Enhancement of anaerobic digestion of sisal leaf decortication residues by biological pre-treatment

By

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DECLARATION

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To

Mrs. Viola J. Munyoki, in recognition of her invaluable assistance and encouragement, which made completion of this research a reality

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ABSTRACT

In recent years, the use of agro-industrial residues as sources of biofuels has gained great attention worldwide due to limited reserves of fossil fuels. The sisal industry in Tanzania generates large quantities of sisal leaf decortications residues (SLDR) with good potential for bio-methanation. However, the process is limited by the lignocellulosic nature of the SLDR. To improve methane production from the residue, pre-treatment is essential prior to anaerobic digestion. In this study, the effect of biological pre-treatment of SLDR with lignolytic and cellulolytic fungi using different inoculum concentrations and incubation periods, singly and in combination was investigated in anaerobic batch bioreactors at a volatile solids (VS) loading rate of 5.84 grams VS. Pre-treatment of SLDR for 4 days with strain CCHT-1 and for 8 days with *Trichoderma reesei* at inoculum concentrations of 10% and 25% separately, gave methane yields of 0.203 m^3 CH_4/kg VS_{added} and 0.192 m^3 CH_4/kg VS_{added}, respectively. In a two-steps pre-treatment using the two organisms in succession, first using strain CCHT-1 followed by *T. reesei*, an increase of about 101% in methane yield was obtained. On the other hand using *T. reesei* first followed by strain CCHT-1, the methane yield increase dropped to 30%. Within the experimental conditions, the results confirmed that biological pre-treatment has the potential to achieve significant improvement in biogas production from SLDR in a two-steps pre-treatment approach using CCHT-1 followed by *T. reesei*. It was concluded that this large reservoir of biomass from the sisal industry could be harnessed for methane production by sisal decortication factories.

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
ADF	Acid detergent fibre
mL	Millilitres
NDF	Neutral detergent fibre
SLDR	Sisal leaf decortication residues
SSF	Solid-state fermentation
TS	Total solids
VS	Volatile solids

CHAPTER ONE

1.0 GENERAL INTRODUCTION

The search for alternative energy sources is an ongoing effort throughout the world to achieve a completely sustainable energy supply. The world is rapidly depleting its supply of natural gas, which is known to be the cleanest of the fossil fuels and various developed countries such as those in European Union are trying to move away from the use of hazardous and waste producing energy sources (van Lier *et al* ., 2001).

Renewable energy sources are often prioritized in efforts to mitigate the greenhouse effect (Chynoweth *et al.,* 2001). Production of methane-rich biogas through anaerobic digestion of organic materials provides a versatile carrier of renewable energy. Methane can be used in replacement for fossil fuels in both heat and power generation and as a vehicle fuel, thus contributing to cutting down the emissions of greenhouse gases and slowing down climate change. Methane production through anaerobic digestion has been identified as one of the most energy-efficient and environmentally benign ways of producing vehicle biofuel, since emissions from the combustion of biogas are lower than those from fossil fuels (Ghosh, 1997).

Residual plant biomass from agriculture, agro-industry and forestry constitutes a major source of renewable energy. Owens and Chynoweth (1993) showed that nearly all organic biomass materials can be digested under anaerobic conditions to produce

methane, and the residual solids which are odour free serve as an excellent soil amendment/compost. Subtropical and tropical countries are the richest sources of residual plant biomass (Svensson *et al.*, 2007). In Tanzania alone, the sisal industry with a production of 45,000 tones of sisal fibre in 2007 generated 4.5 million m^3 of sisal decortications wastewater and 1,125,000 tonnes of sisal solid residues, of which about 900,000 tones is Sisal leaf decortication residues (SLDR), the rest being short fibres residues (information obtained from Hale sisal Estate). At all but one sisal decortication factories in the country, both sisal solid residues and wastewater are disposed of untreated resulting in serious environmental pollution problems. The residues are degraded by microorganisms and in the anaerobic part of the biodegradation process; methane is produced and emitted into the atmosphere.

Methane has a global warming potential that is 21-56 times higher than that of carbon dioxide, and is estimated to contribute to 18-21% of the overall global warming (Ayalon *et al.*, 2001). On the other hand, solid sisal residues have been recently reported to be suitable feedstock for biogas production (Mshandete, 2005). However, the extent of bioconversion of the residues into methane rich biogas is mainly limited by its lignocellulolytic nature and pre-treatment is one way of improving the performance of bioreactors treating it. Pre-treatment can be mechanical, physico-chemical or biological or in combinations (Mshandete *et al.*, 2005, 2006; Björnsson *et al.*, 2005). Biological pre-treatment of lignocelluloses substrates includes the use of white-rot fungi, pre-composting, hydrolytic bacteria and commercial enzymes (van Lier *et al.*, 2001). The

aim of this study was to investigate some biological pre-treatment of SLDR, which could improve the subsequent anaerobic digestion for increased biogas production.

1.1 LITERATURE REVIEW

1.1.3 Fundamentals of the anaerobic biodegradation process

Anaerobic digestion is a multi-step biological process where the organic carbon is converted to its most oxidized carbon dioxide (CO_2) and most reduced methane (CH_4) state (Angelidaki *et al.*, 2003). The main product of the process is biogas which is a mixture of methane and carbon dioxide, as well as trace gases such as hydrogen sulphide and hydrogen. Anaerobic digestion (AD) is a highly promising technology for converting biomass waste into vast quantities of methane. This can directly be used as an energy source, or converted to hydrogen (Albertson *et al.*, 2006). The anaerobic degradation occurs through the synergistic interaction of four different classes of microorganisms; hydrolytic/liquefaction, acidogenesis/fermentative, acidogenic and methanogenic bacteria in a multi-step process (Adney *et al.*, 1991).

1.1.3.1 Hydrolysis

Hydrolysis is an extra-cellular in which organic particulates are broken down to soluble oligomers and monomers. It is an important step prior to fermentation process, as the fermentative bacteria cannot absorb complex organic polymers directly into their cells.

Hydrolytic enzymes include cellulase, cellobioase, xylanase and amylase for degrading polysaccharides into sugars, protease for degrading protein into amino acids, and lipase for degrading lipid into glycerol and long-chain fatty acids (LCFA) (Parawira *et al.*, 2005). The hydrolysis process itself involves several steps, including enzyme production, diffusion, adsorption, reaction and enzyme deactivation (Batstone *et al.*, 2002).

The overall hydrolysis rate depends on organic material size, shape, surface area, enzyme production and adsorption (Batstone *et al.*, 2000). Moreover, competitive adsorption of enzyme on the inert substrate like lignin can also decrease hydrolysis efficiency (Converse and Optekar, 1993). Hydrolysis has been shown to be a rate-limiting step for digestion of highly particulate substrates like agro-industrial residues, municipal solid wastes, swine waste, cattle manure and sewage sludge while methanogenesis is the rate-limiting step for readily degradable substrate, due to inherent slow growth nature of methanogens (Björnsson *et al.*, 2001).

1.1.3.2 Acidogenesis/fermentative

During acidification, sugars, long chain fatty acids and amino acids resulting from hydrolysis are used as substrates by fermentative microorganisms to produce organic acids (Kalyuzhnyi *et al.*, 2000). Glucose fermentative microbes have branched metabolisms, which means they are able to metabolise the substrate via different

pathways which yield different amounts of energy and produce different fermentation products (Dolfing, 1988). The fermentative bacteria can function at high concentrations of hydrogen and formate (Batstone *et al.*, 2002). The dominant pathway depends on several factors such as substrate concentration, pH and dissolved hydrogen concentrations (Rodríguez *et al.*, 2005). Under very high organic load, lactic acid production becomes significant (Mattiasson, 2004). At low pH (< 5) the production of ethanol is increased, while at higher pH (> 6) volatile fatty acids (VFAs) are produced (Horiuchi *et al.*, 1999). At pH lower than 4, fermentation may cease (Hwang *et al.*, 2004).

1.1.3.3 Acetogenesis

During acetogenesis the organic acids produced during acidogenesis are converted into hydrogen (H_2) and acetate by the acetogenic bacteria (Parawira *et al.*, 2005). These conversions of volatile fatty acids are important as these acids, are mainly lethal to the methanogenic bacteria (Forday and Greenfield, 1983). The products from fermentation step consist approximately 51% of acetate, 19% of H_2, and the rest are more reduced products such as higher VFA, alcohols or lactate (Angelidaki *et al.*, 2002). Fermentation products such as fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, and branched-chain and aromatic fatty acids, cannot directly be used in methanogenesis (Stams *et al.*, 2005). In acetogenesis, these products are oxidized to acetate and H_2 by obligated proton reducing bacteria in syntrophic relationship with

methanogenic archaea as low H_2 partial pressure is essential for acetogenic reactions to be thermodynamically favorable ($\Delta G > 0$) (Schink, 1997).

1.1.3.4 Methanogenesis

During methanogenesis, the fermentation products such as acetate and H_2/CO_2 are converted to CH_4 and CO_2 by methanogenic archaea; a unique group of microorganisms, phylogenetically different from the main group of prokaryotic microorganisms (Wheeler and Rome, 2002). Only a limited number of compounds can act as substrate in methanogenesis among these are acetate, H_2/CO_2, methanol and formate (Gujer and Zehnder, 1983). Methanogens live at a pH range of 7-8 (Lastella *et al.*, 2002). The acetoclastic methanogens convert acetate to methane while the CO_2-reducing methanogens convert CO_2 to methane (Novaes, 1986).

Methanogenesis is affected by bioreactor operating conditions such as temperature, hydraulic loading rate, organic loading rate, and feed composition (Fey and Conrad, 2000; Murto, 2003). Moreover, apart from methanogenic reactions, the inter-conversion between hydrogen and acetate catalysed by homoacetogenic bacteria also plays an important role in the methane formation pathway. Homoacetogens can either oxidize or synthesize acetate depending on the external hydrogen concentration (Schink, 2002; Kotsyurbenko, 2005). This makes them able to compete with several different microbes, including methanogens.

In conclusion, the rate limiting steps in anaerobic digestion are hydrolysis and methanogenesis due to the slow growth nature of methanogens. Anaerobic digestion process is also strongly influenced by the prevailing environmental physico-chemical conditions. These include: temperature, pH and buffering capacity (also called alkalinity), retention time, agitation, substrate constituents, nutrients, toxic substances and process inhibitors (Cirn, 2006). These are the main parameters to be optimised in order to make a suitable environment for the microorganisms, which serve as process biocatalysts (Björnsson *et al.*, 2000).

1.2 Anaerobic digestion of organic biomass for methane production

Millions of tones of solid organic waste generated each year from municipal, industrial, agro-industrial and agricultural sources worldwide that are suitable for bio-energy production to date are not fully utilized (Mshandete *et al.*, 2005). Additionally, methane and carbon dioxide released from landfills as a result of microbial activity under anaerobic condition is a major concern for global warming (Baldasano and Soriano, 2000; Chynoweth *et al.*, 2001).

The use of renewable biomass (including energy crops and organic wastes) as an energy resource is not only "greener" with respect to most pollutants, but its use represents a closed balanced carbon cycle regarding atmospheric carbon dioxide (Spencer, 1991). In

addition to the potential environmental hazard, the long term monitoring and mitigation of biogas emissions from landfills is a major financial burden to municipalities and industries (Yu *et al.*, 2002). Thus biological conversion of organic biomass to methane has received increasing attention in recent years (Yadvika *et al.*, 2004). Hand and mechanically-sorted municipal solid wastes and nearly 100 genera of fruit and vegetables solid wastes, leaves, grasses, woods, marine and fresh water biomass have been explored for their anaerobic digestion potential (Gunaseelan, 2004).

Extensive literature under various categories and the influence of several parameters on the methane potential of feedstocks has been reported by a number of workers as reviewed by Gunaseelan (1997). Almost all terrestrial and water based species examined to date either have good digestion characteristics or can be pre-treated to enhance biogas yields (Yadvika *et al.*, 2004). However, considering the fact that plant biomass yield as one of the parameters that make biomass to methane (CH_4) conversion economically and technically feasible, the number of unexplored genera to be screened is still enormous (Gunaseelan, 1997; 2004). Sisal plant (*Agave sisalana*) is amongst the agro-industrial crops, which generates huge quantities of organic biomass. However, there is little published information on biomethanation with respect to methane yield and pre-treatment studies to improve biogas production (Björnsson, *et al.*, 2005; Mshandete *et al.*, 2006). Nevertheless, in East Africa, as in many developing countries there seems to be limited knowledge on biogas production from other substrates other than the traditionally used animal manure employed in low performing traditional Chinese-type

and Indian-type bioreactors which tends to limit the technology to cattle rearing areas (Mshandete *et al.*, 2004).

1.3 Lignocellulose

Lignocellulose is a collective term for the three major components of plant cell wall, namely cellulose (35-45% w/w), hemicellulose (25-45% w/w) and lignin (15-30% w/w) (Betts *et al.*, 1992). In addition to these compounds, plant biomass can contain e.g. non-structural carbohydrates (such as glucose, fructose, sucrose and fructans), proteins, lipids, extractives and pectins (McDonald *et al.*, 1991). The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003).

1.3.1 Cellulose

Cellulose is a linear homopolymer of glucose units linked. It is chemically simple because, it contains repeating units of glucose, but has a complex structure of the long chains of glucose subunits joined together by β–1,4–linkages (Figure 1.1) (Lynd *et al.*, 2002). Cellulose is stabilised by some interactions, which are weak individually but collectively form strong bonds. The chains are in layers held jointly by van der Waals forces and hydrogen–bonds (intra molecular and intermolecular) (Gan *et al.*, 2003). In nature, the cellulose fibres are tightly surrounded by other polymers such as xylan, other hemicellulose components and lignin in a matrix (Pohlschröder *et al.*, 1994). This

interaction is a critical structural characteristic preventing the rate and level of its utilization.

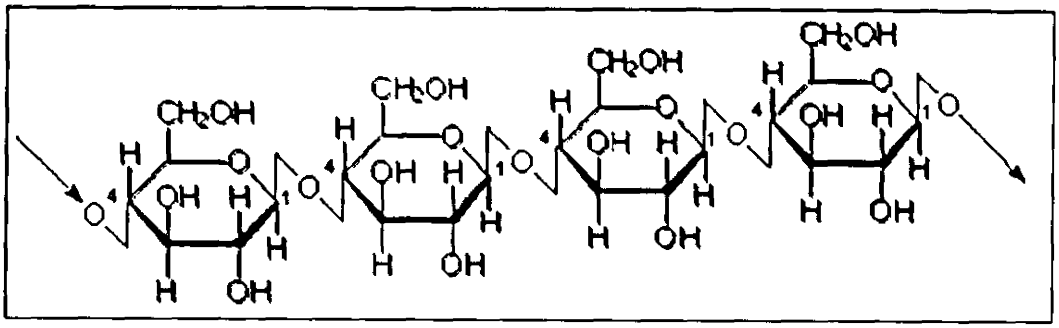

Figure 1.1 Diagram showing the β –1,4–linkages in cellulose (Adapted from Samejima et al., 1998).

1.3.1.1 Enzymatic Hydrolysis of Cellulose

Cellulose is recalcitrant to enzymatic hydrolysis and the degradation of its crystalline structure is a complex process requiring the participation of many enzymes (Schwarz, 2001). Cellulases degrade cellulose to yield a soluble disaccharide called cellobiose which on further hydrolysis results in D-glucose (Chynoweth and Pullammanappallil, 1996).

1.3.2 Hemicellulose

Hemicelluloses are composed of both linear and branched heteropolymers of pentoses (D-xylose, L-arabinose), hexoses (mostly D-mannose, D-glucose, D-galactose and sugar acids (D-glucuronic) (Eriksson *et al.*, 1990). However, only the heteropolysaccharides, those with a much lower degree of polymerisation as compared to that of cellulose are

referred to as hemicelluloses (Howard *et al.*, 2003). Hemicellulose are classified into; xylans, mannans, and galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkage. Xylan is by far the most important component because of its large quantities in the biomass (Hopkins, 1999). It was reported that grasses contain 20-40% of arabinoxylans, while the principal hemicellulose in hardwood is glucomannan and methylglucuronoxylan (Brigham *et al.*, 1996).

1.3.3 Lignin

Lignin is the most abundant biopolymer next to cellulose and contains 1.5 times the carbon content of cellulose; it is the material that confers the qualities of rigidity and durability that make woody plants "woody" (Kent, 1981). It makes up about 15-30 % of wood, and is found in cell walls, in a complex with cellulosic and hemicellulosic polysaccharides. Lignin is aromatic, 3- dimensional and amorphous (Thomson J.A, 1993). In general, lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl and p-coumaryl); it is synthesized from phenyl propanoid precursors by polymerization in higher plants (Sarkanen and Ludwic, 1971). Lignin is further linked to both hemicelluloses and cellulose forming a physical seal around them, which is an impenetrable barrier preventing penetration of solutions and enzymes which makes it hard to be degraded by many microorganisms like other natural polymers (cellulose, starch, proteins, etc.) (Howard *et al.*, 2003). The knowledge of lignocellulolytic activities of microbial organisms is important to the understanding of plant biomass

recycling in nature and the use of enzymes in controlled lignocellulose conversions (Thomson, 1993). The only organisms known to extensively degrade lignin are fungi (Kirk and Farrell, 1987). Because lignin is an insoluble polymer, the initial steps in its biodegradation must be extracellular. Fungi secrete extracellular enzyme systems to metabolize lignin. Though anaerobic bacteria are capable of degrading the monomeric units that make up the lignin molecule, it is doubtful whether lignin can be depolymerized to these monomers under conditions that prevail in anaerobic digesters (Odier and Artaud, 1992). If the associated lignin component is not chemically or biologically modified or removed, cellulose is not available as a carbon source for other microbial decomposers (Boominathan and Reedy, 1992). Many enzymes are involved in the oxidative degradation of lignin, including lignin peroxidases (LiP), manganese peroxidase (MnP), and laccase (Sugiura *et al.*, 2003)

Anaerobic biodegradation of solid lignocellulosic organic biomass starts with microbial enzymatic hydrolysis (Morgenroth *et al.*, 2002). The hydrolysis rate of particulate substrates is lower than that of dissolved polymers, as with the former only part of the substrate is accessible to the enzymes (Veeken and Hamelers, 1999). The performance of a bioreactor digesting a complex lignocellulosic substrate is dependent on the type of microorganisms involved, the enzyme activities, the physicochemical characteristics of the substrate and the environmental conditions in the bioreactor (Sanders *et al.*, 2003). Earlier investigations have shown the potential use of enzyme measurements to characterize anaerobic digestion processes. Protease activity was shown to be a good

indicator of digester stress during overload (Thiel and Hattingh, 1967). Thiel *et al.* (1968) measured four hydrolytic enzymes, namely amylase, protease, cellobiase and phosphatase. Whereby, during normal digestion, the activity of enzymes reached stable levels but exhibited significant variations approaching failure of the bioreactor.

1.4 Pre-treatment

One way of improving methane production from anaerobic digestion of lignocellulosic feedstocks is pre-treatment of the substrate in order to break the polymer chains to more easily accessible soluble compounds (Delgenès *et al.*, 2003). An ideal pre-treatment would increase surface area and reduce lignin content and crystallinity of cellulose. Pre-treatment can be divided into different categories: physical/mechanical (e.g. milling, grinding and irradiation), chemical (e.g. alkali, dilute acid, oxidizing agents and organic solvents), physicochemical (e.g. steam pre-treatment/autohydrolysis, hydrothermolysis and wet oxidation) and biological, or combinations of these (Mshandete *et al.*, 2005, 2006; Björnsson *et al.*, 2005).

1.4.1 Physical /Mechanical Pre-treatment

Physical pre-treatments offering potential for improving methane yields from lignocellulosic materials are steam explosion, thermal hydrolysis, wet oxidation, pre-incubation in water, and treatment with ultrasound or radiation (Sun and Cheng, 2002; Fox and Noike, 2004). Mechanical size reduction of the particles and the resulting

increase in the available surface area, represent an option for increasing the biodegradation yields and accelerating the anaerobic digestion of substrates that have a high fibre content and low bioavailability such as sisal fibres, manures, straw etc. (Angelidaki *et al.*, 2002). However, the cost of the energy renders this method unsuitable in the long term for pre treatment (Takashima *et al.*, 1996).

1.4.2 Chemical Pre-treatment

Chemical pre-treatment include treatment with acids, alkalis, solvents or oxidants (Sun and Cheng, 2002). Alkaline pre-treatment is known to break the bonds between hemicellulose and lignin as well as swell the fibres and increase the pore size, therefore facilitating hydrolysis (Baccay and Hashimoto, 1984; Pavlostathis and Gossett, 1985). Alkali treatments increases microbial digestability and biogas production twice when the plant residues are used as cattle dung supplement (Dar and Tandon 1987). In certain cases, chemical addition may create inhibitory by-products, which disrupt and decrease the performance of anaerobic digestion (Ardic and Tanner, 2005). Furthermore, Na^+ is known to be an inhibitory ion to some methanogenic flora at high concentrations (He *et al.*, 2006). Chemical costs may as well prohibit the economical feasibility of its use as a pre-treatment strategy.

1.4.3 Biological pre-treatment

Biological pre-treatment includes the use of white-rot fungi and pre-composting treatment. Microbes and/or microbial enzymes, enzyme complexes, and digester percolate are also used for partial biodegradation of lignocellulose (Mata-Alvarez *et al.*, 2000). Biological pre-treatment methods have not been developed as extensively as physical-chemical methods for improving hydrolysis of lignocellulosic substrates. However, Biological pre-treatment methods have the advantage that they are simple and do not require major capital investments (Lissens *et al.*, 2003). So far the reported increases in biogas yields by biological pre-treatments of lignocellulosics have been relatively at an average of 20% (Lissens *et al.*, 2003). White-rot fungi are the only known living organisms capable of complete lignin biodegradation, and their application has been suggested for partial delignification to increase digestibility and enhance biogas production from lignocellulosic substrates such as wheat straw (Müller and Trösch 1986; Ghosh and Bhattacharyya, 1999).

Aerobic degradation using soft-rot fungi such as *Chaetomium celluloyticum* a lignolytic fungus has been proposed for efficient anaerobic digestion of recalcitrant organic matter in digested residues (Schober and Trösch, 2000). Moreover, aerobic pre-treatment prior to anaerobic batch digestion of sisal pulp waste increased methane yield by 26% compared to (control) untreated sisal pulp waste (Mshandete *et al.*, 2005). The addition of hydrolytic microorganisms as a method of pre-treatment increases the yield and the

rate of particulate matter solubilization during anaerobic digestion of cellulose-rich materials (Delgenès *et al.*, 2003). In this respect, Del Borghi *et al.* (1999) reported an increase in soluble chemical oxygen demand (COD) of a mixture of sewage sludge and the organic fraction of municipal solid waste as the result of bacterial hydrolysis of polymeric materials using consortia of hydrolytic bacteria from activated sludge. Along a similar line of pre-treatment, Hasegawa *et al.* (2000) reported 50% improvement in methane yield when sewage sludge was solubilised under thermophilic aerobic conditions, prior to conventional anaerobic digestion. This improvement was attributed to bacterial extracellular enzymes.

Hatakka (1983) studied the pre-treatment of wheat straw by 19 white rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus osteatus* in five weeks. On the other hand, contradictory results have been reported recently on biological pre-treatment of grass using white rot and composting methods. The white rot fungi treatment (21 days at 21°C) and short-term composting (7 days) resulted in high losses of organic matter due to biological activity leading to a decrease in methane potential as reported by Lehtomäki *et al.*, 2004. Therefore, when designing appropriate pre-treatment methods for anaerobic digestion of lignocellulosic biomass, the costs, practicability and environmental impacts of pre-treatments, as well as the losses of organic matter and energy content of substrates during pre-treatments, need to be weighed against the overall benefits of pre-treating the biomass (Sun and Cheng, 2002; Lehtomäki *et al.*, 2004).

1.5 Pre-treatment of lignocellulosic biomass intended for anaerobic bioconversion

The bioconversion of lignocellulosic materials (wood, plants, and crop residues) to methane is hindered by their relative resistance to enzymatic hydrolysis. The effect of pre-treatment of lignocellulosic material has been recognized for a long time (McMillan, 1994). Van Lier *et al.*, (2001) postulated that, future developments of anaerobic treatment of lignocellulosic materials would be the enhancement of the process by pre-treatment as a core technology in recycling processes. Therefore, in recent years considerable efforts have been made to further improve the performance of anaerobic digestion of different wastes, especially solid wastes, by means of pre-treatment (Lissens *et al.*, 2003; Yadvika *et al.*, 2004). Application of pre-treatments for facilitating enzymatic hydrolysis and consequent ethanol production from lignocellulosic substrates has been quite intensively investigated as reviewed by Sun and Cheng (2002). However, there is inadequate information on the effects of pre-treating lignocellulosic agro-industrial residues for enhanced methane production. One of the studies on pre-treatment of agro-industrial residues for enhanced methane production applied reduction in particle size. Using sisal decortications residues, a potential increase in methane yield of 23% was recorded for 2 mm fibres compared to untreated control sisal fibres residues (Mshandete *et al.*, 2006). With most substrates, there is a threshold value under which further reduction in particle size becomes uneconomical (Chynoweth *et al.*, 1993).

Very little has been reported in the literature on biological pre-treatment of solid sisal residues for improved biogas production apart from the work by Mshandete *et al.*

(2005). Pre-treatment is capable of jump starting the breakdown of insoluble substrate, allowing for improved availability of cellulose material and increasing the ratio of solubles:insolubles (Tang *et al.*, 1996). Therefore, further studies are needed in the area of enhancement technologies (especially biological pre-treatment) aimed at improving anaerobic digestion of particulate organic matter of sisal residues. In this study, biological pre-treatment using two fungal species *Trichoderma reesei* and strain CCHT-1 was investigated. CCHT-1 is a fungus found growing on piles of composting sisal residues. Its taxonomic status is yet to be elucidated. *Trichoderma reesei* is a versatile filamentous fungus and highly efficient producer of extracellular enzymes such as cellulases and hemicellulases (xylanases) (Li *et al.*, 2005).

1.6 *Trichoderma reesei*

T. reesei is a member of filamentous ascomycetes that is used industrially due to its ability to produce extracellular lignocellulose-degrading hydrolyses in large amounts (Li *et al.*, 2005). *T. reesei* is an acidophilic fungus and most of its secreted cellulases function optimally at around pH 5. Cellulases are very potent industrial enzymes in various processes based on renewable materials. Members of the fungal genus *Trichoderma* have been extensively studied, particularly due to their ability to secrete cellulose-degrading enzymes or to act as biocontrol agents. Most of the work has been carried out on strains of *T. viride, T. reesei and T. harzianum* (Eveleigh, 1987). *T. reesei* and its mutants are recognized as the best strains for the industrial production of

cellulases due to its complete composition of cellulose complex, high productivity and stability during the saccharafication process (Kadam, 1996).

The cellullases from *T. reesei* comprise at least three classes of enzymes: endo-1,4-β-D-glucanase that cleaves the internal glycosidic bonds of cellulose chains at random and produces more chain ends on which exo-1,4-β-D-glucanase may act; 1,4-β-D-glucan cellobiohydrolase or exoglucanase that cleaves cellobiose units from the non reducing end of cellulose chains; and 1,4-β-D-glucosidase that hydrolyses the cellobiose to produce glucose (Medve *et al.*, 1994; Hoshino *et al.*, 1997).

1.7 Solid state fermentation (SSF)

Solid-state fermentation (SSF) is any fermentation process occurring in the absence or near absence of free liquid, employing an inert substrate or a natural substrate as a solid support. SSF produces a high product concentration with a relatively low energy requirement (Yang and Yuan, 1990), and is considered as the most appropriate method for filamentous fungi cultivation and lignocellulolytic enzyme production. This is because, the fungi grow under conditions close to their natural habitats and are capable of producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures (Pandey *et al.*, 1999). Various agricultural substrates, by-products and white rot fungi have been used successfully in SSF for ligninolytic enzyme production (Rodrı´guez-Couto *et al.*, 2005). The hyphal

mode of fungal growth and their good tolerance to low water activity (A_w) and high osmotic pressure conditions make fungi efficient and competitive among natural microflora for bioconversion of solid substrates. SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Tengerdy, 1998).

Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce enzymes. Some of the substrates that have been used include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hulls, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc (Selvakumar *et al.*, 1998). However, so far sisal dust has not been used as a substrate for solid state fermentation. Biological delignification by SSF processes using microbial cultures producing ligninolytic enzymes can have applications in delignification of ligno-cellulosic materials (Pandey *et al.*, 1999), which can be used as the feedstock for the production of biofuels or in paper industry or as animal feedstuff. Currently, industrial demand for cellulases is being met by production methods using submerged fermentation (SmF) processes, employing generally

genetically modified strains of *Trichoderma*. The cost of production in SmF systems is however high, and it is uneconomical to use them in many of the aforesaid processes. This therefore necessitates reduction in production cost by deploying alternative methods, for example the SSF systems.

1.8 Statement of research problem

The sisal industry in East Africa is a high waste industry currently at approximately 2-98% of sisal : fibre waste ratio. In Tanzania alone, the sisal industry with a production of 45,000 tones of sisal fibre in 2007 generated 4.5 million m^3 of sisal decortications wastewater and 1,125,000 tonnes of sisal solid residues (Mshandete *et al.*, 2008). Currently, both sisal solid residues and wastewater are disposed off untreated resulting in serious environmental pollution problems. Although the sisal wastes are a menace to the environment they represent an inexpensive renewable energy source, which, through anaerobic digestion and biogas production, has a very good potential to contribute to sustainable and decentralized energy supply for sisal decortication factories. However, initial hydrolysis of the polysaccharide components of the solid sisal residues used as substrate in anaerobic bioreactors is one of the major limitations in biogas technology. One method of pre-treatment to improve methane production from the residues is by biological pre-treatment. Nevertheless, very little has been reported in the literature on biological pre-treatment of solid sisal residues for improved biogas production.

Therefore the aim of the present study was to enhance biogas production from SLDR by biological pre-treatment using fungal strain CCHT-1 and *Trichoderma reesei.*

1.9 Significance of the study

One of the key development challenges in East African countries is the need to supply energy to drive economic growth and at the same time maintain a clean and safe environment for the present and future prosperity. Therefore there is a clear need for viable survival strategies in order to mitigate climate change and thrive in a post fossil fuel world economy. A particularly desirable option is anaerobic digestion of organic biomass for sustainable energy provision, protection of the ecosystem as well as energy poverty alleviation and production of bio-fuels (biogas). To this effect, the conversion of sisal residues into biogas by anaerobic digestion has been done, though the process needs to be optimized to explore its full potential.

To make a biological delignification process economical, it is essential to maximize both the rate and the specificity of lignin breakdown. This can be by using an organism (wild type or mutant) that requires little carbohydrate to support ligninolysis, and by providing conditions that favour lignin degradation and discourage carbohydrate consumption. Therefore the strategy for enhancement of biogas production from SLDR by biological pre-treatment step using strain CCHT-1 and *Trichoderma* filamentous fungi is novel and will lead to efficient utilization of SLDR for nitrogen recirculation, energy generation

while contributing to reduction of eutrophication due to nitrogen leaching and environmental pollution mitigation. This would be in line with the Millennium Development Goal (MDG) No. 7, which calls for ensuring environmental sustainability. The results of this study are of direct potential benefit to the newly installed Katani Limited biogas plant in Tanzania which is utilizing SLDR. The enhanced anaerobic digestion process has a potential for integration into the current process to improve biogas production. Prior to large scale application of pre-treatment, the results of this study will be used as a basis for designing a pilot study.

1.9.1 General objectives

To enhance biogas production from SLDR by biological pre-treatment using fungal strain CCHT-1 and *Trichoderma reesei.*

1.9.1.1 Specific objectives

1. To determine the effects of different inoculum concentrations of the fungal strains separately on the extent of methane production from SLDR in batch anaerobic bioreactors.
2. To investigate the effect of different incubation periods of SLDR with the fungal strains separately on methane yield.
3. To determine the effect of two-steps pre-treatment of SLDR first by strain CCHT-1 inoculum followed by *Trichoderma reesei* inoculum and vice versa on methane yield.

1.9.1.2 Hypothesis

The study tested the following hypotheses

1. Pre-treatment of SLDR with strain CCHT-1 and *Trichoderma reesei* will improve methane yields with increase of inoculum concentrations up to optima points.
2. Pre-treatment of SLDR with strain CCHT-1 and *Trichoderma reesei* will improve methane yields with increase of incubation periods up to optima points.
3. The effect of two-steps pre-treatment of SLDR with the fungi on methane yield is higher than that of the residue pre-treatment with the individual fungi.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 SOURCE OF SUBSTRATE

Sisal leaf decortication residues (SLDR) used as the substrate in batch anaerobic bioreactors was produced during sisal leaves decortications by the Hammer Mill process. Sisal dust used as fungal inocula carrier were obtained from a sisal processing factory at Hale sisal Estate, owned by Katani Limited, in Tanga, Tanzania. Part of the SLDR was dried for five days in the sun, prior to characterization and pre-treatment. The un-dried SLDR fraction was frozen at -20°C in a WestPoint® Chest Freezer (Model: BP700W, Italy), until used.

2.2 PHYSICO-CHEMICAL CHARACTERIZATION OF SUBSTARATE

2.2.1 Determination of total solids and volatile solids

Total and volatile solids (TS, VS) of the substrate and inoculum were determined by the oven-drying and ignition method, respectively according to standard methods, APHA (1995). Clean empty porcelain crucibles were heated at 550 °C for one hour and cooled in a desiccator to room temperature. The empty crucibles were weighed and the fresh, weighed sample added. They were then oven dried for 24 hours at 105 °C in a Gallenkamp Hotbox Oven (Gallenkamp & Co. Ltd, London, UK).The crucibles were then cooled in the desiccator and their weights recorded. The VS of the samples was determined by the ignition of the oven-dried samples obtained above, at 550 °C for two

hours. The samples were cooled in the desiccator to room temperature before weighing and the following equations used in calculating TS and VS

$$\text{Total solids (\%)} = \frac{(B - A) \times 100}{\text{Sample weight, g}}$$

$$\text{Total volatile solid (\%)} = \frac{(B-C) \times 1000}{\text{Sample weight, g}}$$

Where,

A= average weight of empty Crucible (g).

B= average weight of residue dried at 105 °C + Crucible (g)

C= average weight of residues/ ash after ignition at 550 °C + Crucible (g)

2.2.2 Determination of total carbon

The total carbon was be done by the dry combustion method previously described by Allen (1989). One gram of dried, ground sample was placed in a weighed porcelain crucibles and the crucible contents were heated at 600°C in a muffle furnace for 5 hours. The crucible was cooled to room temperature in a desiccator and the ash weighed. The percentage total carbon was calculated using the following equation:

The denominator 1.8 is used to correct for organic matter lost to organic carbon during combustion.

$$\% \text{ Total carbon} = \frac{100 - \% \text{ ash}}{1.8}$$

2.2.3 Determination of total organic matter content

The organic matter content of the SLDR was done by the dry combustion method previously described by Lyimo *et al.* (2002). One gram of dried, ground sample was placed in a weighed porcelain crucible and the crucible contents were heated at 80 °C until a constant weight, the crucible contents were further heated at 550 °C for 4 h. the total organic matter content was then calculated as the difference in weight between dry weight at (80 °C) and ash weight (550 °C).

2.3 Determination of fibres

SLDR fibres were determined by the permanganate method as Neutral detergent fibre (NDF) and Acid detergent fibre (ADF) in duplicates according to the method of Goering and Van Soest (1970). The procedure is based on the ability of detergent solution to solubilize non-fibrous components and separate the fibre by filtration, as particulate material.

2.3.1 Determination of acid detergent fibre (ADF)

Dried ground SLDR sample of 1 g (W_1) was put in a 250-mL reflux flask fitted with a condenser at the top. 50 mL of acid detergent solution (composed of 49.04 g (26.65 mL) H_2SO_4 95-97% and 20 g cetyltrimethly ammonium bromide (CTAB) and 1 litre of distilled water); 2 mL decahydronaphthalene and one drop of antifoam were added. The reflux fitted with a condenser was placed on a heating mantle in the fume cupboard. The mixture was brought to the boil within 5-10 minutes. Boiling was then maintained for another 60 minutes. The contents of the flask were poured into glass crucibles of porosity 2 (40-100μm pore diameter, which had been dried overnight at 100 °C and weighed while hot), and filtered using a suction pump without letting the sample dry. The sample was washed with hot distilled water (90-100 °C), stirred and left to soak for 5 minutes. The water washed sample was then dried by vacuum, and the above step repeated. The sample was then washed with acetone and vacuum dried for 10 minutes. The crucibles with the sample were oven dried overnight at 100 °C, transferred to a desiccator, cooled to room temperature and weighed (W_2). The percentage ADF was calculated using the formula:

$$\text{Percentage ADF} = (W_2 / W_1) \times 100$$

Where: W_1 = Initial sample weight (g)

W_2 = Oven dried sample weight (g)

2.3.2 Determination of neutral detergent fibres (NDF)

NDF was determined the same way as ADF but an NDF solution instead of ADF solution was used. The NDF solution was composed of 30 g SDS (sodium dodecyl sulphate), 18.61 g ethylene diamine tetra acetic acid disodium salt ($Na_2EDTA.\ 2H_2O$), and 6.31 g Na_2HPO_4 (pH 6.9-7.1).

2.3.3 Determination of Hemicellulose

Hemicellulose was determined as the difference between the percentage NDF and the percentage ADF.

$$\% \text{ Hemicellulose} = \% \text{ NDF} - \% \text{ ADF}$$

2.3.4 Determination of Lignin

Lignin was measured as the weight lost by dissolving away the deposited manganese and iron oxides, resulting from oxidation of lignin by an excess of acetic acid buffered $KMnO_4$. The solutions used in this experiment were prepared as follows: Solution 1 (Lignin buffer) composed of 6 g $Fe(NO_3)_3.9H_2O$) in 100 mL of distilled water, 0.15 g $AgNO_3$, 500mL glacial acetic acid.,5 g potassium acetate and 400 mL tertiary butyl alcohol, Solution 2 was prepared by mixing 50 g $KMnO_4$, 0.05 g Ag_2SO_4 and double distilled water to a final volume of 1 litre. Solution (2) and the lignin buffer (1) were then mixed at a ratio of 2:1 just before use, while the demineralisation solution

composed of 50 g of Oxalic acid ($C_2H_2O_4$.$2H_2O$) in 700 mL of 95% v/v ethanol and 50 mL of 12 N HCL and 250 mL of distilled water.

The crucibles containing the ADF fraction were placed in a pan containing cold water. About 25 mL of the mixture of solutions 1 and 2 were added to the ADF fraction to make the water level of the pan 2-3 cm higher. A glass bar was placed in each crucible to mix the solution. The crucibles were then sucked dry and placed in a clean pan and half-filled with demineralisation solution. The crucibles contents were filtered dry after 5 minutes and the procedure was repeated until the residue was white. The crucibles contents were then filled with 80% ethanol and the content washed thrice and then twice with acetone after which, the glass crucible containing the sample was dried in an oven at 105°C overnight. The oven-dried weight constituted lignin.

2.3.4 Determination of cellulose

The crucibles containing the residues determined as ADF were heated in a muffle furnace at 500°C for 2 hours, and then weighed. The weight loss on ashing was the cellulose.

2.4 Determination of total nitrogen

Total nitrogen was determined by the Kjeldahl method according to standard methods (APHA 1995).

Sample digestion: The sample (0.2g) plus 9.6 g anhydrous sodium Sulphate (Na_2SO_4), 0.5g anhydrous copper Sulphate (Cu_2SO_4) and 0.2 g of selenium powder was placed in a 250-mL Kjeldahl flask. Concentrated sulphuric acid (H_2SO_4) (20mL) was added. The flask was heated at 300 °C until no more frothing and fumes coming out. Heating was continued until a clear yellowish green liquid was obtained. The liquid was cooled to room temperature until it solidified into a mass of crystals.

Distillation: Three hundred millilitres of distilled water was added into the solid cake in small quantities while cooling under the tap. The solution was transferred to a 500-mL distillation flask along with boiling chips. The solution was neutralised by adding 100 mL sodium hydroxide (NaOH) solution into the flask through funnel and the funnel sealed. Ammonia (NH_3) was trapped in the 500-mL receiver with 100 mL saturated boric acid prepared by adding 4 g boric acid to 100 mL of distilled water, and three drops of an indicator prepared by dissolving one part of methyl red mixed with three parts bromocresol green in 95 % ethyl alcohol. Distillation was carried out until when 200 mL distillate was collected, and stopped when the distillate turned universal indicator paper neutral.

Titration; The borate ions formed by the reaction of the liberated ammonia with boric acid (distillate), 300 mL in total was titrated against 0.1 M HCl. NaOH was used to standardise the HCl before each titration. Titration was repeated three times, with the end point of the titration being indicated by a greyish colour at pH around 4.6. Urea $CO(NH_2)_2$, which was used as the standard and a blank (all reagents without the sample) were treated separately in exactly the same way as the sample. The total nitrogen was calculated using the equation:

$$\text{Total nitrogen \%} = \frac{T \times M_a \times 1.4007}{W}$$

Where:

T = Sample titre (mL)

M_a = Molarity of HCL solution used in the titrations

W = weight of sample (g)

1.4007 =milliequivalent weight of N X 100

2.5 Inocula used in bioreactors

2.5.1 Natural Inoculum for bioreactors

The anaerobic inoculum used in this study was obtained from a 10 year old pilot batch manually stirred tank bioreactor digesting SLDR at Hale Sisal Estate. Prior to collecting the inoculum, the digester contents were mixed thoroughly using a manual stirrer.

Twenty-five litres plastic containers with airtight lids were used to carry the inoculum to the laboratory where its characteristics and composition were analysed.

2.5.2 Fungi

2.5.2.1 *Trichoderma reesei*

Trichoderma reesei QM-9414 spores in 20 % glycerol were generously supplied by the Department of Biochemistry, Uppsala University, Sweden. At the Department of Molecular Biology and Biotechnology, University of Dar es Salaam, the spores were sub-cultured on 5 % potato dextrose medium (Agar 15 g/L, dextrose 20 g/L, potato extract 4 g/L with a final pH of 5.6±0.2 at 25 °C) for 7-10 days at 30°C and then maintained on 2% potato dextrose agar plates for 7-10 days.

T. reesei inoculum was prepared as described in the case of mushroom spawn production according to (Stamets, 2000) using sterilized wheat grains. One kilogram of wheat grains was boiled in 1.5 litres of water until the grains were semi soft. The boiled grains were allowed to remain soaked in hot water for about 15 minutes without heating to attain a moisture content of 48-50 %, excess water was filtered drain off and the grains were cooled to room temperature. For every 1 kg of boiled grains, were mixed with 1.35 g of calcium sulphate and 0.35 g of calcium carbonate. The former additive prevents sticking of the grains together and the latter is necessary to adjust pH. One hundred grams wet weights of the boiled grains were put in 250 mL bottles and the lids screwed lightly. Sterilisation was done for one hour at 121 °C and 1.54 kg/cm^2 in a 17 litres autoclave (International pbi Sp, Milano, Italy). After cooling to room temperature,

the bottles were shaken vigorously to avoid clumping of the grains and inoculated with pure cultures of the fungus in a sterile laminar flow hood (Wagtech envair C-flow, UK). The bottle lids were replaced but screwed loosely for gaseous exchange and shaken thoroughly after inoculation to evenly distribute the mycelia and incubated at 28±2 °C for 10 days.

2.5.2.2 Strain CCHT-1 and isolation of pure culture

Starter culture source of strain CCHT-1 were obtained from dumps of decomposing sisal decortication residues at the Hale sisal Estate where they grew naturally. The starter culture source was taken to the lab for pure culture isolation on the same day. Establishment and maintenance of pure mycelium culture of CCHT-1 was done according to Dhouib *et al.* (2005) where by, a healthy starter culture of CCHT-1 was selected and swabbed with 70% v/v ethanol and was broken into two halves. Using a sterile scalpel, a fragment of the interior tissues was used to inoculate a Petri dish containing 5 % malt extract agar (Composition (g/litre) Malt extract 30.0; peptone from soymeal 3.0; agar-agar 15.0). The plate was incubated upside down in the dark at 27±1°C for 7 days. Sub-culturing of the pure isolate was done on 2% malt extract agar at 27±1°C for 7 days. Fungal strain CCHT-1 inoculum was prepared according to Gupta and Sharma (1994) using sterilised wheat grains as described in the case of *T. reesei.*

2.5.2.3 Preparation of fungal inoculum for pre-treatment of SLDR

Sisal fibre dust was used to expand the CCHT-1 fungal inoculum from the wheat grains and used for the pre-treatment of SLDR. Two hundred gram of dry sisal dust was weighed and placed in a clean tray; 150 mL of water was added drop wise while mixing to attain a moisture content of 50-60 %. One hundred grams wet weight was then put in an autoclaving bottles and sterilised for 1 hour at 121°C and 1.54 kg/cm^2. The bottles were allowed to cool at room temperature and later shaken vigorously to loosen the sisal dust particles. Ten gram wet weight of mycelial mats of each fungus which had been grown on wheat grains were used to inoculate the sisal dust in a laminar flow hood. The bottle lids were replaced but screwed loosely for gaseous exchange and incubated at room temperature for 10 days. The colonised sisal dust by CCHT-1 and T. *reesei* was used as an inoculum in the pre-treatment of SLDR.

2.6 Bioreactors

2.6.1 Solid state bioreactors

Rectangular plastic containers measuring 23 cm x14 cm x 9 cm (length, width and height, respectively) (Cello® Domestoware (Mkate), Tanzania) were used as solid state bioreactors in all the pre-treatments. A total of 136 aeration holes of 0.7 cm in diameter and 3cm apart from one another were made in all the sides of each container (bottom, sides and top).

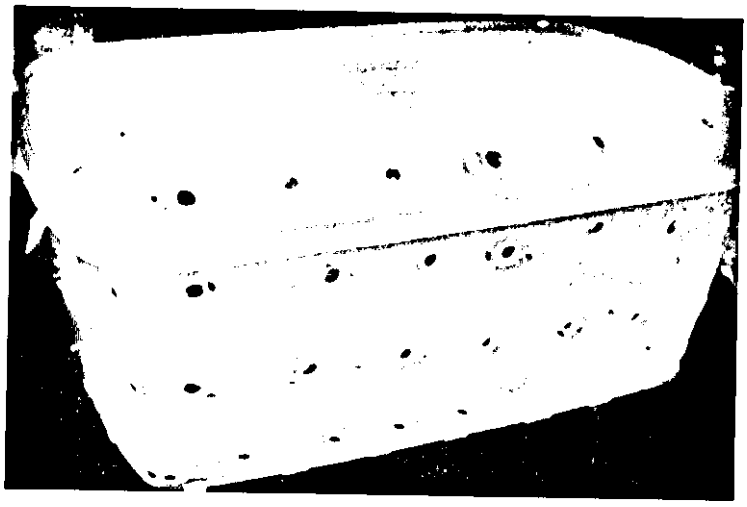

Plate 2.1 solid state bioreactor

2.6.2 Batch anaerobic bioreactors

Biogas production from the substrates was investigated in 500 mL bioreactors consisting of conical flask with a working volume of 260 mL. The biogas produced was led in gas tight-plastic tubes to gas-tight aluminium-reinforced polyethylene bags as illustrated in Plate 2.1. The bioreactors were fitted with gas sampling ports closed with n-butyl stoppers and sealed with aluminium caps (Mshandete *et al.*, 2005).

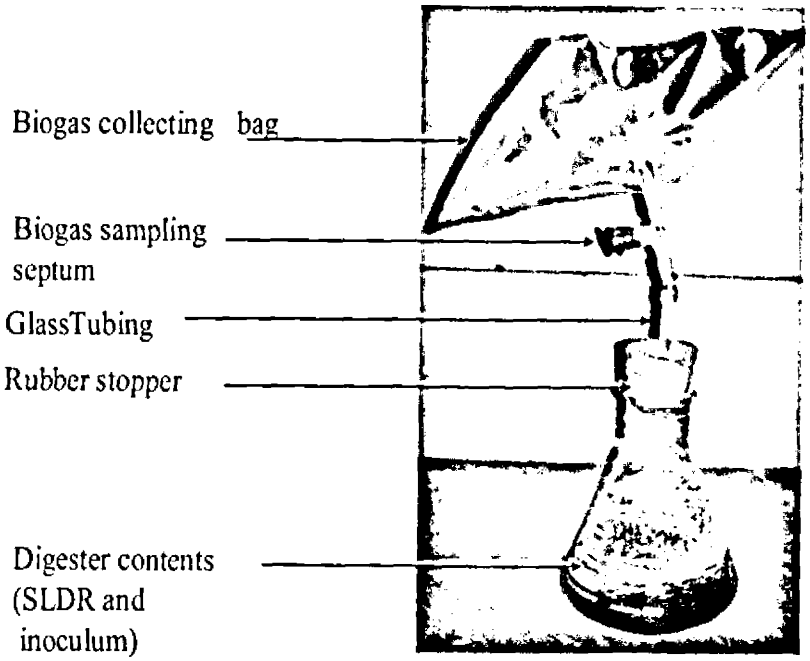

Plate 2.2 Batch anaerobic bioreactor for determining the yield of methane from SLDR

2.7 Determination of the effect of different fungal inoculum concentration on methane production.

In this experiment, batch anaerobic bioreactors with SLDR pre-treated with different inoculum concentrations of fungal strain CCHT-1 and *Trichoderma reesei* were compared to untreated SLDR in terms of methane production potential.

2.7.1 Experimental set up

Pre-treatment of SLDR with different inoculum concentrations of CCHT-1 and *Trichoderma reesei* was done under SSF prior to anaerobic digestion in batch anaerobic bioreactors. Twelve different inoculum concentrations of 0.5, 1, 2, 3, 5, 10, 15, 20, 25, 30, 40, and 50 % of CCHT-1 (inoculum/SLDR wet weight) were used to inoculate 450 gram of dried SDLR (moisture content approximately 50-60%) and incubated for 10 days at 28±2 °C. A repetition of 3, 5, 10, and 15 inoculation percentages were done for 450 grams wet weight of fresh SLDR to investigate pre-treatment of fresh SLDR. Ten different inoculum concentration of 1, 3, 5, 10, 15, 20, 52, 30, 40, and 50 % of *Trichoderma reesei* (inoculum/SLDR wet weight basis) were used to inoculate the residue and incubated for a period of ten days at an ambient temperature of 28±2 °C.

2.7.2 Loading pre-treated SLDR in batch anaerobic bioreactors

To all batch anaerobic bioreactors described in section 2.3.1 above, calculation of the g VS of the inoculum was done with the assumption that 1 gm is equal to 1 mL of the inoculum. From the characterisation of the inoculum and the pre-treated SLDR, the total

solids (TS) and volatile solids (VS) of each pre-treated sample were determined. Two hundred mL of inoculum described in section 2.1 which had total VS of 5.84 was used in the anaerobic batch bioreactors. The amount of pre-treated SLDR added to the bioreactor for each pre-treatment in the ratio of 1:1 (SLDR to inoculum) gram VS per gram wet weight (g VS/g ww) was calculated as illustrated below and tabulated.

VS of SLDR =A%
VS of Inoculum =B%
TS of SLDR =C %
TS of Inoculum =D%

(a) gVS of inoculum =(B)×(D)×(inoculum wet weight)

(b) gVS/g ww of SLDR = (A) × (C) × (Eg)

Where E= amount of SLDR to be loaded into anaerobic batch bioreactor

$$\text{The loading weight} = \frac{\text{g VS wet weight in 200 g of inoculum}}{\text{g VS/g ww of SLDR}}$$

To determine the effect of buffering as well as avoid limitation in case of digester acidification, sodium bicarbonate ($NaHCO_3$) was used to buffer the third series of strain CCHT-1 pre-treated SLDR bioreactors containing pasteurised and pre-treated SLDR and the other set of un-pasteurised pre-treated SLDR together with their controls.

Calculation of the amount of buffer added was done based on the total amount of total gram VS (5.84g VS) of the substrate added in the batch anaerobic bioreactors, as shown below:

It was assumed: 1gVS = 1g acetic acid (CH_3COOH)

But 1 Mol acetic acid = 60g/mol

Hence, 5.84gVS of SLDR ≡ 5.84g of acetic acid

Number of moles of acetic acid = $\frac{5.84g}{60g}$

= 0.097moles

Moles 0.097 of acetic acid would be neutralized by equivalent number of moles of $NaHCO_3$ buffer which is equal to 8.15 g; however for practical reasons 0.7 g $NaHCO_3$ was added since high amount $NaHCO_3$ has inhibitory effects to anaerobic digestion process.

The experimental setup of anaerobic batch bioreactors digesting SLDR pre-treated with CCHT-1 consisted of 108 bioreactors as shown in Table 2.4. Drying of SLDR as well as pasteurisation and buffering of the bioreactors was done to investigate if they had an effect on the overall methane production during anaerobic digestion. Fresh SLDR pre-treated with CCHT-1 was loaded in the final set of bioreactors to investigate the effect of pre-treating fresh SLDR with CCHT-1 without drying and buffering.

Table 2.4 Anaerobic batch bioreactors substrate loading plan

Description of SLDR loaded in the bioreactor	Number of bioreactors
Dried pasteurised and pre-treated	24
Dried pasteurised and un pre-treated	3
Dried un pasteurised and pre-treated	24
Dried un pasteurised and un pre-treated	3
Dried pasteurised, pre-treated in Buffered digester	12
Dried pasteurised, un pre-treated in Buffered digester	3
Dried un-pasteurised, pre-treated, in Buffered digester	12
Dried un-pasteurised, untreated, in Buffered digester	3
Control (anaerobic inoculum only)	3
Fresh residues un pasteurised and pre-treated	18
Control (fresh untreated)	3

Trichoderma reesei pre-treated SLDR (Table 2.5) was loaded in 24 digesters, three other digester contained untreated SLDR and control which had only the inoculum. The bioreactors were kept at an ambient temperature of 28±2°C and shaken manually for one minute thrice daily to provide substrate agitation. The methane content was determined after every 48 hours prior to biogas volume measurement as described in the analytical section. All the digesters were run for 42 days.

2.7.3 Determination of the effect of length of pre-treatment periods on methane production

2.7.3.1 Experimental set up

This experiment was designed to investigate the effect of different pre-treatment periods of SLDR with inoculum concentrations of 10% and 25% for CCHT-1 and *Trichoderma reesei* respectively, on methane production. Five different periods of 2, 4, 6, 8, and 10 days were investigated for both CCHT-1 and *Trichoderma reesei.* Solid state pre-treatment of fresh SLDR was done in solid state bioreactors and incubated at ambient temperature of 28±2°C. The pre-treatment was stopped after every designated day and the pre-treated substrate loaded in batch anaerobic bioreactors. Anaerobic digestion of SLDR pre-treated with strain CCHT-1 and *Trichoderma reesei* at different periods consisted of 21 digesters for each fungus, as illustrated below. The un- pre-treated SLDR was used to mimic the conventional method where no pre-treatment is done, while the control digesters contained only the anaerobic inoculum. All the digesters were sealed

and kept at an ambient temperature of 28±2°C. They were shaken manually for one minute thrice daily to provide substrate agitation and run for 42 days, with the biogas composition and volumes being measured after every 48 hours.

2.8 Determination of the effect of fungal inoculum type pre-treatment on methane production

2.9.1 Experimental set up

This experiment was designed to investigate the effect of a two steps pre-treatment using the two fungi at different times first with CCHT-1 followed by *T. reesei* and vice versa to determine the extent of pre-treatment if either fungi was applied first. In the first step, six different inoculum concentrations of 5, 10, 20, 30, and 50 % of CCHT-1 inoculum (inoculum/SLDR wet weight) were used to inoculate 450 g (wet weight) of fresh SDLR and incubated at ambient temperature of 28±2°C for 4 days. After the fourth day, the SLDR was inoculated with 25% *T. reesei* inoculum (inoculum/SLDR wet weight) and incubated for 8 days at ambient temperature of 28±2°C. Analysis of the pre-treated SLDR was done and the loading weights calculated to load the anaerobic batch bioreactors.

2.9 ANALYTICAL METHODS

2.9.1 Methane analysis

The methane content in the biogas produced from all experimental batch anaerobic bioreactors was estimated by the concentrated alkaline absorption method (Ergüder *et al.* 2001). Five mL of the biogas was injected into entirely closed 11 mL serum bottles containing 8mL concentrated KOH stock solution 20g/L at atmospheric pressure. The bottles were shaken manually for 3-4 minutes. In this method, only CH_4 is determined while other biogas components such as CO_2, H_2S are dissolved in the KOH solution (Mshandete *et al.*, 2005). The volume of biogas formed during the experiment was measured using a graduated 100 mL gas- tight plastic syringe with a sample lock according to Mshandete, *et al.*, 2005 where the volume determinations were done by connecting the tip of syringe to the tip of the biogas bag through the septum and then the syringe plunger was pulled to draw the gas from the bag. The readings were done on the volume of the gas which corresponds to the graduated syringe. Afterwards the lock was opened and the plunger was pushed to withdraw the gas from the syringe, the gas was then released and the process repeated until the bag was almost empty. Methane yield was calculated by subtracting the amount of methane produced by the control from the methane production of each SLRD, expressing this as a function of the mass of volatile solids in the substrate fed in the anaerobic bioreactor.

2.9.2 Determination of pH

The pH before and after anaerobic digestion of the biomass and effluents was determined using a pH 209, meter (Hanna instruments® USA)

CHAPTER THREE

3.0 RESULTS

3.1 Physical and chemical composition of sisal leaf decortication residues

SLDR obtained from the sisal leaf decorticator was green in colour (Plate 3.1) and dominated by sisal juice (liquid). The colour of the residue turned to greyish on drying in the sun for five days (Plate 3.2). The compositions of the fresh and sun dried residues are shown in Table 3.1. The inoculum used in the batch anaerobic digestion of the substrate was partially characterized and had a total solids (TS) content of 2.1±0.1 percent of the fresh weight and a VS content of 67.6±9.1 % of TS.

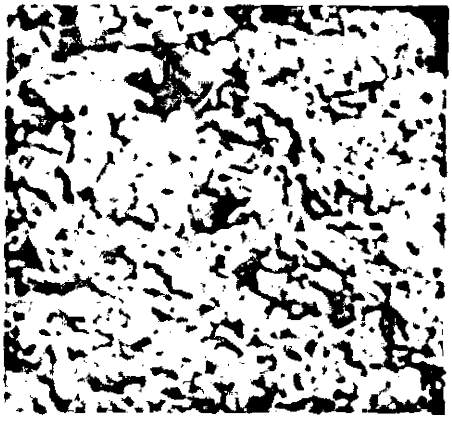

Plate 3.1 Fresh sisal leaf decortication residues SLDR

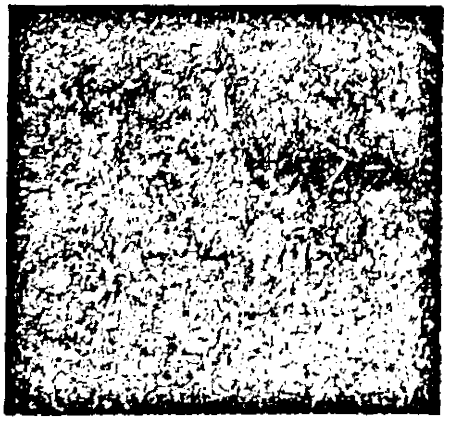

Plate 3.2 Sun Dried SLDR

Table 3.1 Composition of SLDR (mean ± SD)

Determination	Fresh SLDR	Sun dried SLDR
Total solids (TS) %	14.1 ±0.1	16.1±0.1
Volatile solids (VS) (% of TS)	85.5±0.6	84.9±2.0
Organic carbon[a]	48.3±0.2	45.9±1.1
Total nitrogen[a]	1.78±0.9	1.3±0.1
Neutral detergent fibres (NDF)[a]	45.5±0.7	43.5±0.7
Acid detergent fibres (ADF)[a]	43.0±0.2	41.5±0.7
Lignin[a]	9.5.±2.1	5.5±2.1
Cellulose[a]	68.6±1.6	72.3±2.2
Hemicellulose[a]	5.5±0.7	2.0±0.1

All values are averages of triplicates
[a] % of dry weight

3.2 Isolation and culture of fungi

CCHT-1 fungus was found growing on moist decomposing SLDR. For Intellectual Property/Rights (IP/IPR) purposes, the organism was given a code name CCHT-1 and its taxonomic status is yet to be completely elucidated. In pure culture, growth of linear, cotton white thread like mycelia, were observed to originate from the tissue fragment inoculated on malt extract agar when incubated at 28°C (Plate 3.3).

Plate 3.3 CCHT-1 pure mycelia growing on the Malt extract agar after 10 days of incubation

Pure mycelia cultures of *Trichoderma reesei* on malt extract agar plates (Plate 3.4 a) and sisal dust were obtained (Plate 3.4 b) in this study.

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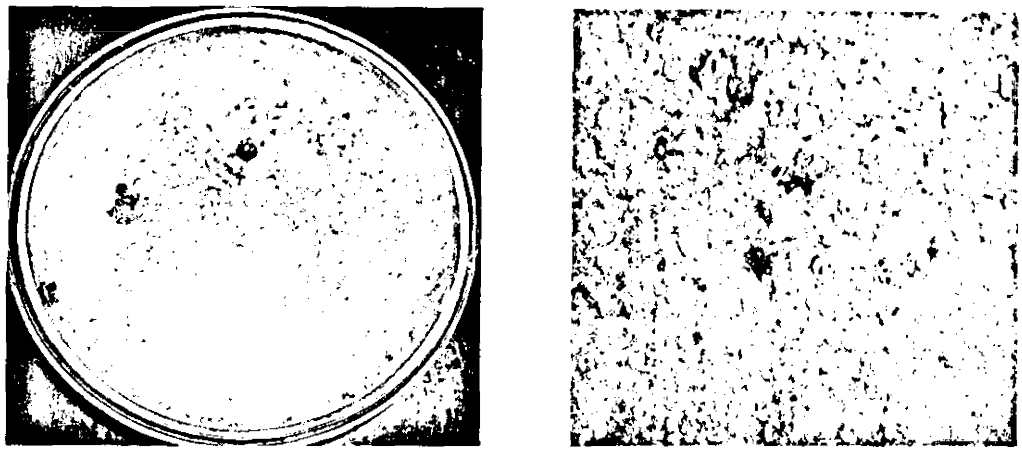

(a) (b)

Plate 3.4 (a) *Trichoderma reesei* growing on potato dextrose agar plate after 10.days of incubation
(b) *Trichoderma reesei* on sisal leaf dust after 10 days of incubation

3.3 Effect of different rates of inoculation with strain CCHT-1 on the extent of methane production from dried SLDR

The methane yields from SLDR pre-treated after drying for five days, with CCHT-1 at different inoculum concentrations, and digested in buffered batch anaerobic bioreactors are shown in Figure 3.1. Methane yields from the un-pasteurised and pre-treated SLDR were in the range of 0.072 and 0.115 m^3 CH_4/kg VS_{added}. The methane yields recorded from the pasteurised, pre-treated SLDR digested in buffered bioreactors were in the range of 0.042 and 0.102 m^3 CH_4/kg VS_{added}.

The methane yields from batch anaerobic bioreactors digesting dried SLDR, after pre-treatment with different rates of CCHT-1 are shown in Figure 3.2. The methane yield increased with an increase in inoculum concentrations used in the pre-treatment. A 10 % inoculum concentration of CCHT-1 on pasteurised and un-pasteurised SLDR gave the highest yields of 0.114 and 0.117 m^3 CH_4/kg VS_{added}, respectively.

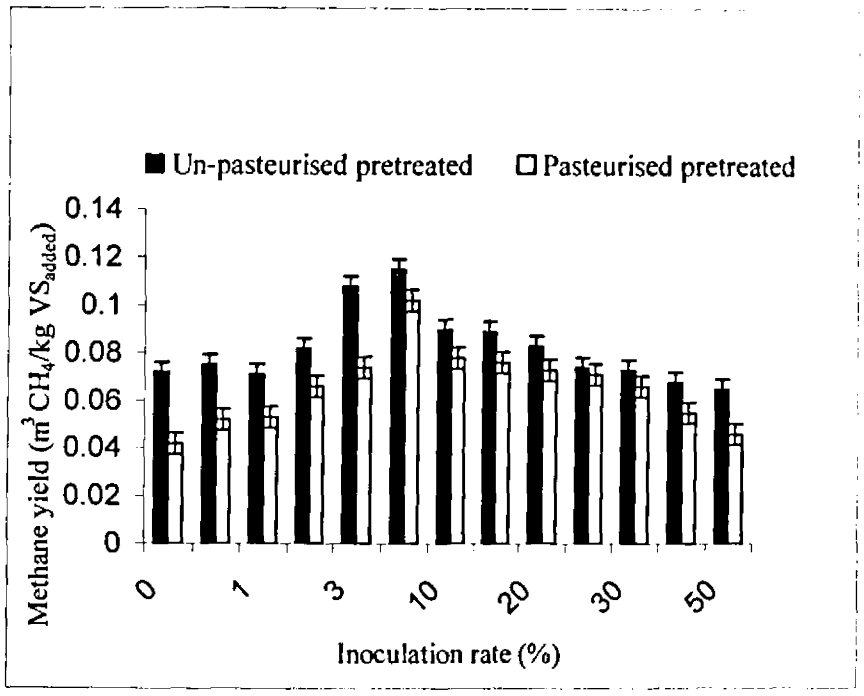

Figure 3.1 Methane yields obtained from un-pasteurised and pasteurised SLDR pre-treated with different inoculum concentrations of strain CCHT-1 and digested in buffered bioreactors

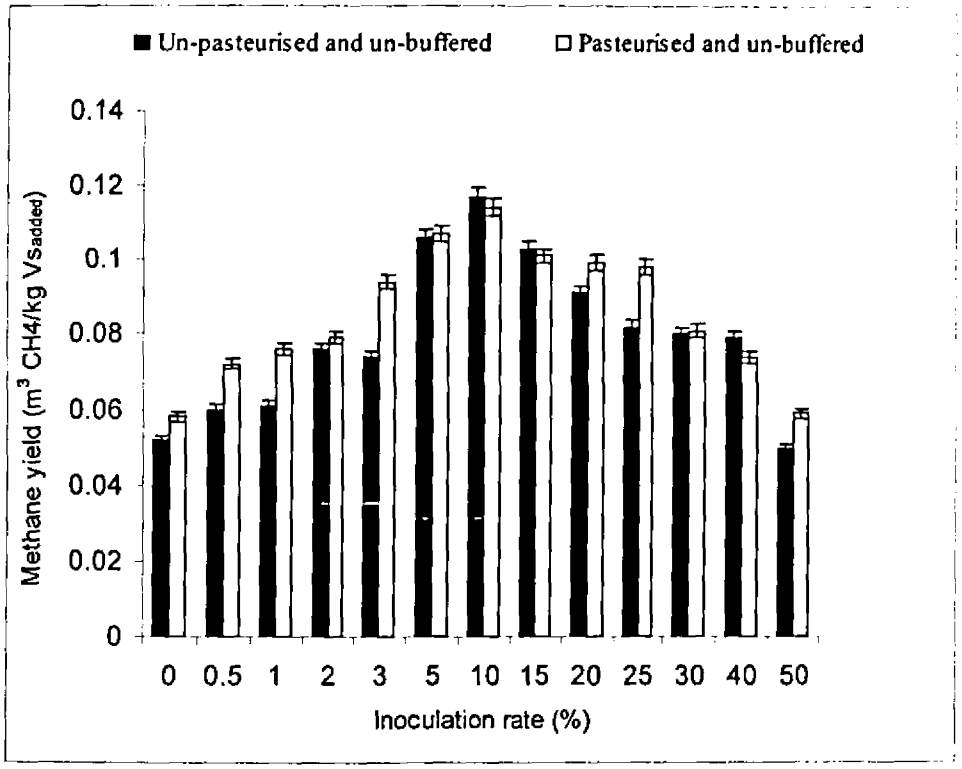

Figure 3.2. Methane yields obtained from un-pasteurised and pasteurised SLDR pre-treated with different strain CCHT-1 inoculum concentrations and digested in un-buffered bioreactors.

The pH values recorded in the batch anaerobic bioreactors before the digestion process were pH 7.9 for the inoculum only and pH 8.5 for both buffered and un-buffered mixture of SLDR and inoculum. The pH value recorded after 7 days in control bioreactors set aside for monitoring the pH changes in the experimental batches revealed a change to 7.12 and 7.73 in the un-buffered and buffered bioreactor, respectively. After 42 days of anaerobic digestion the pH values of the digested SLDR recorded were 7.5 and 8.15 in the un-buffered and buffered bioreactor, respectively.

3.3.1 Effect of different inoculum concentrations of strain CCHT-1 on the extent of methane production from fresh SLDR

Methane yields and composition from anaerobic digestion of fresh SLDR pre-treated with strain CCHT-1 against the methane yield are shown in Figure 3.3 below. The methane yields were in the range of 0.128-0.203 m^3 CH_4/kg VS_{added}, with the highest yield obtained from 10 % inoculum concentration. The yields decreased with increase in inoculum concentrations with 0.074 m^3 CH_4/kg VS_{added}, being recorded in 50 % inoculum concentration. The results indicated the methane yield was high in the fresh substrate and without buffering making it economical for industrial application. The methane content of the biogas produced varied from 51% to 65%.

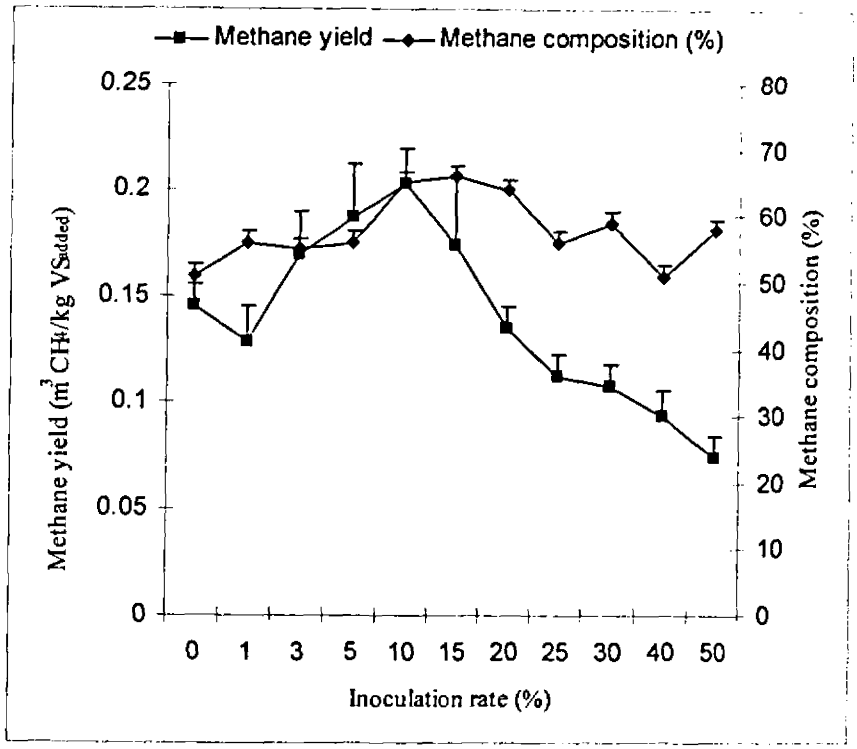

Figure 3.3. Methane yields and methane composition of fresh sisal SDLR pre-treated with strain CCHT-1 at different inoculum concentrations.

3.3.2 Effect of pre-treatment of SLDR with strain CCHT-1 on fibre degradation

The Neutral detergent fibre (NDF) compositions of the pre-treated SLDR prior to loading in the batch anaerobic bioreactors are shown in Figure 3.4, in comparison to methane yields. The fibre content decreased from 44± 0.71 to 37.5±1.4, with the highest inoculum concentration of 50% giving the lowest methane yield. At 10% inoculum concentration NDF recorded was 39.5±0 .5 with the highest methane yield after anaerobic digestion.

3.4 Effect of pre-treatment of fresh SLDR with different inoculum concentrations of *Trichoderma reesei* on methane production

The methane composition and yield for fresh SLDR, pre-treated with different inoculum concentrations of *Trichoderma reesei* are given in Figure 3.5. The methane yield from the batch anaerobic bioreactors varied from 0.088 m^3 CH_4/kg $VS_{added,}$ to 0.192 m^3 CH_4/kg VS_{added}. An inoculum concentration of 25% gave the highest methane yield of 0.192 m^3 CH_4/kg VS_{added}. Further increase in inoculum concentration led to a decrease in methane yield with 0.086 m^3 CH_4/kg VS_{added} being recorded in at 50%. The NDF content (%) decreased from 44.5±1.8 to 38.2±1.1 at 50% inoculum concentration.

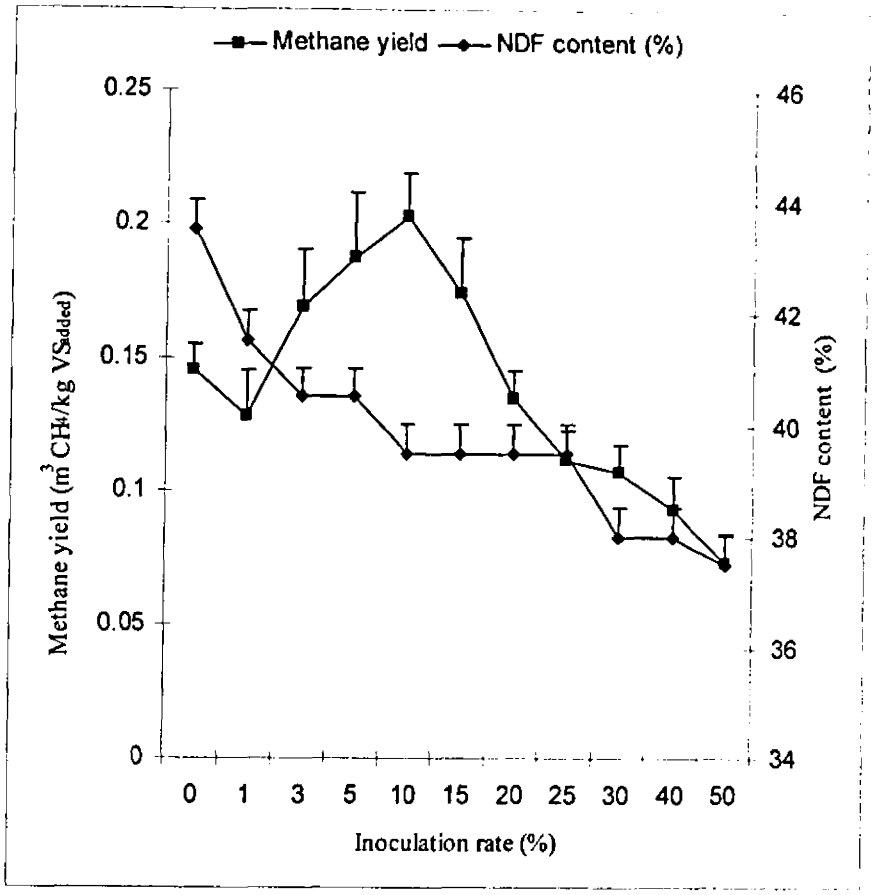

Figure 3.4 Methane yields and NDF content (%) of fresh sisal SDLR pre-treated with strain CCHT-1 at different inoculum concentrations.

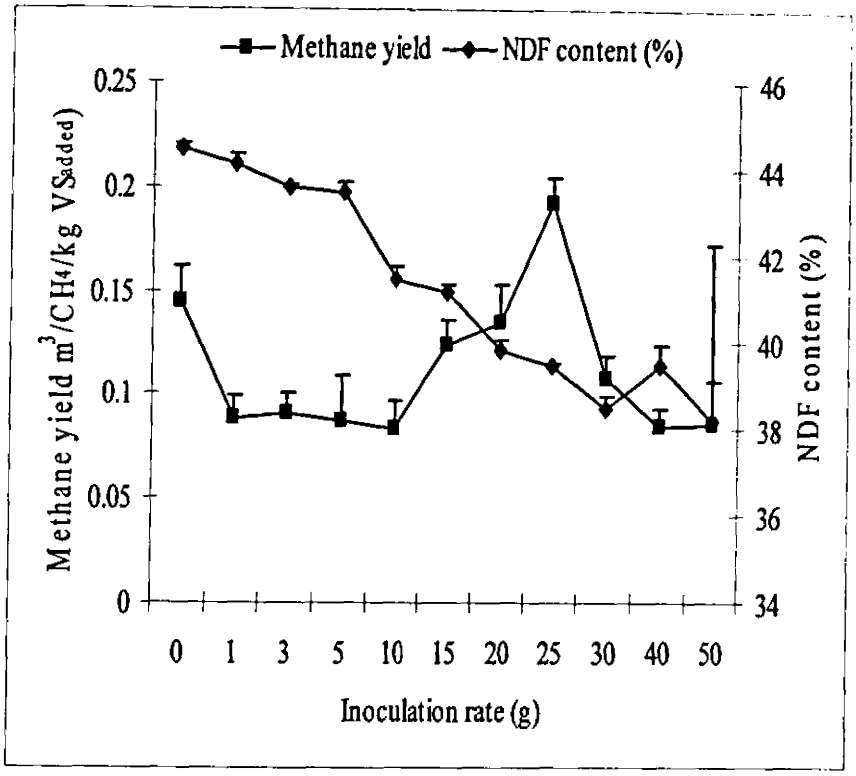

Figure 3.5 Methane yields and NDF content (%) of fresh sisal SDLR pre-treated with *Trichoderma reesei* at different inoculum concentrations.

3.5 Effect of pre-treatment of SLDR with strain CCHT-1 and *Trichoderma reesei* at different incubation periods on the extent of methane production

The methane yield and NDF content (%) obtained for SLDR pre-treated with strain CCHT-1 and *Trichoderma* at different incubation periods are given in Figure 3.6. An inoculum concentration of 10% for CCHT-1 and 25% for *Trichoderma* which were to be the best as determined in previous experiments were used. The methane yields produced varied from 0.110 CH_4 m^3 to 0.192 CH_4 m^3/kg VS_{added}. The highest methane yield was obtained from CCHT-1 after incubation periods of 4 days, on the other hand a methane yield of 0.139m^3 CH_4/kg VS_{added} was obtained from *T. reesei* after 8 days of incubation. The methane content of the biogas produced was in the range of 50-62%. The percentage NDF content of the pre-treated SLDR before loading in the batch bioreactors reveal a decrease in the range of 14-23% with increased incubation period.

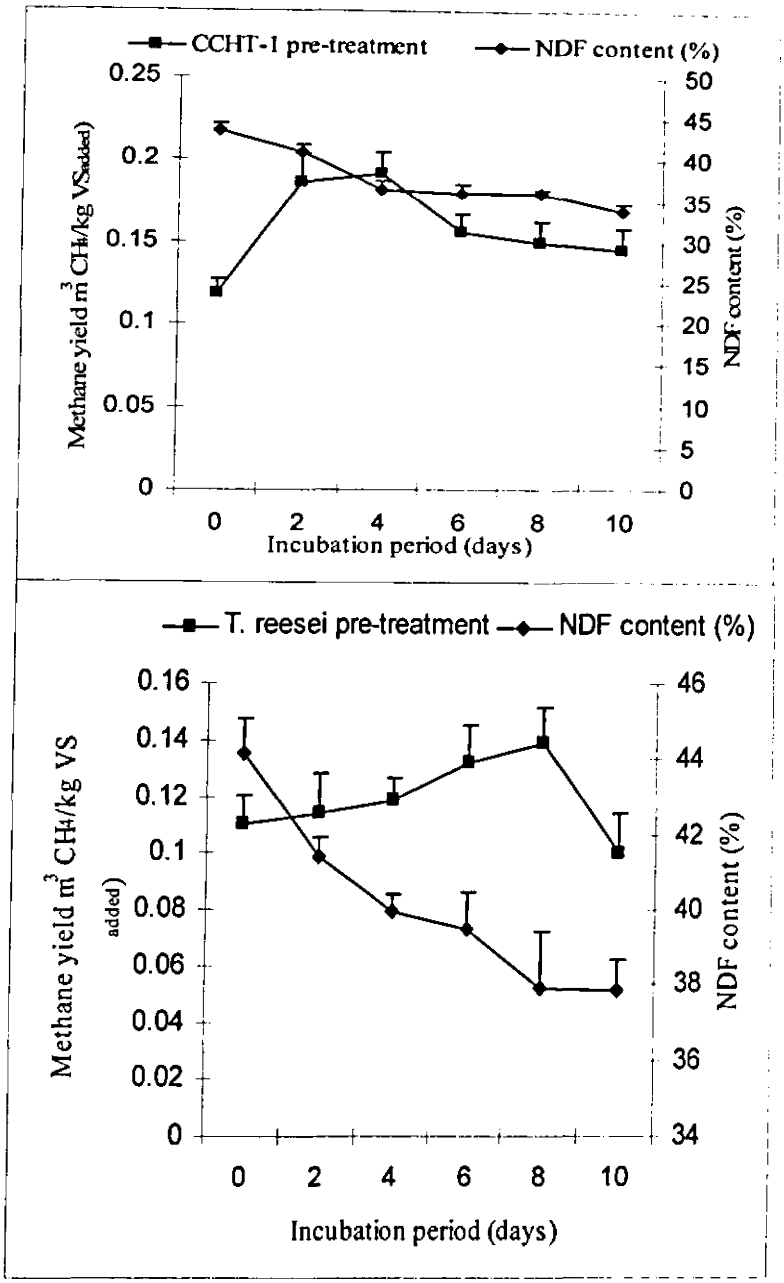

Figure 3.6. Methane yield and NDF content of SLDR pre-treated with strain CCHT-1 and *Trichoderma reesei* at different incubation periods.

3.6 The effect of two-steps pre-treatment of SLDR by strain CCHT-1 and *Trichoderma reesei* on methane production.

The results of methane yield from the anaerobic digestion of SLDR after two steps pre-treatment are given in Figure 3.7. The methane content was in the range of 51 and 59% for the two step pre-treatment of SLDR with the two fungi in succession. In the pre-treatment of SLDR with fixed 10% inoculum concentration (best rate) of strain CCTH-1 incubated for 4 days (best incubation period) followed by different inoculum concentrations of *Trichoderma reesei* incubated for 8 days (best incubation period), the methane yield was in the range of 0.145-0.292 m^3 CH_4/kg VS_{added}, while the NDF content (%) was in the range of 42.7±0.5 to 29.3±0.6 Figure 3.7 (a). On the other hand, in pre-treatment of SLDR with a fixed inoculum concentration of 25% (best rate) by *Trichoderma reesei* for 8 days with varied inoculated rates of CCTH-1 incubated for 4 days, the methane yield was in the range of 0.145-0.189 m^3 CH_4/kg VS_{added} and the NDF content (%) was in the range of 43.7±0.5 to 32.2±0.1.1 Figure 3.7 (b).

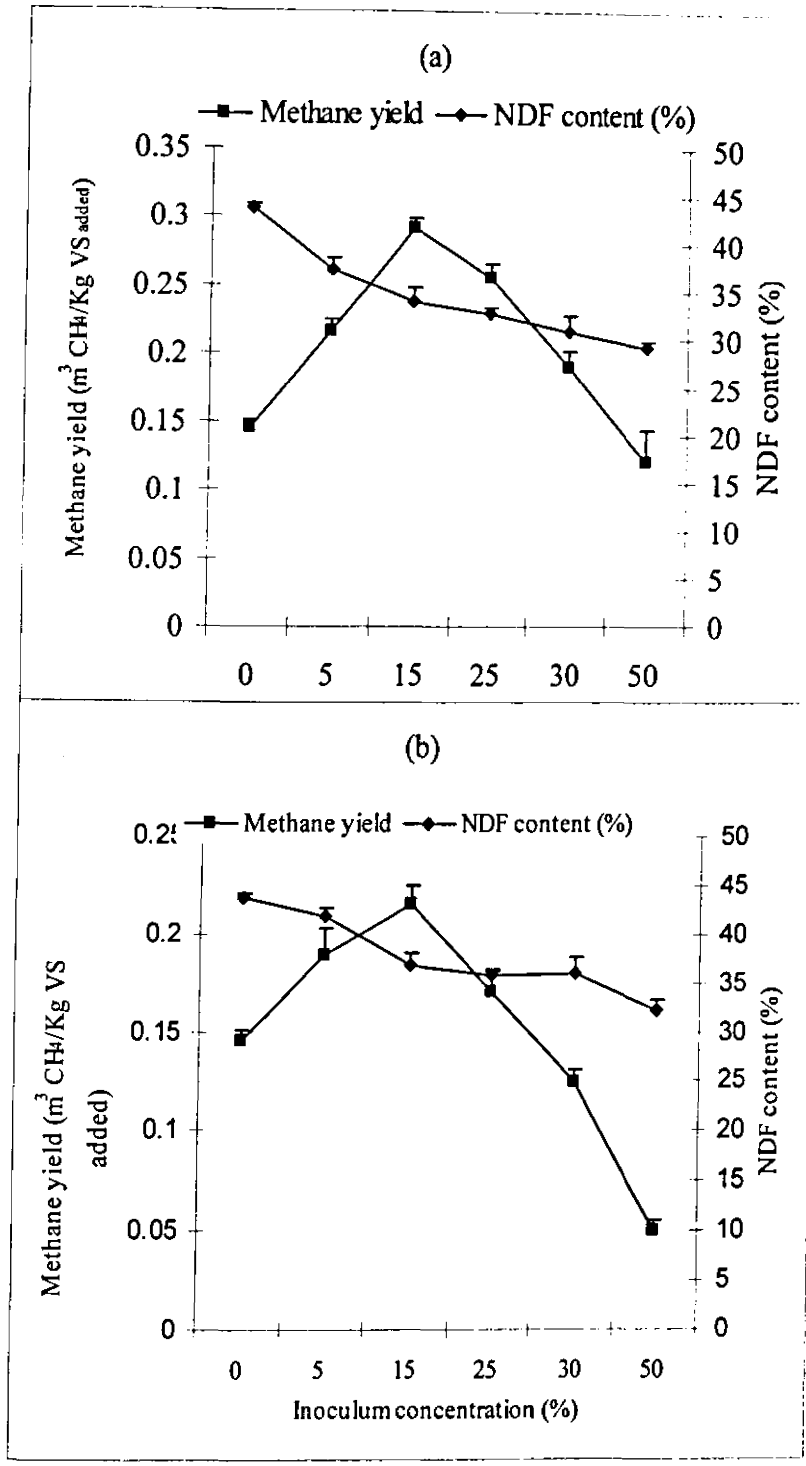

Figure 3.7 Methane yield and NDF content obtained for the two-steps pre-treated SLDR with CCHT-1 (a) and *T. reesei* (b)

CHAPTER FOUR

4.0 DISCUSSION

In the present study, biological pre-treatment and batch anaerobic digestion of SLDR was carried out successfully. The results obtained agree with the early observations that, during pre-treatment, the structural polysaccharides contained in plant material can be partially degraded and intermediates suitable for methanogenic fermentation produced (Egg *et al.*, 1993). This can be attributed to the increased methane yields after pre-treatment.

4.1 Effect of buffering the bioreactors

The pH of the digester is a function of the concentration of volatile fatty acids produced, bicarbonate alkalinity of the system, and the amount of carbon dioxide produced (Nagamani, and Ramasamy, 1991). The pH values at the beginning of digestion were pH 7.9 for the inoculum only and pH 8.5 for the both buffered and un-buffered mixture of sisal leaf decortications residue and inoculum (section 3.3.1). Within 7 days, pH decreased to 7.12 and 7.73 in the un-buffered and buffered bioreactor, respectively and increased to 7.5 and 8.15 in the un-buffered and buffered bioreactor, respectively, after 42 days of anaerobic digestion. The high pH recorded in the buffered bioreactors was as a result of the sodium bicarbonate which was added to buffer the system. Similarly, Mshandete *et al.* (2004) working on sisal pulp waste reported an initial pH range of 7.7-7.8 and a final pH of 7.3-7.7 indicating acidification did not occur in the mixture. Sahota

and Ajit Singh in Nagamani and Ramasamy (1991) reported that the gas production was significantly affected when the pH in the digester went below 5.0. The results suggest the external buffers in the bioreactor were not necessary and accounted for the low methane yields observed in the pasteurisedd and pre-treated SLDR, digested in buffered bioreactors was in the range of 0.042 to 0.102 102m^3 CH_4/kg VS_{added} in comparison to the un-buffered bioreactor where the range was 0.052 to 0.117 m^3 CH_4/kg VS_{added}. Nagamani and Ramasamy (1991) and Gunaseelan (1995) reported that methane is produced within the pH range of 7.5-8.5, thus the batch anaerobic bioreactors were able to produce biogas but with different yields due to variation in the pH levels.

4.2 Pre-treatment of SLDR with different inoculum concentrations of strain CCHT-1

Pre-treatment of dried SLDR using different inoculum concentrations of CCHT-1 and subsequent anaerobic digestion in buffered batch anaerobic bioreactors was carried out in this study. The results (Figure 3.1) show a difference in methane yields for SLDR residues which were pasteurised and un-pasteurised SLDR before pre treatment was done, in buffered bioreactors. The methane yield recorded was 0.102 and 0.115 m^3 CH_4/kg $VS_{added,}$ for the pasteurised and un- pasteurised residues. The un-pasteurised substrate had a fast start up compared to the pasteurised substrate and also gave better results in terms of yield. The pasteurisation was done to enhance the colonization of the residues by the fungi during pre-treatment, but was found to have no impact as revealed

from the methane production in comparison to the un-pasteurised residues. The results for the pre-treatment of un-pasteurised SLDR in un-buffered anaerobic batch bioreactors (Figure 3.2) show that, it is feasible to pre-treat fresh SLDR and digest it anaerobically for methane production without external buffers. The methane yields (Figure 3.3) were in the range of 0.074-0.203 m^3 CH_4/kg $VS_{added,}$ with 0.145 m^3 CH_4/kg VS_{added} being produced from the un pre-treated SLDR. On the other hand, a 10% inoculum concentration gave the highest methane yield of 0.203 m^3 CH_4/kg VS_{added}. This was an increase of 40% in methane yield potential in comparison to the values obtained in the bioreactors where no pre-treatment (control) was employed. Similar yields in the range of 0.19 to 0.240 m^3 CH_4/kg VS_{added} have been previously reported by Mshandete *et al.* (2005) for the mesophilic aerobically pre-treated SLDR. The biogas produced in this study had, the percentage methane content varying greatly within the range of 51% and 66%, with higher percentages being recorded for the pre-treated residues in comparison to where no pre-treatment was employed. Similar methane composition have been reported previously by Mshandete *et al.* (2006) working on mechanically pre-treated sisal fibres decortication residues.

4.3 Pre-treatment of SLDR with different inoculum concentrations of *Trichoderma reesei* on methane

Results of methane yield from the batch anaerobic bioreactors digesting *T. reesei* pre-treated SLDR (Figure 3.5) were in the range of 0.083-0.192 m^3 CH_4/kg VS_{added}. The

methane yield from the un pre-treated substrate was 0.145 m^3 CH_4/kg VS_{added} while 0.192 m^3 CH_4/kg VS_{added} was obtained for SLDR pre-treated with 25% *T. reesei*. This represented an increase of about 32% in methane yield potential recorded for the pre-treated SLDR in comparison to the untreated residues. The increase in methane yield may be attributed to the disruption of the cellulose structure of the substrate by the *T. reesei* extracellular cellulases, which degrade the crystalline cellulose. Mshandete *et al.* (2005) reported 26% higher methane yield from sisal pulp after a 9 h of aerobic pre-treatment prior to anaerobic digestion compared to the un-pre-treatment sisal pulp waste. Mtui and Nakamura (2005) also observed that aerobic pre-treatment with pure culture of *Trichoderma reesei* resulted in improved fibre digestibility which translates to higher methane production. Production of extracellular degrading enzymes is desirable in hydrolysis of sisal residues, which is highly lignocellulosic (Mshandete *et al.*, 2005). Various factors may explain why pre-treatment with *Trichoderma reesei* had varying effect on SLDR in terms of biogas production. In the hydrolysis stage, the cellulase may not have been able to break down cellulosic materials sufficiently due to lignin that surround the cellulose preventing the cellulase from reaching the cellulose fibre. Increase in inoculum concentrations interpreted to increased removal of the hemicellulosic shield, leading to a higher methane yields to an optimum rate of 25%. From the results, pre-treatment with CCHT-1 at different inoculum concentrations had a higher methane yield in comparison to that obtained from the same substrate pre-treated with *Trichoderma reesei*. This observation can most probably be due to the fact that, strain CCHT-1 grows naturally on sisal residues which implies that it is lignocellulolytic

hence degraded the lignin coat in the residues prior to anaerobic digestion process (Hammel, 1997). However in this study, the extent of lignin degradation could not be obtained.

4.4 Strain CCHT-1 and *Trichoderma reesei* pre-treatment periods on the extent of methane production from SLDR

To minimize cellulose degradation during fungus culture incubation and making more cellulose available, the optimization of incubation period was done and the results revealed the best incubation periods to be 4 days with CCHT-1 and 8 days with *Trichoderma reesei*. The methane yield for SLDR pre-treated with CCHT-1 and T. *reesei* at varying incubation periods (Figure 3.7) varied from 0.119m^3 CH_4/kg to 0.192 m^3 CH_4/kg VS_{added} with the best incubation periods for CCHT-1 producing the highest yield. These results are in agreement with the growth pattern of the fungus with a short growth cycle whereby after four days its activity was highest and decreased with extended periods (maturity). The fungus short life cycle and the optimum incubation periods determined concurs with this. White rotted material does not contain much nutrient because white-rot fungi metabolize sugar and starch in preference to lignin and cellulose in cultures (Tripathi *et al.*, 2008). However, negative response of fungal treatment on fermentation is due to the removal of more polysaccharide than lignin (Jung et al. 1992) and also substrate specificity. Possibly, decrease in methane yield with

increase in inoculum concentration led to a decrease in readily available nutrient for biogas production

The NDF% content (Figure 3.6) indicates that, there was a reduction in the total fibre with an increase in the duration of pre-treatment. These results on the pre-treatment periods with CCHT-1 are in agreement with the observation reported by Karunanandaa *et al.* (1992) working on the biodegradability of crop residues colonized by white-rot fungi. The reported increased digestibility after colonization of maize (*Zea maize L.)* and rice straw for 15 and 30 days, respectively by three fungi and a cellulase mutant of *Phanerochaete chrysosporium.* Hadar *et al.* (1993) working on cotton straw reported that biodelignification of cotton straw by the edible "oyster mushroom", *Pleurotus ostreatus*, followed by 36 h of in vivo ruminal digestion removed 2.2 times more organic material than non-fungal pre-treated controls. On the other hand, Lehtomäki *et al.* (2004) reported recently that white rot fungi treatment of lignocellulosic substrates (21 days at 21°C) and short-term composting (7 days) prior to anaerobic digestion resulted in high losses of organic matter due to biological activity. As a result, the increase in methane potential was low or even negative.

4.5 Effect of different inoculum concentrations and pre-treatment periods

The inoculation ratios and periods used in this study were designed to determine the optimal points where the fungal enzyme secreted would be maximal. The results on

methane yields revealed that 10% and 25% inoculum concentrations for CCHT-1 and *Trichoderma reesei,* respectively, were optima. Lower inoculum concentration than the optimum was not sufficient for the disruption of the SLDR structure, as indicated by the low specific methane yields. Increase in inoculum concentration beyond the optimal resulted in decreased methane yields, which can be attributed to the removal of more polysaccharide than lignin and also substrate starch, a similar observation has been reported by Jung *et al.,* (1992).

4.6 The effect of two-steps pre-treatment of SLDR by strain CCHT-1 and *Trichoderma reesei* inoculum on biogas production

A two steps pre-treatment experiment was designed in this study and carried out successfully. The purpose of this experiment was to determine the effect of two fungi employed in sequence using the best inoculum concentrations as was determined in section 3.3 and the best incubation periods as was determined in section 3.5. The highest methane yield recorded when strain CCHT-1 was used followed by *T. reesei* in the pre-treatment of SLDR was 0.292 m^3 CH_4/kg VS_{added}, which is an increase of 101% in comparison to the yield from the un pre-treated residues. This observation is further supported by the decrease in NDF content. When SLDR was pre-treated with *Trichoderma reesei* for 8 days followed by strain CCHT-1, the highest methane yield obtained was 0.189 m^3 CH_4/kg $VS_{added,}$ which was an increase of 30% in comparison to the yields obtained from the un pre-treated residues.

As discussed earlier, the observation that strain CCHT-1 grows naturally on sisal residues implies that it is a good lignocellulosic degrader and hence was able to reduce the limitation of lignin in the anaerobic digestion process (Hammel, 1997). On the other hand *Trichoderma reesei* being a good producer of extracellular cellulolytic enzymes was able to disrupt the crystalline structure of cellulose (Mtui and Nakamura, 2005) resulting in improved methane production. It can therefore be explained that the two-step pre-treatment was more efficient than using CCHT-1 and *T. reesei* separately because of the synergistic activities of the enzymes produced by the two organisms in degrading recalcitrant lignocellulose. However, enzymatic activities were not determined in this study. Besides, there is very little information in the literature on biological pre-treatment of sisal residues for anaerobic digestion and hence direct comparison of the results of this study has been limited.

4.7 SUMMARY AND CONCLUSIONS

The global demand for energy is on the rise, and from an environmental point of view, the best solution to meet the increased energy demand is utilization of renewable sources such as biomass. Biogas production technology has established itself as a technology with great potential for energy generation. The results presented here identify a large reservoir of biomass that can be harnessed in methane production. The Tanzania sisal industry with an annual production of 900,000 tones of SLDR alone, which is currently being disposed of untreated resulting in serious environmental pollution problems can be

pre-treated biologically in two steps prior to anaerobic digestion to generate biogas which can be used for generation of electricity for the sisal decortication factories.

The enzymatic conversion of lignocellulosic material for conversion into biogas has received considerable interest during recent years. This source of raw material is available in abundance. This study assessed the effect of biological pre-treatment of SLDR with lignolytic and cellulolytic microorganisms singly and in combination, and it was found that the mixture of the two organisms resulted in higher methane yields with an increase of 101%, when SLDR was pre-treated with CCHT-1 followed by *T. reesei*. The two-step process of pre-treatment using CCHT-1 followed by *T. reesei* overcomes most of the barriers associated with lignocellulosic biomass, by employing the concerted action of lignolytic, cellulolytic and xylose degrading enzymes.

The total methane yields achieved using pre-treatments employed in this study were higher in comparison to that obtained from the untreated residues, Hence the pre-treated residues responded to anacrobic treatment and produced significant quantities of biogas. Within the experimental conditions, the results confirmed that biological pre-treatment has the potential to achieve significant improvement in biogas production from sisal leave decortication residues. The results indicate that methane potentials per m^3 CH_4/kg VS_{added} increased with increasing inoculum concentrations as well as incubation periods even in two steps pre-treatment, to an optimum, where further increase had a negative impact on the yield.

One possible reason to this observation could be, due to the high inoculum concentrations or prolonged incubation period; the enzymes digested the cellulose in the hydrolysis stage leaving little material to be converted into methane.

The pH values at the beginning of the experiment were pH 8.5 in the un-buffered and buffered bioreactor respectively, the pH values decreased after 7 days to 7.12 and 7.73. This observation could be attributed to accumulation of the acetic acid generated in reactors; there was more pH decrease in the un-buffered reactors than in the buffered, the buffering of systems stabilized the pH. The final pH values were 7.5 and 8.15 in the un-buffered and buffered bioreactor respectively, the methane yields from the un-buffered reactors were higher supporting the fact that use of external buffers was not necessary making application of this system on industrial scale more economical.

5.0 RECOMMENDATION FOR FUTURE RESEARCH

Recommendations for further work include; fine tuning the fungal pre-treatment conditions and time needed to result in significant reduction of total fibre and improvement in cellulase digestibility at small scale, with minimal carbohydrate loss;

Scaling up of the fungal pre-treatment to a pilot scale, so as to evaluate the applicability of this study on an industrial scale is also recommended. At the same time, improvement of existing strains by genetic manipulations can create a basis for attractive industrial biotechnological applications, thus should be considered.

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