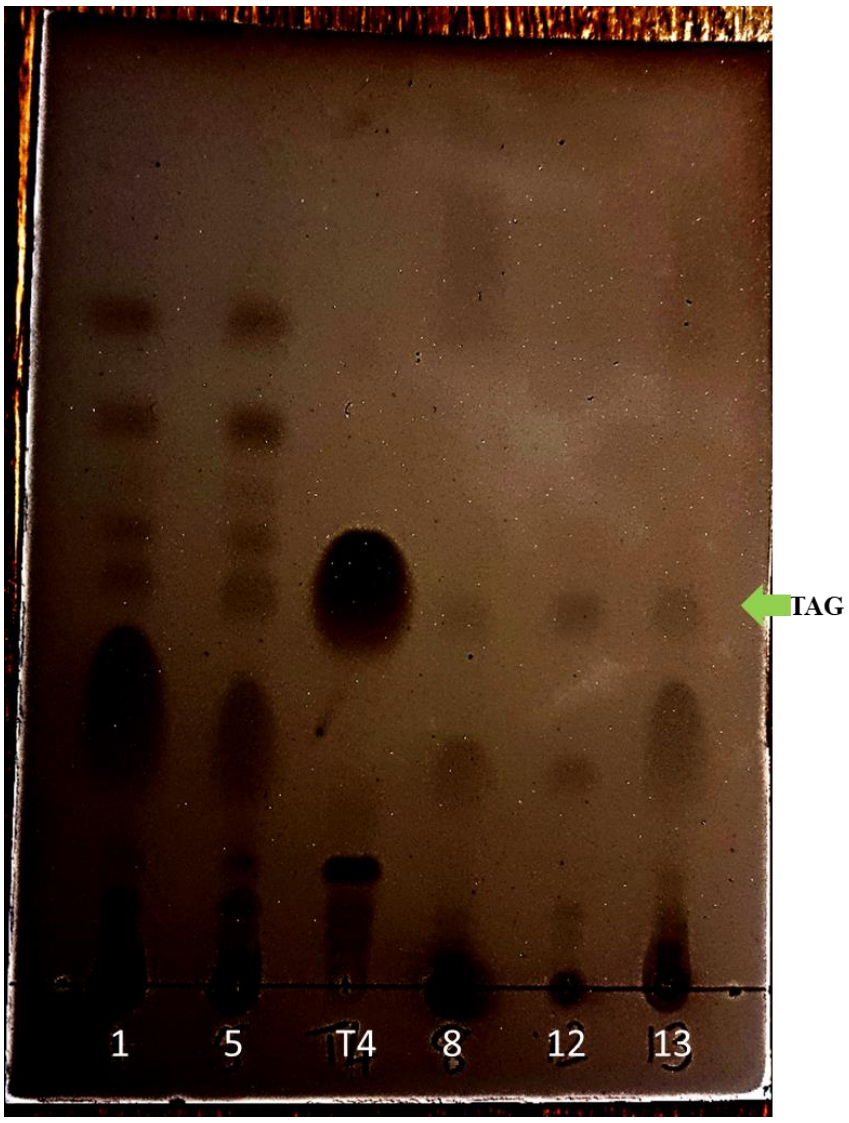
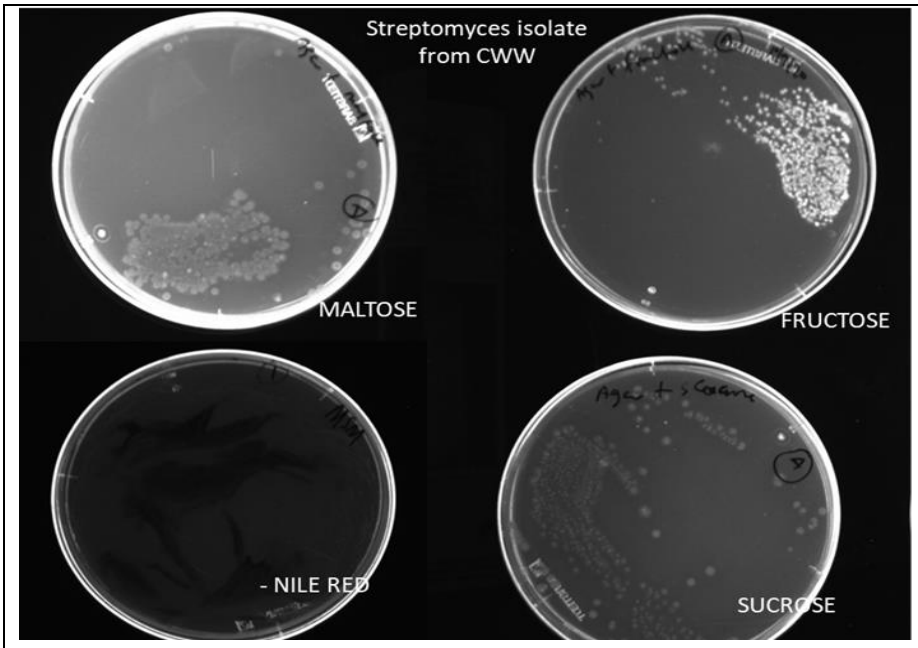


Figure 4.21: TAG accumulation from cassava waste isolate. Samples concentrated in hexane: diethyl ether: acetic acid and run in the same solvent. Sample 1 = 2CPEGATSA4, Sample 5 = 3CPEGATSA1B, Sample 8 = 3CPEGATSA66, Sample 12 =*Micromonospora endolithica*, Sample 13 = *Streptomyces albidoflavus* T4= Control

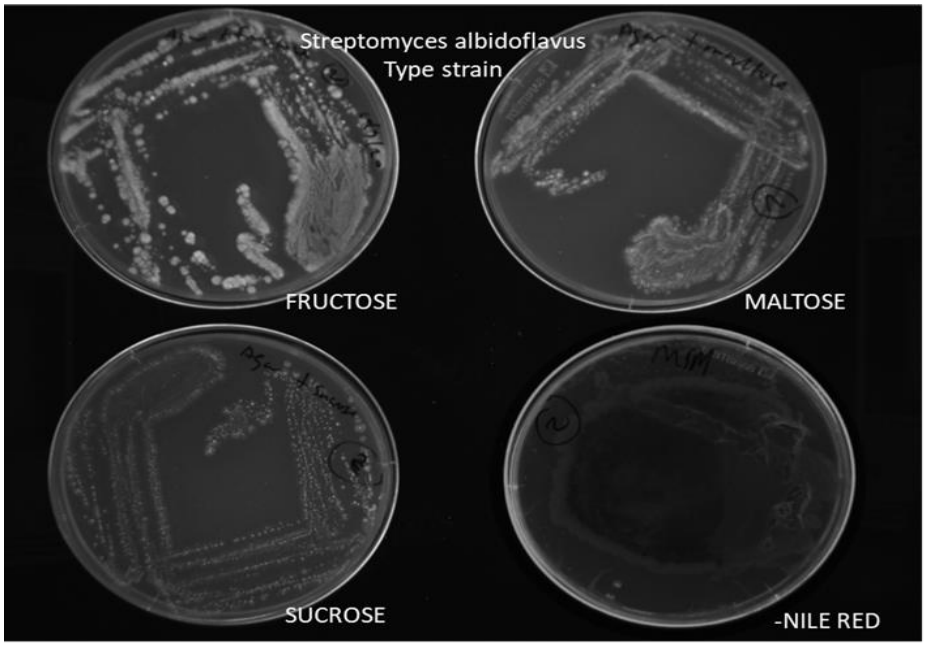
4.3.5. Growth of bacteria in the presence of Nile red on Agar plates for the direct screening for TAG accumulation Isolates

Growth of bacteria on MSM agar plates

The result from the growth of strains on MSM agar and the observation of plates under UV light was captured as seen in Fig.4.22, Fig. 4.23 and Fig.4.24 All Isolates investigated were able to fluoresce in the presence of Nile red. Fluorescence ability was absent in all strains cultured in the absence of Nile red. *R.opacus* strain also showed a similar fluorescence pattern with investigated strains. The fluorescence intensity was most prominent in isolates cultured with fructose as the carbon source.

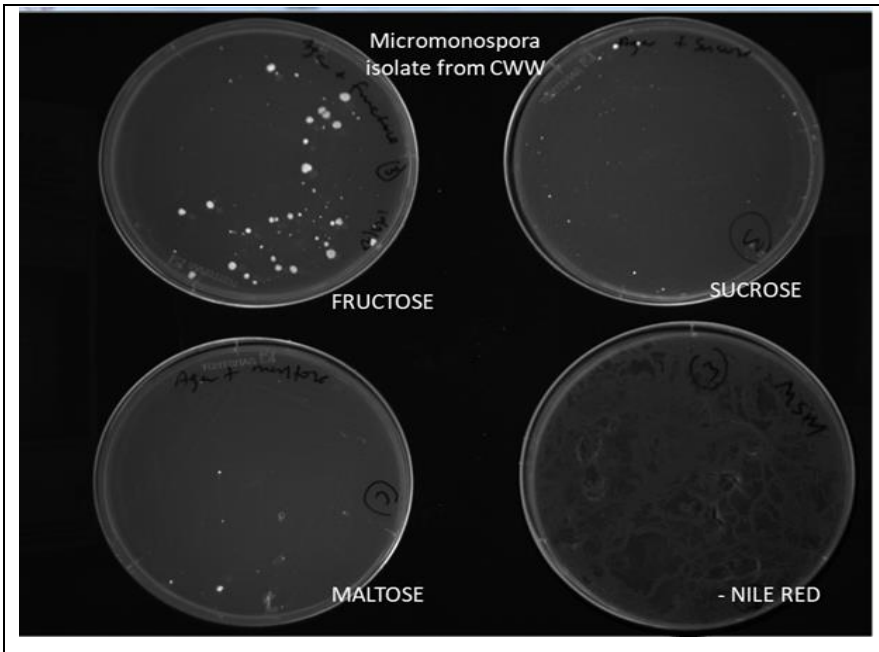


A

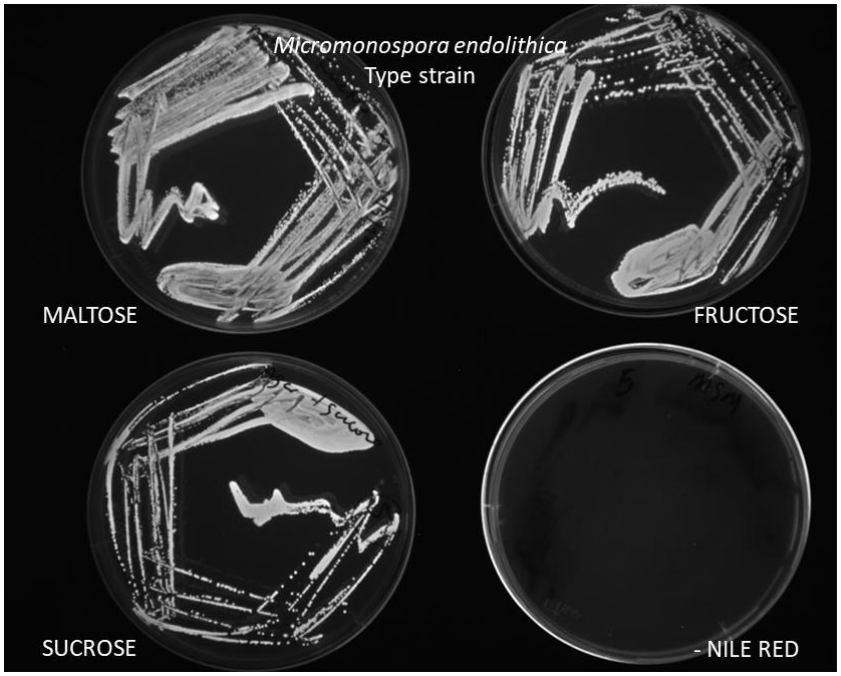


B

Figure 4.22: *Streptomyces* strains grown on MSM agar supplemented with and without Nile red on different carbon sources (Maltose, fructose and sucrose). (A) *Streptomyces* CWW isolate (3CPEGATSA66) grown on the carbon sources (B) *Streptomyces albidoflavus* type strain grown on the carbon sources.



A



B

Figure 4.23: *Micromonospora* strains grown on MSM agar supplemented with and without Nile red on different carbon sources (Maltose, fructose and sucrose). (A) *Micromonospora* CWW isolate (2CPEGATSA4) grown on the carbon sources (B) *Micromonospora endolithica* type strain grown on the carbon sources

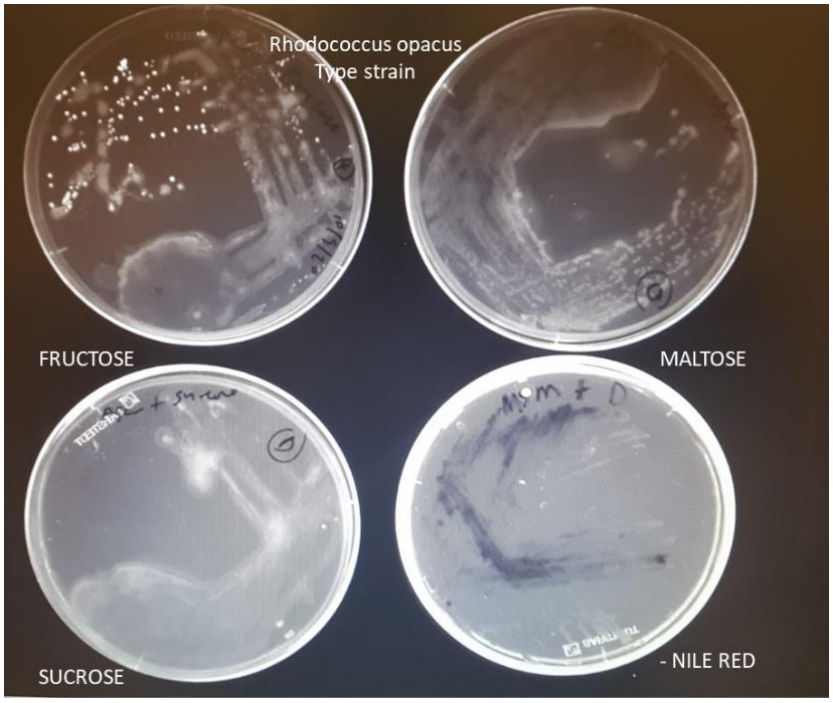


Figure 4.24: *Rhodococcus opacus* grown on MSM agar supplemented with and without Nile red on different carbon sources

4.4. Discussion

A substantial part of the synthesis of TAG is carried out via enzymes of the Kennedy pathway (*de novo* pathway) in which the transfer of an acyl chain from acyl CoA to glycerol-3-phosphate (G3P) at the sn-1, -2 and -3 position occur sequentially (Alvarez *et al.*, 2017). Initial exploration of the TAG pathway mapping using KEGG pathway database revealed the absence of the AGPAT and DGAT/ATF key genes (Fig 4.3 and Fig 4.4). The lack of corresponding entries suggests the latest information from published literature for these genes in representative species are unavailable (Aoki-Kinoshita & Kanehisa, 2007). Representative species adopted in this analysis were the available data for strains closest to the isolated CWW strain. The suggestion of gaps in the available information propelled the investigation into the CWW isolates' ability to accumulate TAG.

Primers were designed to flank the HHXXXDG active region which has been proposed to be essential for the catalytic activity (Arabolaza *et al.*, 2008; Stöveken *et al.*, 2009) to amplify and investigate the DGAT/ATF genes. Another key step involving phosphatidic acid phosphatase (PAP) enzymes were also investigated within *Streptomyces* novel isolate by designing primers to amplify the *lppα* (SCO1102) and *lppβ* (SCO1753) genes. Phenotypic confirmation test was carried out using TLC and a highly sensitive staining method involving the culture of strains in nitrogen limiting condition using Nile red as an indicator.

The application of TLC within this study separated the TAG lipid class present in *Streptomyces* and *Micromonospora* isolated from cassava waste effluent. TAG's ability to migrate through the stationary silica phase when run with a hydrophobic solvent system can be seen on Fig. 4.21. The separation results

obtained from the TLC suggests the presence of TAG. This is based on the bands observed following the solvent runs on silica gel TLC plate across all sample. These bands were at position to the control (Olive oil) used in this experiment. Similar TAG patterns from solvent run on TLC plate for the separation of TAG from lipid constituents has been reported by Arabolaza *et al.* (2008).

A further phenotypic confirmation test was carried out using a highly sensitive staining method involving the culture of strains in nitrogen limiting condition using Nile red as an indicator. This dye was directly added to the growth medium of respective strains allowing the estimation of TAG presence. As seen in Fig. 4.22, Fig 4.23 and Fig 4.24, this dye's presence did not impede the growth of strains but allowed the fluorescing of cells following their development after 5 days. The recognition of a lipid-rich environment resulted in Nile Red's fluorescence intensity as observed in the samples (Spiekermann *et al.*, 1999). The fluorescence of *Micromonospora endolithica* was most prominent across the samples observed suggesting a high lipid inclusion. The *Micromonospora* isolate (2CPEGATSA4) also had a high fluorescence, although the growth was scanty within plates. *Streptomyces* samples investigated did show significant growth and fluorescence when cultured with fructose as the carbon source. This would suggest that the carbon sources which is being used for their growth can also have an impact on the rate of lipid accumulated. The positive fluorescence amongst tested TAG accumulation strains would indicate that these strains can accumulate this neutral lipid (Rumin *et al.*, 2015; Spiekermann *et al.*, 1999). This was inferred from similar flouresencing in the positive *R.opacus* control and the absence of fluorescing in the agar plates which did not contain nile red. Other control which could have been used in this experiment were non-TAG

accumulating bacteria from genera *Pseudomonas*, *Ralstonia*, *Aeromonas*, and *Rhodobacter*. In comparison to TLC method used, both methods inferred the accumulation of TAG. As oppose bright fluorescing in Nile red plates, faint bands were observed in TLC. This faint bands are still indicative of a positive result, however, the faintness can be attributed to the stress level exposed to the samples before freeze-drying. Moreover, the freeze-dried biomass used in TLC analysis was not typically grown in nitrogen limiting condition, due to which the TAG accumulation takes place. This was because strains struggled to grow for analytical purposes when deprived of this nutrient.

For *Micromonospora* sample 1(2CPEGATSA42), 5 (3CPEGATSA1B) and 12 (*M.endolithica)*, the inability to amplify the DGAT gene in samples contrasted with the TLC and Nile red inclusions' suggestion for TAG accumulation. This means an alternative pathway not involving the DGAT might be responsible for TAG synthesis by these isolates. Three different routes which use DAG as a substrate have been suggested for TAG synthesis (Arabolaza *et al.*, 2008). The acylation of DAG in bacteria by DGAT enzymes occurs similarly in eukaryotic TAG biosynthesis, which is catalyzed by DGAT 1 and DGAT 2 protein families. Asides this usual pathway, phospholipid: DAG acyltransferase catalyses an acyl-CoA-independent reaction for the synthesis of TAG. This enzyme which has been found in yeast and plants uses phospholipids as an acyl donor in the esterification of DAG. The third mechanism identified in animals and plants involves a DAG: DAG transacylases that utilize DAG as an acyl acceptor and donor. These alternative methods can be adopted for the synthesis of TAG by these samples. However, the nature of the alternative pathways in *Micromonospora* is not yet known at this point. Insilico data of the

acyltransferase genes that can support this synthesis's function has also not been reported for *Micromonospora*. Hence, it is not possible to correlate the putative acyltransferase gene homology to that of eukaryotic enzymes responsible for TAG synthesis. Since the physically observed agar and silica plates suggest TAG presence, it can also be inferred that a potential DGAT isoenzyme could be responsible for the synthesis of TAG. DGAT isoforms have been previously suspected to be present in some cells, which can catalyze TAG synthesis (Kaddor *et al.*, 2009). It is also imperative to state that the alternative DGAT/ATF pathway or isoenzymes might not be responsible for this TAG accumulation. Still, the DGAT enzymes might be poorly active or require other methods of investigation. The prospective alternative pathway which can be present in *Micromonospora* was not examined further within this current study. In the *Streptomyces* sample 8 (3CPEGATSA66) and 13 (*S.albidoflavus*), TAG accumulation based on DGAT was not corroborated with TLC and Nile red results. Similarly, to *Micromonospora*, the suggestion of the DGAT gene was not inferred from the amplification of the active HHXXXDG region. The available data present for another TAG synthesizing species (*Streptomyces coelicolor*) was used to investigate the TAG accumulation in *Streptomyces* samples further. The presence of PAP multifunctional enzymes found in *S.coelicolor* Sco1102 and Sco1753 was investigated further. The second key step in the synthesis of TAG via the Kennedy pathway (*de novo* pathway) involves the activity of phosphatidic acid phosphatase (PAP) enzymes. Exploring the available pathway for TAG in this study via the KEGG database also suggests this key enzyme's absence. Both PAP gene were observed in *S.albidoflavus,* but only a single *lppα* (SCO1753) was inferred from our

Streptomyces isolate (3CPEGATSA66). The dephosphorylation of phosphatidate, catalyzed by PAP enzymes, can be suggested within this experiment in DAG synthesis. The TAG observation from TLC bands and inference from lipophilic dye inclusion study also suggest an alternative pathway for TAG synthesis in these samples equally. Surprisingly, there was no significant difference in the TLC band size between the two *Streptomyces* samples to suggest these genes had a significant impact on the TAG accumulated. This, implying that a knockout of either gene might not have a considerable effect on the TAG accumulation. It has been suggested that the disruption of either *lppα* or *lppβ* has no alteration to the intracellular TAG level. The inactivation of both genes was also reported to cause a reduction in TAG, while the overexpression of these genes increased the intracellular TAG levels (Comba *et al.*, 2014).

In retrospect, *S.coelicolor* could have been used as an additional positive control to support the positive result obtained from the experimental procedures adopted. Overall, the findings from this study suggest TAG accumulation in CWW isolates of *Streptomyces* and *Micromonospora*. Although not conclusive at this point, further work is required on the PAP enzymes, Lppα (Sco1102) and Lppβ (Sco1753) amplicon from *S.albidoflavus* and Lppβ (Sco1753) amplicon from CWW *Streptomyces* isolate 8 (3CPEGATSA66) from this study. The successful sequencing of amplicons within this study and annotation of CWW *Streptomyces* isolate 8 from this study can provide more information on the TAG synthesis pathway. Further tests are needed to be concluded equally on *Micromonospora* based on the TAG result and the absence of available pathway information on TAG accumulation for *Micromonospora*.

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CHAPTER 5: General discussion and conclusion

5.1. General discussion

Biofuel production provides a sustainable alternative to fossil fuels. However, growth of biofuel industry has mainly been affected by several uncertainties, including the sustainability of production processes. Finding an economically viable approach and the right substrates have been a source of constant debate. This research investigates the metabolic potential of oleaginous actinomycetes in biodiesel production, utilizing cassava wastewater as a substrate.

The initial investigation involved utilizing culture-dependent and independent strategies to investigate the microbial diversity in cassava wastewater effluent obtained from different processing time points, with actinobacteria as the target phylum. Previous studies highlight the microbial diversity from cassava effluent (Elijah *et al*., 2014; Miambi *et al*., 2003). However, there remains a gap in understanding the microbial diversity in cassava effluent and the different processing stages. This particular study fulfils this with further investigation in identifying the potential of industrially significant actinobacteria for the purpose of generating biodiesel.

The strategies adopted to investigate microbial diversity suggested the dominance of firmicutes, proteobacteria, cyanobacteria, and actinobacteria. The observation of a host of phyla justifies this effluent's potential to support bacterial growth. Moreso, across the effluent stages, the diversity was rich. Suggesting that each stage of processing can impact positively when explored as low-cost waste for growth of microorganisms in biodiesel purpose. The isolation of genera *Micromonospora*, *Micrococcus,* and *Streptomyces* provide physical evidence to suggests the presence of useable actinobacteria for biodiesel production. The differences in observed results from the culture-

dependent and culture-independent approaches can be attributed to various biases related to culture methods (Giraffa & Neviani, 2001; Jany & Barbier, 2008).

The combination of both microbial diversity approach from this study provided information on the potential of this effluent. Although little information is available at the moment on the role of some of the actinobacteria observed, *Streptomyces*, as inferred from culture-dependent approach, has been related to TAG accumulation with the potential application in biodiesel production (Comba *et al.*, 2014). Other genera observed from culture-independent approach such as *Corynebacterium* has also been suggested to be important in biofuel (Zhang *et al.*, 2019). Further work needs to be carried out to optimize the isolation conditions if this genus is to be considered.

Similar diversity was observed from the different stages, although the washing and pressing effluent seemed to possess the most interesting actinobacteria. The results from the phyla abundance and culture methods support this claim. This inference is interesting as it provides a clearer picture of the importance of these stages as stand alone stages for end use.

The success of biodiesel production from this waste depends primarily on finding suitable microorganisms with properties that can be exploited for end-use. The strategy adopted to investigate CWW isolates biodiesel potential involved various functional examinations. Novel isolates of genera belonging to *Micromonospora* and *Streptomyces* were investigated accordingly. The temperature function on isolates investigated revealed that these isolates developed best in a moderate temperature. In the context of research objective, this property of isolates tallies with the default condition in which the cassava

effluent is processed, suggesting that no added cost for temperature control is required if these isolates are cultivated on cassava effluent. This temperature effect on isolates is important as energy cost can negate the biodiesel process's sustainability if not adequately considered.

In this study, the effect of pH suggests that the uniform consideration of all cassava effluents for growth of novel *Micromonopora* and S*treptomyces* would require pH optimisation of effluent. Moreover, pH of cassava effluents at each processing stage was acidic, varying slightly across the effluent sample points. This was in contrast to the adequate growth of isolates in neutral pH. The growth in neutral pH is a common characteristic of isolates' genera, hence their proper development at this pH (Barka *et al.*, 2016). The inability of our *Streptomyces* isolate (3CPEGATSA66) in particular to develop properly in acidic condition does not conclusively suggest that they are intolerant to acidic pH. It only suggests that a possible slow rate of growth was the impact of this acidic physiological condition on strain. Moreso, it has been reported that *Streptomyces* can grow at an acidic pH of 3.5 (Kim *et al.*, 2003). Furthermore, this isolate was obtained from cassava effluents with acidic pH.

It could be hypothesized that to utilize cassava effluent for biodiesel production successfully, some further optimization asides pH might be required. These optimizations could be to ensure that the microorganisms inoculated for the end-use perform accordingly. The optimization of cassava effluent could require fortification with carbon or nitrogen sources. Investigation of CWW isolate growth on carbon and nitrogen source in this research suggested isolates ability to utilize a range of compounds. Particularly, glucose and valine were the most effective carbon and nitrogen base for the growth of isolates. This would suggest

that these compounds can be succinct candidates to improve the effluent further if needed.

The ability of isolates to degrade a host of complex organic compounds further supports the importance of this CWW isolate explored. This ability suggests that pure strains could utilize some of the other waste associated with cassava processing, or other potential low-cost wastes for biodiesel production. One such low cost and available waste as an alternative to cassava effluent is the cassava peel. The ability of all isolates to degrade starch as seen in the phenotypic results could suggest the peels of cassava can be optimized equally for the growth of isolates.

The enzymatic profile and isolates' resistance to various antibiotics were explored. The result provided information on the enzymatic profile of isolates and the susceptibility of isolates to different antibiotics. This information can be useful in optimizing isolates for biodiesel production. Particularly, the information on the antibiotics susceptibility and resistance could be important in controlling other unwanted bacteria that may compete with isolates for nutrients during biodiesel production.

Investigating the FAME (Fatty acid methyl esters) profile of *Micromonospora* and *Streptomyces* revealed that isolates are characterized by a significant percentage of branched-chain fatty acids (BCFAs). These result's suggest the importance of fuels generated from these isolates could have improved properties, including lower cloud point, better cold flow, and lower freezing point than biofuels comprising straight-chain fatty acids (Knothe, 2005, 2009; Knothe *et al.*, 2005). The biodiesel obtained from these isolates is more suitable for existing engines. Particularly, these BCFA's are suggested to be potentially

more suited in powering jet engines. The biosynthesis, regulation and engineering of microbially produced BCFAs have been extensively reviewed (Bai *et al.*, 2019).

Reconfirmation of novel isolates' ability to grow on cassava effluent is important for confirming the hypothesis on growth of actinobacteria on CWW. The devising of a buffered medium and the utilisation of specific actinobacteria incubation conditions were used to cultivate isolates. Furthermore, the ability of non-indigenous actinobacteria (*Rhodococcus opacus*) to grow on CWW agar suggests the adequacy of this effluent for biofuel. The growth of high TAG accumulating *Rhodococcus opacus* PD630 on cassava effluent can indicate that these waste effluent could potentially support a wider variety of potentially useful bacteria in biodiesel production.

Following the isolates' functional assessment, this study sought to investigate the TAG accumulation in these novel isolates. The TAG accumulation information in this study could be significant in creating strategies to further improve its accumulation for biofuel purposes in our isolates. TLC run and viable colony staining method based on Nile red inclusion in growth constituent suggested TAG accumulation. It is known that the accumulation of TAG occurs via the Kennedy pathway (Alvarez *et al.*, 2017). However, limited data is available to confirm the route of TAG accumulation in isolates. Moreover, further investigation by the attempt to amplify the DGAT gene in our isolate was unsuccessful. The presence of phosphatidic acid phosphatase enzymes Lppα (Sco1102) and Lppβ (Sco1753) investigated in *Streptomyces* suggests that DAG could be produced. Hence it could be hypothesized that an alternative route not involving DGAT enzymes could be involved in the TAG accumulation

observed. TAG accumulating ability via other routes not involving ATFA/DGAT has been previously inferred (Arabolaza *et al.*, 2008). It is imperative to state that the initial trials predicted the absence of the DGAT gene in all CWW isolates of *Micromonospora* and *Streptomyces* explored. The optimization in terms of primer sets and conditions could have affected the results observed.

5.2. Conclusion

This study suggests that microbial diversity analysis using culture-dependent and independent approach is essential in revealing the nature of indigenous cassava effluent microflora at the different stages. Highlighting the significance of combining the two approaches in conclusively investigating microbial diversity. Furthermore, this study revealed that indigenous cassava effluent microorganisms possess functional properties that allow their sustainable use in biodiesel production. The properties of these isolates also support the production of more efficient FAME. The metabolic characteristic of novel isolates can propel the accumulation of TAG as inferred from this study. Although at this stage, the extent of TAG accumulation requires further investigation. Overall, this research findings suggest that CWW isolates of *Streptomyces* and *Micromonospora* can be interesting candidates for the biotechnological production of biodiesel from cassava waste effluent.

5.3. Future work

Understanding the nature of isolates and their potential role within the different cassava effluent stages in this study provided more knowledge on novel CWW isolates' potential. Exploring these isolates further, suggested they could play a crucial role in the production of biodiesel. The extent to which these roles can

be played requires further investigations, especially when cultivated on low-cost cassava waste medium.

From the diversity study within this research, the culture-independent approach suggested some other potentially useful actinobacteria that were not isolated within the adopted culture-dependent methods. Further work could be done to optimise the isolation protocols to target these inferred genera. The potential functions of these species could broaden the importance of this waste in biofuel production.

Shotgun metagenomic sequencing is an alternative approach which could be applied to further explore the unculturable microbiota in our cassava effluent samples. This sequencing approach can be used in the profiling of the taxonomic composition, investigating further functional potentials and in the recovery of whole genome sequences of the microbial communities within the effluent samples. This approach can also provide a unique snapshot of indigenous microbial community with an option to compare taxonomic and functional properties across all sampling point within this study. A classical workflow for the taxonomic analysis of cassava effluent shotgun metagenomics data would includes quality trimming and the comparison of reference databases of selected marker gene or whole genomes to obtain a taxonomic profile for each of our isolates.

In light of the TAG accumulation results, the alternative pathways which could be used in TAG synthesis by CWW isolates need to be explored in full. The enzymes and the genes which code for these enzymes equally need to be defined. This information is required to update the available databases which hold such information.

Metabolic engineering technologies to improve lipid productivity of the isolate is an aspect that can be further explored based on the results from this study. The engineering to improve the lipid profiles of these isolates within cassava effluent can improve lipid yields. The overexpression of enzymes such as phosphatidate phosphatase Lppβ suggested within this study in streptomyces isolate is one such example that can increase TAG accumulation. The enhancement of fatty acid synthesis by the overexpression of Acetyl-CoA carboxylase could also be an aspect that can propel an increased TAG production. Another strategy that can be adopted in our isolates involves blocking competing pathways to enhance the metabolic flux channelled to TAG biosynthesis. This can be carried out via the repression of oxidation, phospholipid biosynthesis and repression of TAG degradation. The initial steps to carrying out these modifications would be the prediction of cellular response to constraints as stated above. The use of insilico modelling tools such as genome-scale metabolic models and constraint-based models can quantify our CWW isolate's metabolic potential there by allowing the probe into new phenotypes and metabolic network states that can result in the improvement of lipid yields.

The reproducibility of TAG accumulation by isolates grown in CWW medium on a larger scale needs to be defined in future studies. After which, the most effective lipid extraction method for lipid recovery from these isolates also needs to be explored. Furthermore, the most effective transesterification process from CWW isolates' TAG needs to be investigated. The assessment of these processes in the large scale biodiesel production has to be made considering the reproducibility in low to middle-income countries like Nigeria.

5.4. Reference

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Appendix

Cassava waste effluent isolate sequence for *Micromonospora* and *Streptomyces*

Sequences as seen below for *Micromonospora and Streptomyces* have been deposited in Genbank. Accession numbers and details of sequence is given below

3CPEGATSA1 Accession number:MWA26314

Isolated from cassava pressing effluent Reputable allied food processing

ATGCAGTCGAGCGGAAGGCCCTTCGGGGTACTCGAGCGGCGAACG
GGTGAGTAACACGTGAGCAACCTGCCCCAGGCTTTGGGATAACCCC
GGGAAACCGGGGCTAATACCGAATATGACCTTGCACCGCATGGTGT
TTGGTGGAAAGTTTTTCGGCTTGGGATGGGCTCGCGGCCTATCAGCT
TGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCC
TGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGC
CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAG
AAGCGCCGGCCAACTACGTGCCAGCAGCCGCGGTAAGACGTAGGG
CGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGG
CTTGTCGCGTCGACTGTGAAAACCCGCAGCTCAACTGCGGGCCTGC
AGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGACTGGAATTCC
TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGG
CGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAA
CGTTGGGCGCTAGGTGTGGGGGGCCTCTCCGGTTCCCTGTGCCGCA
GCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATG
CGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACAT
GGCCGCAAAACTGTCAGAGATGGCAGGTCCTTCGGGGGCGGTCACA
GGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTCGTTCGATGTTGCCAGCGCGTTA
TGGCGGGGACTCATCGAAGACTGCCGGGGTCAACTCGGAGGAAGG
TGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCAC
GCATGCTACAATGGCCGGTACAATGGGCTGCGATACCGTGAGGTGG
AGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAAC
TCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACG
CTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGT
CACGAAAGTCGGCAACACCCGAAGCCGNG

3CPEGATSA1B Accession number:MWA26315

Isolated from cassava pressing effluent Reputable allied food processing

GGGTACTCGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGC
CCCAGGCTTTGGGATAACCCCGGGAAACCGGGGCTAATACCGAATA
TGACCTCCGATCGCATGGTTGGTGGTGGAAAGTTTTTCGGCTTGGG
ATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCA
AGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC
GTAAGTGACGGTACCTGCAGAAGAAGCGCCGGCCAACTACGTGCC
AGCAGCCGCGGTAAGACGTAGGGCGCGAGCGTTGTCCGGATTTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAAC
CCGCAGCTCAACTGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGT
TCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGAT
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGGGGG
CCTCTCCGGTTCCCTGTGCCGCAGCTAACGCATTAAGCGCCCCGCCT
GGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGA
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CGTTCGATGTTGCCAGCGCGTTATGGCGGGGACTCATCGAAGACTG
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GCCCCTTATGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAA
TGGGCTGCGATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCT
CAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCG
CTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACGTCACGAAAGTCGGCAACACCC

CMESCA4TSA11 Accession number:MWA26316

Isolated from Matna cassava starch effluent

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GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
GGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGTAAGTG
ACGGTACCTGCAGAAGAAGCGCCGGCCAACTACGTGCCAGCAGCC
GCGGTAAGACGTAGGGCGCGAGCGTTGTCCGGATTTATTGGGCGTA
AAGAGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAACCCGCAGC

TCAACTGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAG
GGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG
GAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
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GGTTCTCTGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACC
TTACCTGGGTTTGACATGGCCGCAAAACTGTCAGAGATGGCAGGTC
CTTCGGGGGCGGTCACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCG
ATGTTGCCAGCGCGTTATGGCGGGGACTCATCGAAGACTGCCGGGG
TCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTT
ATGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGCTG
CGATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCG
GATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAA
TCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTAC
ACAC

3CPEGATSA70 Accession number:MWA26317

Isolated from cassava pressing effluent Reputable allied food processing

CGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCAGGC
TTTGGGATAACCCCGGGAAACCGGGGCTAATACCGAATATGACCTT
GCATCGCATGGTGTTTGGTGGAAAGTTTTTCGGCTTGGGATGGGCTC
GCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACG
ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACG
GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGTAAGTGA
CGGTACCTGCAGAAGAAGCGCCGGCCAACTACGTGCCAGCAGCCG
CGGTAAGACGTAGGGCGCGAGCGTTGTCCGGATTTATTGGGCGTAA
AGAGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAACCCGCAGCT
CAACTGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGG
GGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTA
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TTCCCTGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTA
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AGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTA
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CGGGGGCGGTCACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
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ACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATG
TCCAGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGCTGCGA
TACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGAT
CGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCG
CAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
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3CWEGATSA52 Accession number:MWA26318

Isolated from cassava washing effluent

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GCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGG
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GCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
GCAACGCGAAGAACCTTACCTGGGTTTGACATCGCCGGAAATCCTG
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CGGTACAATGGGCTGCGATACCGTGAGGTGGAGCGAATCCCAAAA
AGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAG
TCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGCA
ACACCC

3CPEGATSA12 Accession number:MWA26314

Isolated from cassava washing effluent Reputable allied food processing

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TGGTGGAAAGTTTTTCGGCTTGGGATGGGCTCGCGGCCTATCAGCTT
GTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCC
TGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
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2CPEGATSA42 Accession number:MWA26320

Isolated from cassava pressing effluent Bilatrade food processing

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GTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
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TGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAGA
AGCGCCGGCCAACTACGTGCCAGCAGCCGCGGTAAGACGTAGGGC

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2CFEGATSA32 Accession number:MWA26321

Isolated from cassava fermentation effluent Bilatrade food processing

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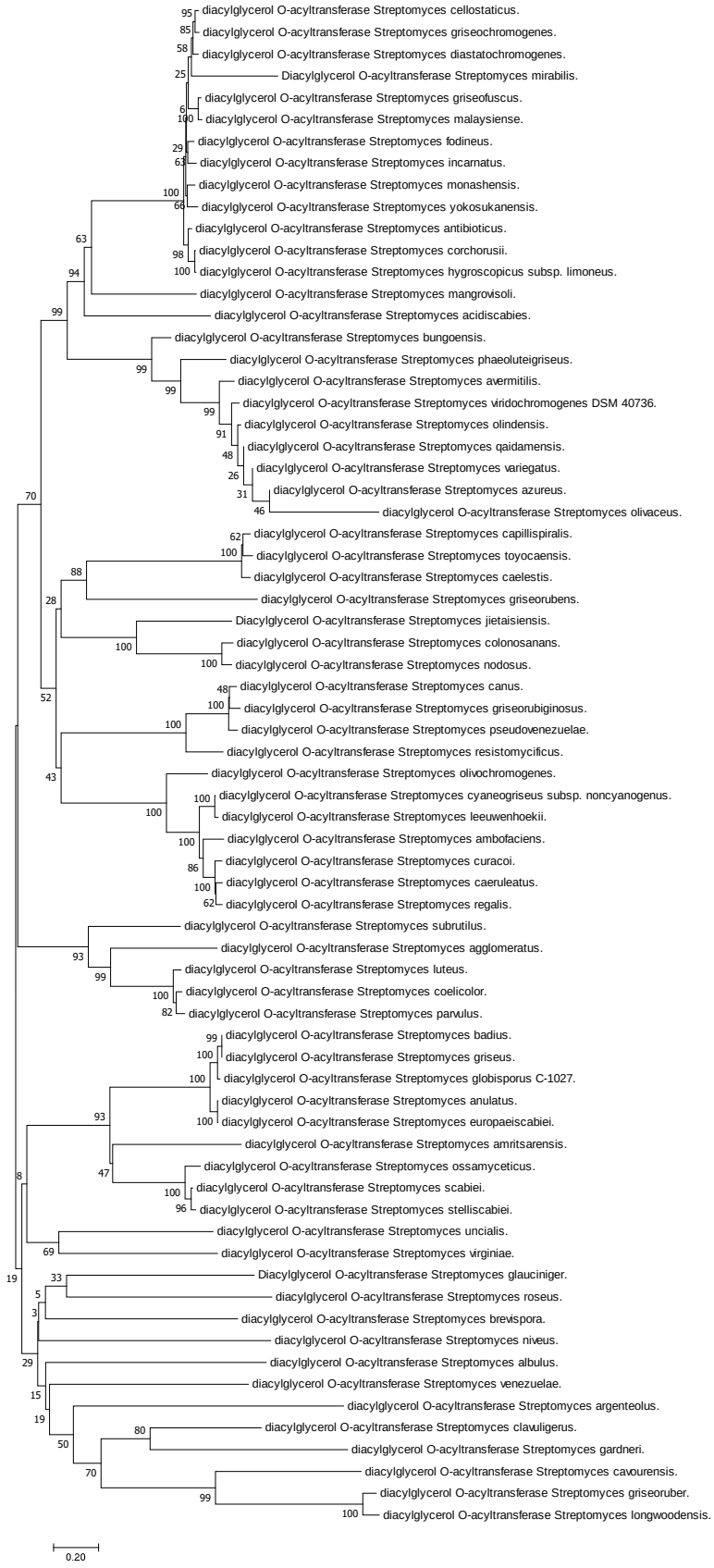
GAGCGAATCCCAAAAAGCCGGTNTCAGTTCGGATCGGGGTCTGCAA
CT

Streptomyces STRAIN 3CPEGATSA66 (Accession number:MWA26323).

Isolated from cassava pressing effluent Reputable allied food processing

TGCAGTCNNCGATGAACCGCNTTNNNNGGCGGGGATTAGTGGCGA
ACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAG
CCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGG
TGGATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTAT
CAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
AAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT
TGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGC
AGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTA
GGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGG
TCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGA
ATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
GTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTG
CCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGA
GCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTT
GACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGC
AGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAAC
TCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTC
TTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATA
CCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTG
GGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA
GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACGTCACGAAAGTCGGTAACACCCGAANCCGG

DGAT PRIMER DESIGN



PHYLOGENETIC TREE

ATF phylogenetic tree attached

The evolutionary history was inferred using the Neighbor-Joining method The optimal tree with the sum of branch length = 7.19803315 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction methodand are in the units of the number of amino acid substitutions per site. The analysis involved 72 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 193 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

PRIMERS

Primer was designed from the alignment of DGAT consensus sequence from available DGAT data available on NCBI website

SEQUENCE FROM ALIGNMENT

ELPPGDFAEEASRLAGELMERPLERGIPPWEMYVLAGGPTEGGAPGGR

PFAVLVKLHHALADGMRAVAIGAGIFDEIA

BACK TRANSLATED SEQUENCE

GAGTTGCCGCCGGGGGATTTTGCGGAGGAGGCGAGGAGGTTGGCG

GGGGAGTTGATGGAGAGGCCGTTGGAGAGGGGGATACCGCCGTGG

GAGATGTATGTGTTGGCGGGGGGGCCGACGGAGGGGGGGGCGCCG

GGGGGGAGGCCGTTTGCGGTGTTGGTGAAGTTGCATCATGCGTTGG

CGGATGGGATGAGGGCGGTGGCGATAGGGGCGGGGATATTTGATG

AGATAGCG

PRIMER PICKING RESULTS FOR ATF primers Streptomyces

Template masking not selected
No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	46	19	59.47	63.16	0.00	0.00	0.00	**GGGGAGTTGATGGAGAGGC**
RIGHT PRIMER	222	20	59.09	55.00	0.00	0.00	0.00	**AAATATCCCCGCCCCTATCG**

SEQUENCE SIZE: 234
INCLUDED REGION SIZE: 234

PRODUCT SIZE: 177, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

1
GAGTTGCCGCCGGGGGATTTTGCGGAGGAGGCGAGGAGGTTGGCG
GGGGAGTTGATGGAG
>>>>>>>>>>>>>>>

61
AGGCCGTTGGAGAGGGGGATACCGCCGTGGGAGATGTATGTGTTGG
CGGGGGGGCCGACG
>>>>

121
GAGGGGGGGGCGCCGGGGGGGAGGCCGTTTGCGGTGTTGGTGAAG
TTGCATCATGCGTTG

181
GCGGATGGGATGAGGGCGGTGGCGATAGGGGCGGGGATATTTGAT
GAGATAGCG
<<<<<<<<<<<<<<<<<<<

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

SEQUENCE FROM ALIGNMENT

RRTAGVSVDLDDVHHVRKTTGGTVNDVLIAVVAGALRRWLDERGDG
SE

BACK TRANSLATED SEQUENCE

cgccgcaccgcgggcgtgagcgtggatctggatgatgtgcatcatgtgcgcaaaaccacc
ggcggcaccgtgaacgatgtgctgattgcggtggtggcgggcgcgctgcgccgctggctg
gatgaacgcggcgatggcagcgaa

PRIMER PICKING RESULTS FOR ATF primers Streptomyces

Template masking not selected
No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	16	20	59.54	55.00	0.00	0.00	0.00	**gtgagcgtggatctggatga**
RIGHT PRIMER	135	18	60.59	61.11	13.39	0.00	0.00	**atcgccgcgttcatccag**

SEQUENCE SIZE: 144
INCLUDED REGION SIZE: 144

PRODUCT SIZE: 120, PAIR ANY_TH COMPL: 14.52, PAIR 3'_TH COMPL: 14.52

```
  1 cgccgcaccgcgggcgtgagcgtggatctggatgatgtgcatcatgtgcgcaaaaccacc
              >>>>>>>>>>>>>>>>>>>>
 61 ggcggcaccgtgaacgatgtgctgattgcggtggtggcgggcgcgctgcgccgctggctg
                                                  <<<
121 gatgaacgcggcgatggcagcgaa
    <<<<<<<<<<<<<<<<
```

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

SEQUENCE FROM ALIGNMENT

PRSAHPQGNRLSGYLMRLPVGDPDPLARLGTVRAAMDRNKDAGPGR

GAGAVALLADHVPALGHRLGGPLVSGAARLWFDLLVTSVPLPSLGLR

LGGHPLTEVYPLAPLARGHSLAVAVSTYRGRVHYGLLADAKAVPDLD

RLAVAVAEEVETLLTACRP

BACK TRANSLATED SEQUENCE

ccgcgcagcgcgcatccgcagggcaaccgcctgagcggctatctgatgcgcctgccggtg
ggcgatccggatccgctggcgcgcctgggcaccgtgcgcgcggcgatggatcgcaacaaa
gatgcgggcccgggccgcggcgcgggcgcggtggcgctgctggcggatcatgtgccggcg
ctgggccatcgcctgggcggcccgctggtgagcggcgcggcgcgcctgtggtttgatctg
ctggtgaccagcgtgccgctgccgagcctgggcctgcgcctgggcggccatccgctgacc
gaagtgtatccgctggcgccgctggcgcgcggccatagcctggcggtggcggtgagcacc
tatcgcggccgcgtgcattatggcctgctggcggatgcgaaagcggtgccggatctggat
cgcctggcggtggcggtggcggaagaagtggaaaccctgctgaccgcgtgccgcccg

PRIMER PICKING RESULTS FOR ATF primers Streptomyces

Template masking not selected
No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	230	20	58.83	55.00	0.00	0.00	0.00	**ggtttgatctgctggtgacc**
RIGHT PRIMER	455	20	59.41	55.00	0.00	0.00	0.00	**gtttccacttcttccgccac**

SEQUENCE SIZE: 477
INCLUDED REGION SIZE: 477

PRODUCT SIZE: 226, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

1 ccgcgcagcgcgcatccgcagggcaaccgcctgagcggctatctgatgcgcctgccggtg

61 ggcgatccggatccgctggcgcgcctgggcaccgtgcgcgcggcgatggatcgcaacaaa

121 gatgcgggcccgggccgcggcgcgggcgcggtggcgctgctggcggatcatgtgccggcg

181 ctgggccatcgcctgggcggcccgctggtgagcggcgcggcgcgcctgtggtttgatctg
>>>>>>>>>>>

241 ctggtgaccagcgtgccgctgccgagcctgggcctgcgcctgggcggccatccgctgacc
>>>>>>>>>

301 gaagtgtatccgctggcgccgctggcgcgcggccatagcctggcggtggcggtgagcacc

361 tatcgcggccgcgtgcattatggcctgctggcggatgcgaaagcggtgccggatctggat

421 cgcctggcggtggcggtggcggaagaagtggaaaccctgctgaccgcgtgccgcccg
<<<<<<<<<<<<<<<<<<<<