EVALUATION OF *CHLORELLA VULGARIS* AS A SOURCE OF FERMENTABLE SUGAR USING AMYLASE PRODUCING *BACILLUS CEREUS*

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ABSTRACT

Bacillus cereus was isolated from the soil using mannitol egg York polymixin B agar (MYP) and used for hydrolysis of *Chlorella vulgaris* starch content. The Dinitrosalicilic acid method was used to determine the amount of reducing sugar produced. Maximum reducing sugar yield of 2.10mg/ml was obtained for pre-treated *C. vulgaris* biomass, 2.06mg/ml for untreated biomass and 2.56mg/ml for corn starch (control) after 24 hours incubation.When the production parameters where optimized, maximum reducing sugar yield was obtained at a pH of 6.5, temperature of 35^0C, incubation time of 24 hours and 4% inoculum size for pretreated *C. vulgaris* biomass. Also, a 1.3 fold reducing sugar yield was enhanced when compared with non-optimized conditions indicating the usefulness of optimization on biochemical processes. The maximum reducing sugar obtained for hydrolysis of Pretreated *C.vulgaris* biomass is significantly comparable to the reducing sugar yield of corn starch. Also, the carbohydrate content of *C. vulgaris* was 66% which is comparable to the carbohydrate content of corn (about 73%). *Chlorella vulgaris* has the potential of being an alternative substrate for fermentable sugar production.

CHAPTER ONE

1.0 INTRODUCTION

The effect of global warming, due to accumulation of carbon dioxide in the atmosphere has become a major concern to climate scientists. Also increase in prices of petroleum products along with depletion of global oil reserves have motivated scientists to search for alternatives to conventional fuels. The commitment of several nations to reduce the carbon dioxide emissions, has given a great impulse to the development of alternative energy sources not based on fossil sources but rather on renewable resources. Scientists have focused on biofuels (bioethanol, biodiesel) from raw plant material and agricultural waste products (Farrell, 2006.). However, if the biofuel industry is to expand in future, researchers have recognized that non-food resources should be used so that the biofuel industries will not compete with the food market in terms of land for cultivation (Doucha *et al.*, 2008).

Algae are good candidates, as alternative energy sources, as they obtain their energy from the sunlight and build up their biomass by removing carbon dioxide from the atmosphere through photosynthesis. In this way, anytime a fuel that originated from plants or algae is burnt, the carbon dioxide emitted is the very same that was previously removed by those organisms. However, unlike plants, algae cultivation does not compete for land space. Microalgae are particularly attractive as they are photoautotrophic organisms that grow in simple inorganic medium and contrary to higher plants; each cell is photosynthetically competent so that the amount of carbon dioxide fixed is much higher in a biomass base (John *et al.*, 2011).

Moreover, its cell wall structure does not contain lignin, which can be advantageous to the enzymatic hydrolysis of its polysaccharide components (Marcoaurelio and Elba, 2011).

One of the key steps of development and implementation of an alternative method of biofuel production is an effective conversion of starch from unicellular algae to ethanol. These photoautotrophic microorganisms have the ability to enjoy faster growth than higher plants as well as lower water and fertilizer consumption (Barbora *et al*., 2010).

The genus *Chlorella* comprises of species in which the cell wall is composed of polysaccharides formed mainly by glucose and mannose and those in which this structure is composed of polysaccharides formed mainly by glucosamine. The cell wall sugar composition of *C. zofingiensis* is 70% glucose and 30% mannose in its cell wall and 65% mannose, 30% glucose, plus minor amounts of rhamnose and galactose in its matrix cell wall (Takeda, 1993b).

Chlorella cell wall is a good source of fermentable sugars, consisting of starch and cellulose (Marcoaurelio and Elba, 2011), provided these polysaccharides are properly hydrolysed.

Although there are several macro-algae which contain intracellular carbohydrates and have a potential for production of biofuels e.g. bioethanol and bio-oil, *Chlorella vulgaris* is among the macro-algae which contains appreciable amounts of starch and cellulose. However, cellulose is a high molecular weight crystalline polymer which is highly stable and recalcitrant to enzymatic hydrolysis (Taherzade and Karimi, 2007). Therefore, a pre-treatment process is typically necessary for efficient conversion of cellulose to simple sugars. Dilute-acid treatment has been successfully developed for pre-treatment of

cellulosic materials. The pre-treatment can enhance the carbohydrates available for enzymatic hydrolysis and fermentation (Taherzade and Karimi, 2007).

Many microbial enzymes are commercially exploited and successfully used on industrial scale to catalyse several chemical processes. These enzymes proved to be better, cheaper and environment friendly compared to the use of chemicals. Enzymes have also been exploited in bioremediation of complex waste substances (Whiteley and Lee, 2006). Microbial enzymes, involved in the degradation and transformation of plant cell-wall polysaccharides, have found many biotechnological applications (DeVries and Visser, 2001). Ligninases, hemicellulases, cellulases, pectinases and amylases are the enzymes which are required to degrade not only the plant biomass to its completion but also have found applications in different industries and pharmaceutical preparations (Bhat, 2000).

Amylases are produced by a variety of living organisms, ranging from bacteria to plants and humans. Alpha-amylases (endo-1, 4-α-D-glucan glucohydrolase,) are extracellular enzymes that randomly cleave the 1,4-alpha-D-glucosidic linkages between adjacent glucose units in the linear amylose chain (Pandey *et al.*,2003). Among various extracellular enzymes, alpha-amylase ranks first in terms of commercial exploitation. Spectrum of applications of alpha-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industries (Anto *et al*., 2006).

Bacteria and fungi secrete amylases to the outside of their cells to carry out extra-cellular digestion. When they have broken down the insoluble starch, the soluble end products such as glucose or maltose are absorbed into their cells. Although many microorganisms produce this enzyme, the ones most commonly used for industrial productions are *Bacillus subtilis*,

Bacillus cereus, *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger* (Sivakumar *et al*., 2012).

1.1 Statement of research problem

Fossil fuels contain high percentages of carbon and include coal, petroleum, and natural gas. The burning of fossil fuels produces around 21.3 billion tonnes of carbon dioxide (CO_2) per year, but it is estimated that natural processes can only absorb about half of that amount, so there is a net increase of 10.65 billion tonnes of atmospheric carbon dioxide per year (one tonne of atmospheric carbon is equivalent to 3.7 tonnes of carbon dioxide) ("US Department of Energy on greenhouse gases",2007). Carbon dioxide is one of the greenhouse gases and contributes to global warming, causing the average surface temperature of the earth to rise in response, which the vast majority of climate scientists agree will cause major adverse effects.

In the United States, more than 90% of greenhouse gas emissions come from the combustion of fossil fuels. Combustion of fossil fuels also produces other air pollutants, such as nitrogen oxides, sulphur dioxide, volatile organic compounds and heavy metals.

(Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990–1998).

Combustion of fossil fuels generates sulphuric, carbonic, and nitric acids, which fall to earth as acid rain, impacting both natural areas and the built environment. Monuments and sculptures made from marble and limestone are particularly vulnerable, as the acids dissolve calcium carbonate.

Harvesting, processing, and distributing fossil fuels can also create environmental concerns. Coal mining methods, particularly mountain top removal and strip mining, have negative environmental impacts, and offshore oil drilling poses a hazard to aquatic organisms. Oil

refineries also have negative environmental impacts, including air and water pollution (American coal ash Association (ACAA), 2004).

Fossil fuels are non-renewable resources because they take millions of years to form, and reserves are being depleted much faster than new ones are being made. The production and use of fossil fuels raise environmental concerns (O'Driscoll and Vergano, 2007).

1.2 Justification

The idea of using microalgae as a source of fuel is not new (Kapdan and Kargi, 2006) but it is now being taken more seriously because of the escalating price of petroleum and, more importantly, the emerging concern about global warming that is associated with burning fossil fuels (Sawayama *et al*., 1995). Evidence from literature and past studies has revealed that the recent global warming has influenced agricultural productivity leading to declining food production (Lobell *et al*, 2008; Apata, 2009).

Bioethanol is currently being produced by fermentation of sugars found in plants such as sugarcane and corn. Many social concerns have barred the adoption of their future use, so other feedstocks are being considered as substitutes. Algae are one of such alternative. Algae do not produce as much starch as corn, and do not have firm agricultural practices. However, there is reason to believe that algae will play a role in the future bio-ethanol market. In order to lower the cost of producing this fuel, other products from algae will have to be processed and bio-ethanol is one of such products (Berhens *et al*., 1985).

Chlorella vulgaris is known as one of the fastest growing microalgae and the cell wall of microalgae belonging to the Chlorophyte division may reach up to 80% in carbohydrate content, as is the case for *Chlorella fusca* (Loos and Meindl, 1982).

Algae and aquatic biomass have the potential to provide a new range of third generation biofuels, including jet fuels. Their high oil and biomass yields, widespread availability,

absent (or very reduced) competition with agricultural land, their efficient use as a means to capture carbon dioxide and their suitability for wastewater treatments and other industrial plants makes algae and aquatic biomass one of the most promising and attractive renewable resources for a fully sustainable and low carbon economy portfolio.

1.3 Scope of the study

This research work was restricted to the isolation and characterization of amylase producing *Bacillus cereus* from the soil as well as hydrolysis of *Chlorella vulgaris* biomass to fermentable sugar (glucose).

1.4 Aim of the study

This work was aimed to evaluate the use of *Chlorella vulgaris* a green microalga, as a source of fermentable sugar (glucose), through the enzymatic hydrolysis of its cell wall intracellular starch Content

1.5 Specific objectives of the study

1. To Isolate and characterize *Bacillus cereus* from the soil;
2. To screen *B. cereus* isolates for amylase production;
3. To pre-treat and hydrolyse pre-treated and un-treated *C. vulgaris* biomass to fermentable sugar (glucose) and to analyse the resultant reducing sugar;
4. To optimize the conditions for *C. vulgaris* biomass hydrolysis;
5. To estimate *C. vulgaris* carbohydrate

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Effects of global warming

The effects of global warming are the ecological and social changes caused by the rise in global temperatures. There is a scientific consensus that climate change is occurring, and that human activities are the primary driver. Evidence of climate change includes the instrumental temperature record, rising sea levels, and decreased snow cover in the Northern Hemisphere (Intergovernmental Panel on Climate Change (IPCC), 2007)

According to IPCC, most of the observed increase in global average temperatures since the mid-20th century is very likely due to the observed increase in human greenhouse gas concentrations (Solomon, 2009).

Carbon dioxide is one of the greenhouse gases and contributes to global warming, causing the average surface temperature of the Earth to rise in response, which the vast majority of climate scientists agree will cause major adverse effects.

Human activities have contributed to a number of the observed changes in climate. This contribution has principally been through the burning of fossil fuels, which has led to an increase in the concentration of green house gases in the atmosphere. Another human influence on the climate are sulphur dioxide emissions, which are a precursor to the formation of sulphate aerosols in the atmosphere (Committee on the Science of Climate Change, US National Research Council, 2001)

Human-induced warming could lead to large-scale, irreversible, and/or abrupt changes in physical systems. An example of this is the melting of ice sheets, which contributes to sea level rise. The probability of warming having unforeseen consequences increases with the rate, magnitude, and duration of climate change.

2.1.1 Effects on agriculture

Evidence from literature and past studies has revealed that the recent global warming has influenced agricultural productivity leading to declining food production (Lobell *et al.*, 2008). In order to meet the increasing food and non-food needs due to population increase, man now rapidly depleting fertile soils, groundwater, biodiversity, and numerous other non-renewable resources to meet his needs. This resource depletion was linked with other human pressures on the environment. Possibly the most serious of human impacts is the injection of greenhouse gases into the atmosphere. The reality of the impact of climate change on agricultural development has started showing signs (Adams *et al*., 1998). A substantial body of research has documented these wide-ranging effects on many facets of human societies (Wolfe *et al.*, 2005; Apata *et al*., 2009).

Rough estimates suggest that over the next 50 years or so, climate change may likely have a serious threat to meeting global food needs than other constraints on agricultural systems (Building Nigeria's Response to Climate Change (BNRCC), 2008). Specifically, population, income, and economic growth could all affect the severity of climate change impacts in terms of food security, hunger, and nutritional adequacy. If climate change adversely affects agriculture, effects on human are likely to be more severe in the poorer world. Wolfe *et al* (2005) and Stige, (2006)) worry that rising demand for food over the next century, due to population and real income growth, will lead to increasing global food scarcity, and a worsening of hunger and malnutrition problems particularly in developing countries.

Recently, international tensions and concerns are heightening over what the impact of climate will have on the environment and agricultural produce (Nigerian Environmental Study Team (NEST), 2004; BNRCC, 2008; Apata *et al.*,2009). Also, how agricultural and

food-distribution systems will be further stressed up by the shifting of temperatures and precipitating belts, especially if changes are rapid and not planned for (NEST, 2004). The crucial issue in this study is whether agricultural output supply can keep pace with population increase under this climate variability. This will depend; both on the scope for raising agricultural productivity (including reducing waste during distribution), availability of inputs used in the agricultural sector (land, labour, machinery, water resources, fertilizers among others) and having sufficient information on climatic variables for possible effective adaptation and mitigation strategies.

2.1.2 Effects on the ocean

The role of the oceans in global warming is a complex one. The oceans serve as a sink for carbon dioxide, taking up much that would otherwise remain in the atmosphere, but increased levels of CO_2 have led to ocean acidification. Furthermore, as the temperature of the oceans increases, they become less able to absorb excess CO_2. The ocean has also acted as a sink in absorbing extra heat from the atmosphere. The increase in ocean heat content is much larger than any other store of energy in the Earth's heat balance over the two periods 1961 to 2003 and 1993 to 2003, and accounts for more than 90 % of the possible increase in heat content of the Earth system during these periods (Shaffer *et al*., 2009). Global warming is projected to have a number of effects on the oceans. Ongoing effects include rising sea levels due to thermal expansion and melting of glaciers and ice sheets, and warming of the ocean surface, leading to increased temperature stratification.

About one-third of the carbon dioxide emitted by human activity has already been taken up by the oceans. As carbon dioxide dissolves in sea water, carbonic acid is formed, which has the effect of acidifying the ocean, measured as a change in pHs. The uptake of human carbon emissions since the year 1750 has led to an average decrease in pH of 0.1 units.

The effects of ocean acidification on the marine biosphere have yet to be documented. Laboratory experiments suggest beneficial effects for a few species, with potentially highly detrimental effects for a substantial number of species. With medium confidence, (Fischlin *et al.* 2007) projected that future ocean acidification and climate change would impair a wide range of planktonic and shallow benthic marine organisms that use aragonite to make their shells or skeletons, such as corals and marine snails (pteropods), with significant impacts particularly in the Southern Ocean. The amount of oxygen dissolved in the oceans may decline, with adverse consequences for ocean life.

2.2 Biofuels

Biofuels are liquid fuels made from plant materials that can be used as a substitute for petroleum-derived fuel. Biofuels can include relatively familiar ones, such as ethanol made from sugar cane or diesel-like fuel made from soybean oil, to less familiar fuels such as dimethyl ether (DME) or Fischer-Tropsch liquids (FTL) made from lignocellulosic biomass (International energy Agency, 2006). Biofuels are drawing increasing attention worldwide as substitutes for petroleum-derived transportation fuels to help address energy cost, energy security and global warming concerns associated with liquid fossil fuels. (Goldenberg *et al*., 2004)

2.2.1 Biofuel classification

2.2.1.1 First-generation biofuels

The most well-known first-generation biofuel is ethanol made by fermenting sugar extracted from sugar cane or sugar beets, or sugar extracted from starch contained in maize

kernels or other starch-laden crops. Similar processing, but with different fermentation organisms, can yield another alcohol, butanol. Commercialization efforts for butanol are ongoing (Dupont, 2007), while ethanol is already a well-established industry.

Global production of first-generation bio-ethanol in 2006 was about 51 billion litres , with Brazil (from sugar cane) and the United States (from maize) each contributing about 18 billion litres, or 35 % of the total. China and India contributed 11 per cent to global ethanol production in 2006, and production levels were much lower in other countries with feedstock that include cane, corn, and several other sugar or starch crops (sugar beets, wheat, potatoes).

Many countries are expanding or contemplating expanding their first-generation ethanol production, with Brazil and the United States having by far the largest expansion plans.

2.2.1.2 Second-generation biofuels

Second-generation biofuels share the feature of being produced from lignocellulosic biomass, enabling the use of lower-cost, non-edible feedstock, thereby limiting direct food versus fuel competition. Second-generation biofuels can be further classified in terms of the process used to convert the biomass to fuel: biochemical or thermochemical. Second-generation ethanol or butanol are made via biochemical processing, while all other second-generation fuels are made via thermo-chemical processing (Collins, 2007).

Second-generation thermochemical biofuels may be less familiar to most readers than second-generation ethanol, because there are no first-generation analogs. On the other hand, many second-generation thermochemical fuels are fuels that are already being made commercially from fossil fuels using processing steps that in some cases are identical to

those that would be used for biofuel production. These fuels include methanol, refined Fischer-Tropsch liquids (FTL), and dimethyl ether (DME) (Coelho, 2006).

Mixed alcohols can also be made from fossil fuels, but there is no commercial production today due to the immature state of some components of systems for producing these. The other thermochemical biofuel is green diesel, for which there is no obvious fossil fuel analog. Unrefined fuels, such as pyrolysis oils, are also produced thermochemically, but these require considerable refining before they can be used in Engines.

2.2.1.3 Second-generation biochemical biofuels

The fuel properties of second-generation ethanol or butanol are identical to those of the first generation equivalents, but because the starting feedstock is lignocelluose, fundamentally different processing steps are involved in producing them. Second-generation biochemically-produced alcohol fuels are often referred to as "cellulosic ethanol" and "cellulosic biobutanol". The basic steps for producing these include pre-treatment, saccharification, fermentation, and distillation. Pretreatment is designed to help separate cellulose, hemicelluloses and lignin so that the complex carbohydrate molecules constituting the cellulose and hemicelluloses can be broken down by enzyme catalyzed hydrolysis (water addition) into their constituent simple sugars (Jeffries, 2006).

Cellulose is a crystalline lattice of long chains of glucose (6-carbon) sugar molecules. Its crystallinity makes it difficult to unbundle into simple sugars, but once unbundled, the sugar molecules are easily fermented to ethanol using well-known micro-organisms, and some micro-organisms for fermentation to butanol are also known. Hemicelluloses consists of polymers of 5-carbon sugars and is relatively easily broken down into its constituent sugars such as xylose and pentose. However, fermentation of 5-carbon sugars is more

challenging than that of 6-carbon sugars. Some relatively recently developed micro-organisms are able to ferment 5-carbon sugars to ethanol (Zhang and Lynd, 2005).

2.2.1.4 Second-generation thermo-chemical biofuels

Thermo-chemical biomass conversion involves processes at much higher temperatures and generally higher pressures than those found in biochemical conversion systems. The key intrinsic characteristics distinguishing thermochemical from biochemical biofuels are the flexibility in feed stocks that can be accommodated with thermochemical processing and the diversity of finished fuels that can be produced (Stricklen, 2006).

Thermochemical production of biofuels begins with gasification or pyrolysis. The former is generally more capital-intensive and requires larger scale for best economics, but the final product is a clean finished fuel that can be used directly in engines. This focuses on gasification based processing, by which a variety of different biofuels can be produced, including Fisher-Tropsch liquids (FTL), dimethyl ether (DME), and various alcohols.

During gasification, biomass (with 10–20 % moisture content) is heated (typically by combusting a portion of the biomass in oxygen) to cause it to be converted into a mixture of combustible and non-combustible gases. Contaminants in the gas are removed, followed in some cases by adjustments (using the "water-gas shift" reaction) of the composition of the gas (also called synthesis gas, or syngas) to prepare it for further downstream processing. Carbon dioxide (CO2) is a diluent in the syngas and so is then removed to facilitate subsequent reactions downstream. The major components of the now-clean and concentrated syngas are carbon monoxide (CO) and hydrogen (H2), usually with a small amount of methane (CH4). The CO and H2 react when passed over a catalyst (the CH4 is inert) to produce liquid fuel. The design of the catalyst determines what biofuel is produced (Devi *et al*., 2003).

In most plant designs, not all of the syngas passing over the catalyst will be converted to liquid fuel. The unconverted syngas typically would be burned to make electricity to provide some or all of the power needed to run the facility and in some cases to export electricity to the grid.

In 2008, fossil fuels accounted for 88% of the global primary energy consumption (Brennan *et al*, 2010). However, the use of fossil fuels is now widely accepted as unsustainable due to depleting resources and the accumulation of greenhouse gases in the environment.

It has become increasingly important to develop abatement techniques and adopt policies to promote those renewable energy sources to minimize the dependency on fossil reserves and also to maintain environmental and economic sustainability (Brennan *et al*, 2010 and Singh *et al*., 2010).

Biofuels are expected to reduce dependence on imported petroleum with associated political and economic vulnerability, reduce greenhouse gas emissions and other pollutants, and revitalize the economy by increasing demand and prices for agricultural products (Balat, 2010). Biofuels are an attractive alternative to current petroleum based fuels as they can be utilized as transportation fuels with little change to current technologies and have significant potential to improve sustainability (Carere *et al.*, 2008). There are two global liquid transportation biofuels: bioethanol and biodiesel, respectively (Demirbas, 2011). Bioethanol is a good alternate fuel that is produced almost entirely from food crops (Hammond *et al*., 2008, Tan *et al*., 2009, Börjesson, 2009).

All automobile manufacturers produce vehicles that can readily use 10% ethanol or 85% ethanol (E85) blends for fuel, and ethanol can replace diesel in heavy vehicles as well.

Nearly all fuel ethanol is produced by fermentation of corn glucose in the United States or sucrose in Brazil (Rosillo-Calle *et al*., 1998).

Generally, ethanol can be derived from any material that contains sugar. Today, raw materials used in the manufacture of ethanol by fermentation are classified as sugars, starches and cellulosic materials (Bailey and Ollis, 1986). Sugars can be converted to ethanol directly, while starches first have to be hydrolyzed to fermentable sugars by the action of enzymes. Cellulose must likewise be converted to sugars before further fermentation, generally by the action of mineral acids (Bashir *et al.*, 1994).

2.2.1.5 Third generation biofuels

Third generation biofuels, also termed advanced biofuels, are produced from fast growing microalgae and are potential replacements for conventional fuels. Microalgae are being promoted as an ideal third generation biofuel feedstock because of their rapid growth rate, low requirements for fertilizer and land use, high CO_2 fixation ability, lack of competition with food crops, and production possibilities on non-arable land. Microalgae have broad bio-energy potential as they can be used to produce biodiesel and bio-ethanol. For example, *Chlorella vulgaris* has been considered as a potential raw material for bio-ethanol production because it can accumulate high levels of starch (Hirano *et al*., 1997). Production of bioethanol by using microalgae can also be performed via self-fermentation. As reported by Ueno *et al*., 1998, dark fermentation of marine green algae *Chlorococcum littorale* at 30°C could produce 450 μmol of ethanol g/1 biomass.

Marine microalgae, or phytoplankton, are mostly represented in ocean populations: the best known are the diatoms (Bacillariophyta), the dinoflagellates (Dinophyta), the green algae (Chlorophyta) and the blue-green algae (Cyanophyta). The unicellular marine microalgae

were considered to be an abounding resource for carotenoids, lipids, and polysaccharides, and were widely investigated in the fields of food supplements and bio-fuel production (Liau *et al*., 2010). While terrestrial plants in temperate climates can achieve a photoconversion efficiency of only below 1 %, microalgae can convert up to 5% of the solar energy into chemical energy (Rosch *et al.*, 2012). Microalgae can be converted to biodiesel, bioethanol, bio-oil, biohydrogen and biomethane via thermochemical and biochemical methods **(**Demirbas, 2011). Using microalgae as a source of biofuels is not a new idea (Chisti, 1980), but it is now being taken seriously because of the increasing cost of petroleum and, more significantly, the emerging concern about global warming arising from burning fossil fuels (Sawayama *et al*., 1995).

2.2.1.6 Fourth generation biofuels

Fourth generation biofuels are a step ahead from third generation biofuels. In fourth generation systems, the feedstock is tailored to improve the processing efficiency and designed to capture more carbon dioxide than normal. The processing methods are also coupled to "carbon capture and storage (CCS)" technologies which funnel the generated carbon dioxide into geological formations or mineral storage systems. In this way, fourth generation biofuels are better at reducing greenhouse gas emissions than other generation biofuels (Chandrashekhar *et al*., 2012).

Fourth generation biofuel crops include high biomass crops, trees with increased carbon storage capacity, drought-tolerant energy crops, grass species that are able to grow on acidic soils, and new plants with particular properties catering to a specific bioconversion process (for example, low lignin trees, maize with embedded enzymes for rapid conversion). Recent examples include designed *Eucalyptus* trees with a low-lignin content which allows for easier conversion into cellulosic ethanol.

2.3 Utilization of bioethanol and biodiesel as transportation fuels

Plant and algae-derived ethanol can be used as a pure fuel or as a mixture with gasoline in certain proportions to replace conventional fuels, without affecting the performance of the machine. Being an oxygenated fuel (contains 35 % of oxygen), use of bioethanol reduces exhaust emissions such as CO and unburned hydrocarbons in to atmosphere (Balat, 2009). The most common blend of bioethanol with petrol is gasohol, also called E10 containing 10 % bioethanol and 90 % gasoline. E10 could be burned in the internal combustion engines (ICEs) of most modern cars (Demirbas, 2008). Bioethanol could also be used in blended fuels such as E85 (containing 85 % ethanol and 15 % gasoline).

The biologically produced ethanol contains about 5 % water and by using an appropriate emulsifier this hydrated ethanol could be mixed with diesel. A mixture of hydrated ethanol with diesel with the emulsifier is called diesohol. This contains 84.5 % diesel, 15 % hydrated ethanol and 0.5 % of emulsifier. In Brazil, ethanol is used as clean or as gasohol (24 % ethanol and 76 % gasoline) (Balat, 2009). While in the European Union, according to the EN228 standard, bioethanol could be used as a 5 % blend with gasoline. On the other hand, biodiesel is the most common biofuel in Europe. Pure biodiesel (B100) is the lowest emission fuel. Biodiesel can be used in any diesel engine when mixed with diesel. Biodiesel is safe to handle and transport as it is biodegradable and has a high flash point compared to diesel.

2.4 Microalgae

Microalgae are microscopic algae, typically found in freshwater and marine systems (Thurman, 1997). They are unicellular species which exist individually, or in chains or groups. Depending on the species, their sizes can range from a few micrometers (µm) to a few hundreds of micrometers. Unlike higher plants, microalgae do not have roots, stems

and leaves. Microalgae, capable of performing photosynthesis, are important for life on earth; they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically.

Microalgae are generally more efficient than land based plants in utilizing sun light, CO_2, water and other nutrients which translate to higher biomass yields and higher growth rates .They can also grow in a variety of aquatic environments and can be grown without the use of fertilizers and pesticides which results in less waste and pollution (Richmond *et al*., 1982).

The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about 200,000-800,000 species exist of which about 50,000 species are described (Starckx, 2012). Over 15,000 novel compounds originating from algal biomass have been chemically determined. Most of these microalgae species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols.

The chemical composition of microalgae is not an intrinsic constant factor but varies over a wide range, both depending on species and on cultivation conditions. It is possible to accumulate the desired products in microalgae to a large extent by changing environmental factors (Tredici and Materassi , 1992) like temperature, illumination, pH, CO_2 supply, salt and nutrients.

Microalgae can provide several different types of renewable bio-fuels. These include methane produced by anaerobic digestion of the algal biomass (Spolaore *et al*., 2006) biodiesel derived from micro algal oil (Thomas, 2006; Rorssler *et al*., 1994 and Banerjee *et al*., 2002) bioethanol and photo biologically produced bio-hydrogen.

2.4.1 Ethanol production from microalgae

Seaweeds are classified into three groups: green, brown, and red, and they contain various types of glucans which are polysaccharides composed of glucose, though the concentration of these glucans is known to be relatively low. Seaweed was proposed as one of the most promising biomass materials that can be easily converted to ethanol, since seaweeds are known to contain a low concentration of lignin or no lignin at all. Three types of seaweed including sea lettuce, chigaiso, and agar weed were used as representatives of green, brown, and red seaweeds, respectively, and methods for obtaining high concentrations of ethanol (bioethanol) from these seaweeds were investigated (Chandrashekhar *et al*., 2012). An ethanol yield of more than 3 % was obtained from these seaweeds.

Today, the most important microorganisms applied for ethanol production are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*. However, both these ethanol producers have a very narrow substrate range. Horn *et al* (2000) have reported that *Zymobacter palmae* could produce ethanol from mannitol in seaweed extract, if some supply of oxygen was provided. In addition, the possibility of ethanol production from *Laminaria hyperborean* extracts was evaluated, focusing on the yeast *Pichia angophorae* and its potential of utilising both mannitol and laminaran as substrates. Initial experiments with seaweed extract showed that *Pichia angophorae* was able to utilise both mannitol and laminaran for ethanol production (Horn, 2000). Ethanol can be produced from the mannitol and laminaran contained in extracts from *L.*

hyperborea fronds. *Z. palmae* was able to produce ethanol from mannitol, while *P. angophorae* could utilise both carbohydrates for ethanol production.

Bioethanol from algae holds significant potential due to their low percentage of lignin and hemicellulose as compared to other lignocellulosic plants (Harun *et al.*, 2010). While

having low lignin content, macroalgae contain significant amount of sugars (at least 50 %) that could be used in fermentation for bioethanol production (Wi *et al*., 2009). However, in certain marine algae such as red algae the carbohydrate content is influenced by the presence of agar, a polymer of galactose and galactopyranose. Current research seeks to develop methods of saccharification to

Unlock galactose from the agar and further release glucose from cellulose leading to higher ethanol yields during fermentation (Wi *et al*., 2009; and Yoon *et al*., 2010).

2.4.2 Factors affecting microalgal growth

2.4.2.1 Nutrient composition of media

Media composition has an immense effect on both the growth rate and the final concentration of microalgae. Microalgae are known to grow more abundantly in nutrient rich (eutrophic) waters leading frequently to algal blooms (Schenk *et al*., 2008). In a comparison study of the growth of *Chlorella sorokiniana*, in TAP (Tris-Acetate-Phosphate) media, with either 15 g/L of NH_4Cl or 1.5 g/L of $NaNO_3$, it was found that the maximum biomass concentration (4.4 g/L) was similar on both cases when using 5 % CO_2 enriched air but the pH decline was more drastic in the case of the original TAP formulation (with NH^4Cl) (Kumar and Das, 2012). (Feng *et al*., 2011) observed a depletion of ammonium (as NH_4Cl) in artificial wastewater within 3 days of culture. The growth of *Chlorella vulgaris* was not limited, even after the depletion of ammonium,which leads to confirm that microalgae utilized ammonium first and then the nitrate present in the inoculum.

A change of the concentration of nitrates (as KNO_3) in the culture medium, when growing different strains of *Chlorella*, had little effect on the biomass concentration and lipid production possibly due to the fact that the lowest concentration tested (1.24 g/L) was already high enough to promote growth (Sirisansaneeyakul *et al*., 2011)

A study published in 1982 showed that relatively low concentrations of $NaHCO_3$ (0.5M or 4.2 g/L) and bubbling with CO_2 enriched air (1.5 %), favored the growth of *Chlorella vulgaris* in a mixed culture with *Spirulina platensis*, this being independent of the pH, which ranged between 8.5 and 9 (Richmond *et al*., 1982). Another study by (Zheng *et al*., 2011), showed similar results where the growth of *Chlorella vulgaris*, with $NaHCO_3$ at a concentration of 40mmol/L (3.36 g/L), yielded 0.67 g/L of dried biomass. Addition of $NaHCO_3$ to Basal media, showed an increase of biomass production by *Chlorella vulgaris* in a concentration range of 0.1-1.6 g/L, as the concentration of $NaHCO_3$ increased so did the biomass production, reaching its maximum (0.6 g/L of biomass) at a concentration of 1.2 g/L of $NaHCO_3$ (Yeh *et al*., 2010). The fixation of dissolved CO_2, in the form of bicarbonate using an alkali NaOH, has shown to increase the biomassproduction of *Chlorella* sp. in comparison to the addition of CO_2 alone (Aishvarya *et al*., 2012).

It has been noted that the lipid content of some microalgae increases with the supplementation of $NaHCO_3$ to the media at different concentrations and bubbling with CO_2 enriched air. Maximum growth rate and lipid production in the case of *Chlorella* was seen at a $NaHCO_3$ concentration of 75 ppm (75 mg/L) and with a concentration of CO_2 of 4758 ppm (Devgoswami *et al*., 2011).

2.4.2.2 Light

Duration of light periods is an important criterion to be considered in the design of a photobioreactor set-up. Photoperiods have a significant effect on the photosynthetic activity and growth rates in a typical microalgae cultivation system but it should be noted that excessive light exposure can lead to waste of electricity and cell growth inhibition. With a photoperiod of 12 h nearly doubling the microalgae biomass concentration in comparison

with 3 h, 6 h, and 9 h light periods (Lam *et al*., 2012). It has been found that growth rates and maximum cell density of microalgae increase proportionally to light period duration, even at different light intensities (Amini *et al*., 2012; and Jacob-Lopes *et al*., 2009). An exception to this was discovered with a 12:12 h (light:dark) photoperiod, in which higher productivities and maximum cell density values where obtained, in comparison with the 14:10 h (light:dark) (Jacob-Lopes *et al*., 2009).

2.4.2.3 pH

Initial culture pH can have a major effect on biomass concentration and lipid production of Some strains of microalgae, it can also cause the precipitation of some calcium salts if it exceeds a value of 9.0 (Becker, 1994; Sirisansaneeyakul *et al*., 2011). Mayo (1997) found that *Chlorella vulgaris* can tolerate a pH as low as 3.0. At the same time, high final pH (pH=11) caused negative effects on the growth of another strain of *Chlorella vulgaris* (Yeh *et al*., 2010).

2.4.3 Microalgae cultivation

Different approaches can be taken when looking to grow microalgae in large volumes. Mainly outdoor ponds, with light supplied by the sun, and photo-bioreactors, which can be outdoor or indoor and light, can be supplied by electric lights. Species control is better achieved under closed conditions that are very common in laboratory setting. Closed systems provide a better opportunity, meet specific demands and in the control and optimization of cell growth parameters. In the case of photo-bioreactors, many designs, considerations need to be made depending on what the end goal is. There are basic design features that should be considered regardless of the configuration; like the materials used for the set-up, source of light, how will the algae circulate through the reactor, how to provide CO_2, and how to control other parameters such as pH, temperature and nutrient

concentration in the media (Andersen, 2005 and Chisti, 2007). A study on *Chlorella vulgaris* by (Lam *et al.*, 2012) found that the most adequate growth was under indoor conditions and utilizing inorganic nutrient media. There are various configurations that have been built and tested for the growth of microalgae.

2.4.4 Microalgae growth kinetics.

To estimate growth rates, one must have a series of measurements, at different times, that will permit the calculation of the rate of change in biomass concentration. Cell number should be counted, either through a direct method, as through light microscopy with a hemacytometer, or indirectly through biomass concentration (as dry weight) or optical density, as long as this measurements correlate linearly with the number of cells (Andersen, 2005 and Becker, 1994)

Under a typical homogenous batch regime (in a closed system), microalgae will pass through the following growth phases that include Adaptation (lag phase), Exponential growth phase (log phase), Stationary phase, Logarithmic death phase (Becker, 1994).

2.5 *Chlorella*

Chlorella is a genus of single-cell green microalgae, belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 μm in diameter, and is without flagella. *Chlorella* contains the green photosynthetic pigments chlorophyll-a and-b in its chloroplast. Through photosynthesis, it multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce (Zelitch,1971). The genus is comprised of four species that consist of *C. vulgaris, C. Pyrenoidosa, C. munitissima*, and *C. variabilis.*

2.5.1 *Chlorella* as a source of food / feed

Chlorella is an attractive potential food source because it is high in protein and other essential nutrients; when dried, it contains a good proportion of protein, fat, carbohydrate, fibre, minerals and vitamins. Mass-production methods are now being used to cultivate it in large artificial circular ponds. It is also abundant in calories, fat, and vitamins (Belasco, 1997).

When first harvested, *Chlorella* was suggested as an inexpensive protein supplement to the human diet. Advocates sometimes focus on other supposed health benefits of the algae, such as claims of weight control, cancer prevention, and immune system support (Belasco, 1997).

A small (35 participant) study suggested *Chlorella* supplementation has a positive effect on the reduction of dioxin levels in breast milk and it may also have beneficial effects on nursing infants by increasing the IgA levels in breast milk (Nakano *et al*.,2007).

Following global fears of an uncontrollable population boom, during the late 1940s and the early 1950s *Chlorella* was seen as a new and promising primary food source and as a possible solution to the then-current world hunger crisis. Many people during this time thought hunger would be an overwhelming problem and saw *Chlorella* as a way to end this crisis by providing large amounts of high-quality food for a relatively low cost (Belasco, 1997).

2.5.2 Wastewater treatment

Microalgae like those belonging to the genus *Chlorella* have been successfully used as part of the treatment of different types of wastewater; they can then help in the removal of pollutants including some carcinogenic chemicals. In some cases pre-treatment may be

necessary since dark color and high fat content of the wastewater has been found to inhibit the growth of microalgae but this makes it possible to effectively use microalgae in the treatment of different animal farm effluents (Heidari *et al*., 2011; Hodaifa *et al*., 2012; Munoz *et al*., 2006; Munoz *et al*., 2005; Schenk *et al*., 2008).

2.5.3 Use in medicine

Chlorella is consumed as a health supplement primarily in the United States and Canada and as a food supplement in Japan. *Chlorella* has a number of health effects believed to be associated with it (Shim *et al*., 2007).

Because of its ability to bind with mercury, lead, and cadmium, *Chlorella vulgaris* has been increasingly used in alternative medicine to detoxify the body of certain heavy metals. Several studies demonstrate that it has an ability to safely draw toxic metals that accumulate in the gut and intestinal tract (Inthon *et al*., 2002, Shim *et al*., 2007 and Blasvaldivia *et al*., 2010). Like blue-green algae, clinical studies on *Chlorella* suggest effects including polychlorinated dibenzodioxins detoxification in humans (Nakano *et al*., 2005) and animals (Tekekoshi *et al*., 2005) healing from radiation exposure in animals (Singh *et al*., 1995) and the ability to reduce high blood pressure, lower serum cholesterol levels, accelerate wound healing, and enhance immune functions in humans (Merchant *et al*., 2001).

There are several other health effects believed to be associated with *Chlorella*. *Chlorella* has been found to have anti-tumor properties when fed to mice (Tanaka *et al*., 1990, Miyazawa *et al*., 1988 and Konishi *et al*., 1985). Another study found enhanced vascular function in hypertensive rats given oral doses of *Chlorella* (Sansawa *et al*., 2006). However, the use of *Chlorella* for healing effects has received criticism (Berker, 1994).

The sugar composition of the algae cell wall has been studied. The cell wall of microalgae belonging to the Chlorophyte division may reach up to 80 % in carbohydrate content, as is the case for *Chlorella fusca* (Marcoaurelio *et al*., 2011). The composition and the cell wall structure is species dependent and may be used as taxonomical markers. As such, the genus *Chlorella* comprises of species in which the rigid cell wall is composed of polysaccharides formed mainly by glucose and mannose and those in which this structure is composed of polysaccharides formed mainly by glucosamine. The sugar composition of the cell wall matrix of the first group is dominated by mannose and fructose whereas in the second group galactose, fructose, and sometimes xylose are the main sugars found (Tekeda, 1991). The cell wall sugar composition of *C. zofingiensis* is 70 % glucose and 30 % mannose in its "rigid cell wall" and 65 % mannose, 30 % glucose, plus minor amounts of rhamnose and galactose in its matrix cell wall (Tekeda, 1991). Concerning cell wall sugar composition of *C. homosphaera*, whose synonymy with *Chlorella minutissima* has been recently established (Kienitz *et al*., 2011), it presents 85 % glucose 15 % mannose in its "rigid cell wall" and 70 % mannose, 20 % glucose and 10 % galactose in its matrix cell wall.

Accordingly, *Chlorella* cell wall is a good source of fermentable sugars, mostly cellulose derived, not to mention its starch content (Behrens *et al*., 1985) provided these polysaccharides are properly hydrolyzed. The microalgae *Chlorella vulgaris*, particularly, has been considered as a promising feedstock for bioethanol production because it can accumulate up to 37 % (dry weight) of starch. However, higher starch contents can also be obtained for optimized culture conditions (Hirano *et al*., 1997).

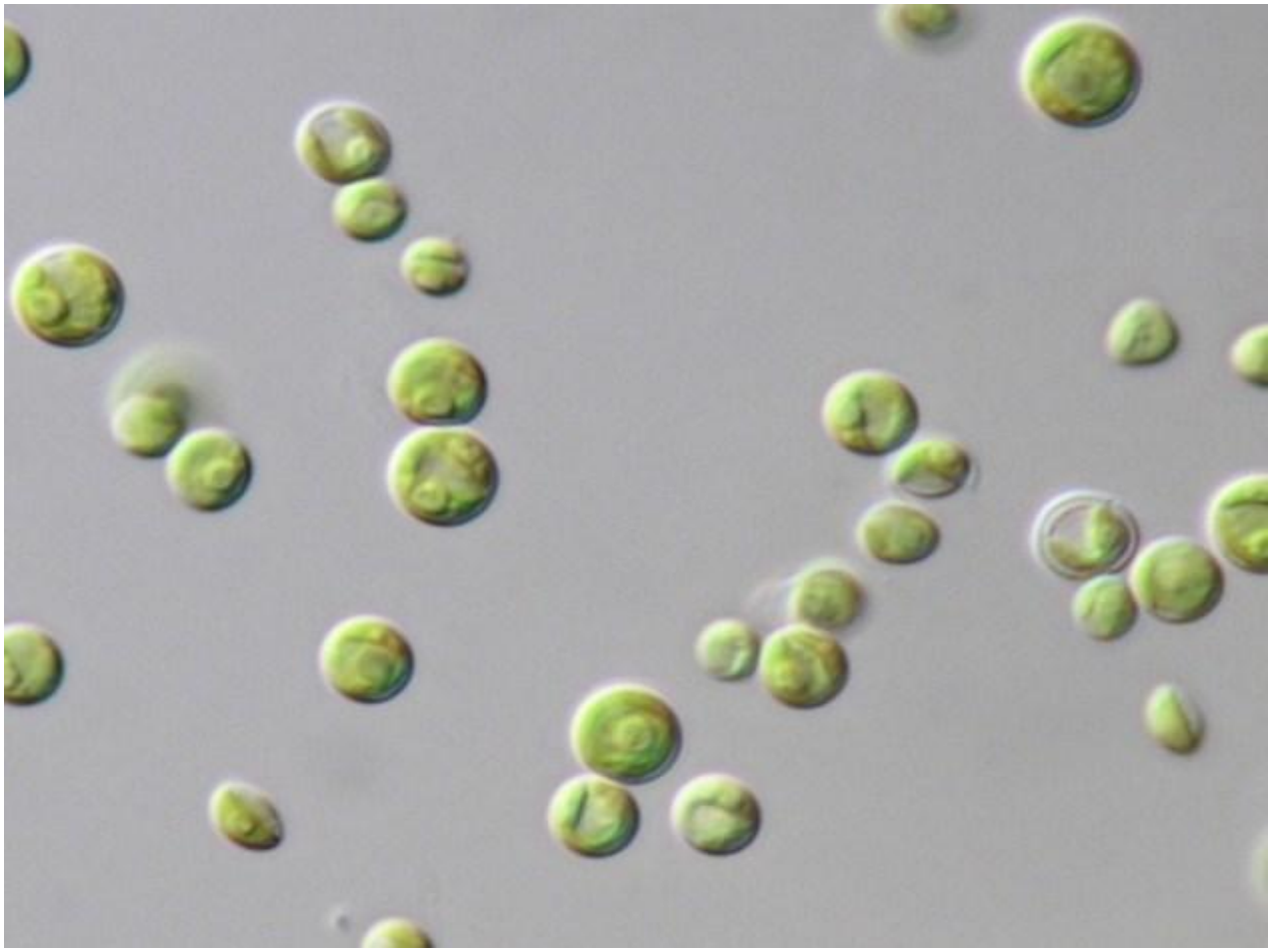

Plate 2.1 Microscopic images *of chlorella vulgaris* ((Elumalai *et al*., 2011)

2.6 *Bacillus* sp

Bacillus refers to a specific genus of bacteria. The family *Bacillaceae* are all Gram-positive, rod-shaped bacteria which form endospores, with two main divisions: the anaerobic spore-forming bacteria of the genus *Clostridium* and the aerobic or facultatively anaerobic spore-forming bacteria of the genus *Bacillus (*Vilian *et al*., 2006)

Characteristically, *Bacillus* cultures are gram-positive when young, but may become Gram-negative as they age. *Bacillus* species are aerobic, sporulating, rod-shaped bacteria which are ubiquitous in nature. Gram-stained cells, 1 µm wide, 5-10 µm long, arranged singly or in short chains.

The genus *Bacillus* consists of the best known species such as *Bacillus subtilis, Bacillus anthracis, Bacillus cereus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus sphaericus* and *Bacillus thuringiensis* with other 50 valid species. Studies with 16SrRNA have now allowed a start to be made on breaking the genus down into more manageable and better-defined groups (Rossler *et al*., 1991). Many of these species now fall into several apparently distinct rRNA sequence groups such as *Bacillus subtilis* group, *Bacillus cereus* group, and *Bacillus sphaericus* group.

2.6.1 Bacillus cereus description and significance

Bacillus cereus is a large, 1 x 3-4 µm, soil dwelling, Gram-positive, rod-shaped, beta hemolytic bacterium (Vilian *et al*., 2006). Some strains are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals (Ryan *et al*., 2004). *Bacillus cereus* bacteria are facultative anaerobes, and like other members of the

genus *Bacillus* can produce protective endospores. Its virulence factors include cereolysin and phospholipase C.

It was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient and was cultured from the blood and pleural fluid (Hoffmaster *et al*., 2006). 16s rRNA comparison reveals *Bacillus cereus* to be most related to *Bacillus anthracis*, the cause of anthrax, and *Bacillus thuringiensis*, an insect pathogen used as pesticide (Delvecchio *et al*., 2006). Although they have similar characteristics, they are distinguishable as *B. cereus* is most motile, *B. thuringiensis* produces crystal toxins, and *B. anthracis* is nonhemolytic .

B. cereus is mesophilic, growing optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions. It is distributed widely in nature and is commonly found in the soil as a saprophytic organism (Vilian *et al*., 2006) *B. cereus* is also a contributor to the microflora of insects, deriving nutrients from its host, and is found in the rhizosphere of some plants (Vilian *et al*., 2006).

2.6.2 Bacillus cereus genome

B. cereus has a circular chromosome measuring 5,411,809 nt in length and was completely sequenced using the shotgun sequencing method (Rasco *et al*., 2005). The genome structure of *B. cereus* consists of 5481 genes, 5234 protein coding, 147 structural RNAs, and 5, 366 RNA operons. An interesting gene cluster found within its genome encodes for arginine deiminase metabolic pathway. This cluster is predicted to have a role in its survival, enabling it to be resistant to acidic conditions in a similar manner as *Streptococcus pyogenes* (Rasco *et al*., 2004). Additionally, *B. cereus* has a nine gene urease gene cluster that encodes for proteins, blasticidin S deaminase, and an S-layer protein. The urease

enzyme increases its vigor in acidic conditions and is similar to the urease found in other bacteria that is required for colonization of the human stomach.

2.6.3 Cell structure

Bacillus cereus is a 1 x 3-4 µm, rod shaped, Gram- positive bacterium. Its cell structure consists of an inner membrane and a thick peptidoglycan which functions to maintain cell shape (Ticknor *et al*., 2001). The polysaccharide portion makes up 50 % of the cell wall and consists of a neutral polysaccharide composed of N-acetylglucosamine, N-acetylmannosamine (ManNac), N-acetylgalactosamine and glucose in a molar ratio of 4: 1: 1: 1 (Amano *et al*., 1977). The acidic portion of the cell wall is characteristic in having a repeating tetrasaccharide unit. Five (5 %) of the cell wall is made up of techoic acids consisting of N-acetylglucosamine, galactose, glycerol, and phosphorus in a molar ratio of 1: 1.4: 1: 1. The linkage between the polysaccharide and peptidoglycan is a muramic acid 6-phosphate (Amano *et al*., 1977). The peptidoglycan of some *B. cereus* strains are unique with only a few oligomers present, the cross-linked muropeptides are dimmers, and many of the muropeptide lack the N-acetly group (Severin *et al*., 2004). These distinguishing features affect cell surface charge which contributes to the attachment of an outer capsule or an S-layer in pathogenic strains.

Clinical isolates of *B. cereus* have a glycoprotein S-layer over its peptidoglycan which consists of proteinaceous para-crystalline arrays and covers the cell surface. The S-layer is involved in the virulence of *B. cereus* and functions to promote interactions with human polymorphonuclear leucocytes (Mignot *et al*., 2001). It also enables *B. cereus* to adhere to laminin, type I collagen, fibronectin, and fibrinogen of the epithelium, and thus has a role in

increasing interaction between *B.cereus* and its host (Mignot *et al*., 2001). In addition, this proteinaceous layer enhances its resistance to radiation.

2.6.4 Motility

B. cereus is motile by means of flagella and exhibits two types of motility including swimming and swarming, depending on the environment. Single cells exhibit swimming motility by means of short flagellated rods (Senesi *et al*., 2002). On the other hand, swarming is a collective movement of swarm cells with flagellum that is observed to be three to four times longer, and also forty times more flagellated than single swimming cells (Senesi *et al*., 2002).

2.6.5 Spore structure

B. cereus spore formation occurs when nutrients are scarce within the environment and germinate into vegetative cells once they are available (Wijnands *et al*., 2006). Therefore, spore structure is important to the survival of this bacterium. *B. cereus* spores consist of an inner core surrounded by the inner membrane, and outer cortex surrounded by the outer membrane with an additional exterior coat (Kutima *et al*., 1987). The spore coat is made of proteins and small amounts of lipids and carbohydrates which contribute to its resistance to oxidizing agents and chemicals by blocking toxic molecules. In addition, the outer spore structure allows them to be heat and γ-radiation resistant (Pol *et al*., 2001). Spore germination is commonly in response to L-alanine which stimulates germination events including hydrations of spores, loss of Ca2+ and dipicolinic acid, and Metabolism (Pol *et al*., 2001).

2.6.6 Metabolism

B. cereus is a facultative aerobe so it can utilize oxygen as a terminal electron accepter, but also has methods of anaerobic respiration as a mechanism of energy release. Whole genome sequencing revealed genes encoding for metabolic enzymes such as NADH dehydrogenases, succinate dehydrogenase, complex III, non-proton-pumping cytochrome bd quinol oxidases, and proton-pumping oxidases such as cytochrome c oxidase and cytochrome aa3 quinol oxidase (Duport *et al*., 2006).

In aerobic respiration, reducing equivalents produced from glycolysis and the Krebs cycle are reoxidized by the electron transport chain, creating a proton motive force and ATP by ATP synthase (Duport *et al*., 2006). In anaerobic respiration, *B. cereus* utilizes fermentation to generate energy. Fermentation recycles NAD+ by reducing pyruvate and produces lactate and ethanol . ATP is generated by substrate level phosphorylation.

B. cereus can metabolize a variety of compounds including carbohydrates, proteins, peptides and amino acids for growth and energy. Some of the major products produced from carbon sources such as sucrose or glucose during anaerobic respiration include L-lactate, acetate, formate, succinate, ethanol, and carbon dioxide (Mols *et al*., 2007). During nitrate respiration, nitrate reductase converts nitrate into nitrite which is converted to ammonium by nitrite reductase .

2.6.7 Ecology

B. cereus interacts with other microorganisms in the rhizosphere, the region surrounding plant roots. Plants benefit from the presence of *B. cereus* since it is capable of inhibiting

plant disease caused by protest pathogens and also enhances plant growth (Jenson *et al*., 2003). It naturally produces the antibiotics zwittermicin A. and Kanosamine which inhibit the growth of plant pathogen, oomycetes, certain fungi, and a few bacterial species (Silo-suh *et al*., 1994). The presence of *B. cereus* in the rhizosphere also increases nodulation of soybean plants by *Bradyrhizobium japonicum* (Margulis *et al*., 1998). In addition, *Cytophaga-Flavobacterium* group (CF) benefit from *B. cereus* by utilizing its peptidoglycan as a carbon and energy source. CF bacteria attain *B. cereus* peptidoglycan by hydrolyzing the outer layer. The relationship between the two organisms is commensal since the growth of *B.cereus* is not affected by the presence of CF bacteria.

B. cereus is also found in the gut microflora of invertebrates and is an intestinal symbiont of arthropods where it exhibits filamentous growth in sow bugs, roaches, and termites (Margulis *et al*., 1998). Arthropods consume feces or soil with spores or cells of *B. cereus*. This intestinal stage of *B. cereus*, also known as the *Arthromitus* stage, involves the attachment of endospore fibers released from a parent cell. After attachment of spores, they begin motile and filament growth stages and attach to the epithelium. *B. cereus* cells are then defecated back into the soil where they can continue to grow.

As a ubiquitous bacterium, small amounts are consumed by humans from foods. Therefore, it is a contributor to the human intestinal microflora (Jenson *et al*., 2003). In addition *B cereus* is widely known to affect humans by causing food poisoning and infections as an opportunistic pathogen.

2.6.8 Application of *Bacillus cereus* to biotechnology

2.6.8.1 Biological control agent

Biological control agents are alternatives to chemical pesticides that are capable of suppressing plant pests and can also enhance plant growth. Most strains of *Bacillus cereus* produce toxins known to cause food-borne illnesses. However, there are strains that do not produce the HBL enterotoxin. Thus, antifungal compounds of *Bacillus cereus* strains have been developed as a useful biological control agent in the suppression of fungi and crop disease.

2.6.8.2 *Bacillus cereus* strains

Bacillus cereus B4 is used as a pesticide to deter fungi from rotting seedling plants. This strain produces three types of metabolites that enable it to suppress certain plant diseases and promote plant growth. The metabolites include Kanosamine, 3, 4-dihydroxy benzoate, and 2 keto-4 methylthiobutyrate. The metabolite 3,4-DOHB improves crop strength and results in a healthier and larger root system (Sunaina,2005).

Bacillus cereus DGA34 was isolated from the environment and is useful in fighting off fungal damping disease in crops. It naturally produces the antibiotic zwittermicin A. by fermentation which is found in the supernatant fluid of its culture medium. This antibiotic is effective in fighting a wide range of fungi and bacteria, and reduces symptoms of damping-off disease and root rot (Handelsman *et al*., 2007).

Bacillus cereus UW85 produces two antibiotic toxins including zwittermicin A. and antibiotic B. *Bacillus cereus* UW85 is used to protect alfalfa seeds from dampening off

caused by *Phytophthora medicaginis*, cucumber fruits from *Pythium aphanidermatum*, peanuts from *Sclerotinia minor*, and tobacco seedlings from *Phytophthora nicotianae* (Silo-suh *et al*., 1994).

These are just a few *B. cereus* strains with current United States patents as biocontrol agents for crop diseases. There are currently many *Bacillus cereus* strains that are still under review.

2.6.9 Growth limitation of *Bacillus cereus*

2.6.9.1 Effect of temperature on growth.

Mesophilic strains of *B. cereus* can grow between 10ºC and 42ºC, some strains being able to grow at 50-55ºC, with an optimal growth temperature between 30 and 37ºC. Phychrotrophic strains would grow below 10ºC, at temperatures as low as 4ºC (Andersen *et al*., 2001). Psychrotrophic strains have optimal growth temperatures between 30 and 37 ºC. Maximum growth temperature ranged from between 37 and 42°C. Refrigeration reduces growth of *B. cereus* by increasing the generation time. For instance, doubling times of a cocktail of 5 strains of *B. cereus*, including psychrotrophic and mesophilic strains in laboratory media were: 1.6 h at 19.5°C, 2.9 h at 14.2°C, 4 h at 9.6°C and 6.7 h at 6.5°C (Choma *et al*. 2000). Refrigeration also considerably increase lag time. Valero *et al* (2000) reported lag times of respectively 148.77 h and 1.96 h at 5 and 30 ºC. Growth of some strains of *B. cereus* was observed at 55°C, but growth kinetics have not been characterised. At 50°C and below, important variations were observed among strains. Generation times for *B. cereus* in laboratory media were: 0.3 to 3.6 h at 50°C (for strains able to grow), 0.3 to 0.7 h at 40°C, 0.4 to 1.3 h at 35°C (Rajkowski *et al.*, 1987; Johnson *et al*. 1983). These generation times are similar or slightly lower than those reported for *Clostridium*

perfringens: 0.5 h at 51°C and 0.2 h at 40°C in cooked meat (Willardsen *et al.*, 1978), 0.3 to 0.4 h at 35°C in laboratory media (Beuchat *et al*. 1980).

2.6.9.2 Effect of pH on growth

B. cereus is not a particularly acid tolerant bacterium. pH limit for growth in carrot substrate acidified with citric acid was between 4.5 and 4.75 (Valero *et al*. 2000). In milk acidified with HCl, slight growth was observed at pH 4.1 at 37°C (Clavel *et al*. 2004). At 25°C, growth rate of a strains of *B. cereus* was approximately constant between pH 7 and pH 5.5 but dropped dramatically below this level (Lindsay *et al*. 2000)

2.6.9.3 Effect of water activity on growth

Bacteria are more sensitive to low water activity than yeast and moulds. In the particular case of *Bacillus cereus*, the water activity must be higher than 0.92 for growth. For other *Bacillus* spp, at 20°C and pH 6.4 in Tryptose-Caseine-Soja broth, a mixture of 9 strains isolated from bakery products had lag times of 10 days and 15-18 days respectively for aw of 0.92 and 0.91 (Quintavalla, 1993).

2.6.9.4 Effect of sodium chlorine

Regarding the effect of NaCl concentration on the growth of *Bacillus cereus* there are inconsistent results. Raevuori (1975) and Claus (1986) reported that 11-89 % of strains of *B. cereus* grew in 7 % of NaCl. Mossel *et al* (1967) suggested that 5 % NaCl included in isolation agars was a useful selective agent for *Bacillus cereus* but that 10 % was too inhibitory. Peters *et al* (1991) identified the temperature range over which *Bacillus cereus* could grow at different NaCl concentrations. These ranged from growth at all temperatures examined (14-41ºC) at NaCl concentration of 0.5 % (w/v) with pH 4.7 the lowest permitting growth. At 5 % (w/v) NaCl, the temperature range supporting growth was

observed only between 21-39 ºC and the minimum pH allowing growth was 5.5, while at 7 % (w/v) NaCl no growth was recorded at any temperature.

2.6.9.5 Effect of modified atmosphere packaging

Bennink *et al*. (1995) studied the effect of CO2 and O2 concentrations on the growth of foodborne pathogens. For *B. cereus*, a strong reduction in maximum population density was observed only under 50 % CO2. Effect of CO2: O2 ratio on growth of foodborne pathogens including *Bacillus cereus* was studied by Ogihara *et al.* (1993). Growth of *B. cereus* was not completely inhibited by the CO2, O2 concentrations tested, however growth rate was reduced as the proportion of CO2 in the gas mixture increased.

2.6.9.6 Effect of heating

Heat is the more common method used to kill bacterial spores in foods. However, spores of *B. cereus* have a broad range of heat resistance, being a problem for producers to develop consistent cooking or pasteurization processes. For instance, among a set of strains from various origins, time to reduce 10 fold the number of cultivable spores at 90°C, pH 7 (D value) varied from a few min to >100 min (Dufrenne *et al*. 1994). Strains from foodborne outbreaks had D values at 100°C ranging from 6 to 27 minutes (Rajkowski, 1987). Strains isolated from spoiled canned vegetables had D values at 130°C around 0.3 min (Bradshaw *et al*. 1975). Spores isolated from vegetables showed a D105ºC value of 0.63 min in reference substrate (pH7) . Heat resistance of *Bacillus cereus* spores can be modified by the pH. Survival of *Bacillus cereus* spores at 95 ºC decreased by three fold when the pH of the heating substrate was decreased from 6.2-4.7 (Fernandez *et al* 2002). Mazas *et al* (1998) found that acidification from pH 7-4 produced a fivefold decrease in D103ºC values. According to those results, pasteurisation or cooking would not eliminate *Bacillus cereus* spores. In low acid foods, the process used to eliminate *Clostridium botulinum*, 121°C for

30 min would also eliminate spores of *B. cereus*. Foods subjected to less severe heat treatments will occasionally carry spores of *B. cereus*.

2.6.9.7 Effect of other processes

The effect of non thermal technologies on bacterial spores seems to be variable. Very high pressures are needed to inactivate spores. The inactivation of bacterial spores by High Pressure combined with moderate heat occurs in two steps. First pressure causes spore germination and/or dipicolinic acid release, which permit spore inactivation (Margosch *et al*. 2004). However, always remains an ungerminated fraction "super-dormant fraction" that under temperature abuse could germinate and grow. Electric Pulsed Fields does not kill bacterial spores and their effect on germinated forms is very limited. Spores of *B. cereus* have a resistance to irradiation similar to that of *C. perfringens*. The dose needed to cause a 10-fold reduction was around 1.6 kGy with a shoulder (Minimum dose to apply before starting to kill the spores) of 2 kGy (WHO, 1999).

2.6.9.8 Effect of food additives

Food additives such as nisin produce the inactivation of *Bacillus cereus* vegetative cells while others as carvacrols have a very little effect (Periago *et al*., 2001).

2.7 Enzymes

Enzymes are large biological molecules responsible for the thousands of chemical interconversions that sustain life (Grisham *et al*., 1999). They are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions, from the digestion of food to the synthesis of DNA. Most enzymes are proteins, although some catalytic RNA molecules have been identified. Enzymes adopt a specific three-dimensional structure, and may employ organic and inorganic cofactors to assist in catalysis.

In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, called products. Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

Like all catalysts, enzymes work by lowering the activation energy for a reaction, thus dramatically increasing the rate of the reaction. As a result, products are formed faster and reactions reach their equilibrium state more rapidly. Most enzyme reaction rates are millions of times faster than those of comparable un-catalysed reactions. As with all catalysts, enzymes are not consumed by the reactions they catalyse, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts in that they are highly specific for their substrates. Enzymes are known to catalyze about 4,000 biochemical reactions (Bairoch, 2000). A few RNA molecules called ribozymes also catalyze reactions, with an important example being some parts of the ribosome (Cech, 2000). Synthetic molecules called artificial enzymes also display enzyme-like catalysis (Groves, 1997).

Enzyme activity can be affected by other molecules. Inhibitors are molecules that decrease enzyme activity; activators are molecules that increase activity. Many drugs and poisons are enzyme inhibitors. Activity is also affected by temperature, pressure, chemical environment for example pH, and the concentration of substrate. Some enzymes are used commercially, for example, in the synthesis of antibiotics. In addition, some household products use enzymes to speed up biochemical reactions for example enzymes in biological

washing powders break down protein or fat stains on clothes; enzymes in meat tenderizers break down proteins into smaller molecules, making the meat easier to chew.

2.7.1 History

As early as the late 17th and early 18th centuries, the digestion of meat by stomach secretions and the conversion of starch to sugars by plant extracts and saliva were known. However, the mechanism by which this occurred had not been identified.

In 1833, French chemist Anselme Payen discovered the first enzyme, diastase. A few decades later, when studying the fermentation of sugar to alcohol by yeast, Louis Pasteur came to the conclusion that this fermentation was catalyzed by a vital force contained within the yeast cells called "ferments", which were thought to function only within living organisms. He wrote that "alcoholic fermentation is an act correlated with the life and organization of the yeast cells, not with the death or putrefaction of the cells (Dubos, 1951).

2.7.2 Structures and mechanisms

Enzymes are in general globular proteins and range from just 62 amino acid residues in size, for the monomer of 4-oxalocrotonate tautomerase (Chen *et al*.,1992) to over 2,500 residues in the animal fatty acid synthase. A small number of RNA-based biological catalysts exist, with the most common being the ribosome; these are referred to as either RNA-enzymes or ribozymes. The activities of enzymes are determined by their three-dimensional structure (Anfinson, 1973). However, although structure does determine function, predicting a novel enzyme's activity just from its structure is a very difficult problem that has not yet been solved (Dunaway-Mariano, 2008).

Most enzymes are much larger than the substrates they act on, and only a small portion of the enzyme (around 2–4 amino acids) is directly involved in catalysis. The region that contains these catalytic residues, binds the substrate, and then carries out the reaction is known as the active site. Enzymes can also contain sites that bind cofactors, which are needed for catalysis. Some enzymes also have binding sites for small molecules, which are often direct or indirect products or substrates of the reaction catalysed. This binding can serve to increase or decrease the enzyme's activity, providing a means for feedback regulation.

Like all proteins, enzymes are long, linear chains of amino acids that fold to produce a three-dimensional product. Each unique amino acid sequence produces a specific structure, which has unique properties. Individual protein chains may sometimes group together to form a protein complex. Most enzymes can be denatured, that is, unfolded and inactivated by heating or chemical denaturants, which disrupt the three-dimensional structure of the protein. Depending on the enzyme, denaturation may be reversible or irreversible.

Structures of enzymes with substrates or substrate analogs during a reaction may be obtained using Time resolved crystallography methods.

2.7.3 Specificity

Enzymes are usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity (Jaeger *et al*., 2004).

Some of the enzymes showing the highest specificity and accuracy are involved in the copying and expression of the genome. These enzymes have "proof-reading" mechanisms. Here, an enzyme such as DNA polymerase catalyzes a reaction in a first step and then checks that the product is correct in a second step (Shevelev *et al*., 2002). This two-step process results in average error rates of less than 1 error in 100 million reactions in high-fidelity mammalian polymerases. Similar proofreading mechanisms are also found in RNA polymerase ,(Zenkin *et al*., 2006) aminoacyl tRNA synthetases and ribosomes.

Some enzymes that produce secondary metabolites are described as promiscuous, as they can act on a relatively broad range of different substrates. It has been suggested that this broad substrate specificity is important for the evolution of new biosynthetic pathways.

Enzymes are very specific, and it was suggested by the Nobel laureate organic chemist Emil Fischer in 1894 that this was because both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another. This is often referred to as "the lock and key" model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve.

2.7.4 Industrial applications

Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required. However, enzymes in general are limited in the number of reactions they have evolved to catalyse and also by their lack of stability in organic solvents and at high temperatures. As a consequence, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or *in vitro* evolution (Renugopalakrishnan *et al*., 2005, and

Hult *et al*., 2003) and These efforts have begun to be successful, and a few enzymes have now been designed "from scratch" to catalyse reactions that do not occur in nature.

Ligninases, hemicellulases, pectinase and amylases are the enzymes that have found applications in different industries and pharmaceutical preparations.

The microorganisms of *Bacillus* genus are known to be one of the most important sources of enzymes and other bio-molecules of industrial interest, being responsible for the supply of about 50% of the market for enzymes (Scallmey *et al*., 2004). The world market for enzymes is estimated at 1.6 billion dollars, 29% for the food industry, animal feed 15% and 56% in other applications (Outtrup *et al*., 2002). *Bacillus subtilis* produces a variety of extracellular enzymes including proteases, amylases and lipolytic enzymes of great importance in industrial processes, such as pharmaceutical, leather, laundry, food and waste processing industries (Machius *et al*., 1995 and Scallmey *et al*., 2004).

Among the different categories, the hydrolase enzymes are among the largest of industrial application and, among these, the alpha-amylase and beta-galactosidase have received special attention (Konsoula *et al*.,2006). These enzymes catalyse the hydrolysis of starch and are produced by a wide variety of microorganisms, however, for commercial applications they are basically derived from the genus *Bacillus* (Scallmey *et al*., 2004). The major amylases produced by Bacillus are heat resistant, which is commercially interesting because many processes require high temperatures, so the thermosensibility ceases to be a limiting factor (Konsoula *et al*., 2006).

2.7.5 Amylases

Amylases are the hydrolytic enzymes that cleave the α- 1-4 glucosidal linkage of complex polysaccharides (Pandey *et al*., 2000). Amylases have potential applications in the food, fermentation, textile, paper and pharmaceutical industries. Amylases have been most widely reported to occur in microorganisms, although they are also found in plants and animals. Currently, they comprise about 30% of the world enzyme production (Vander *et al*., 2002). The main microbial sources for amylase production are *Bacillus* species (Nurmatov *et al*., 2001, Deutch, 2002, Dey *et al*. 2002) and *Asperigillus* species (Gigras *et al.*, 2002). Considerable information is available on the microbial production of α-amylase in solid media (Ramachandran et al. 2004, Xu et al. 2008) and liquid media under stationary and submerged conditions (Francis *et al.*, 2003, Kaur *et al.*, 2003).

Two major classes of amylases have been identified in microorganisms, namely alpha-amylase and glucoamylase. Alpha-Amylases (endo-1,4-a-D-glucan glucohydrolase, are extracellular enzymes that randomly cleave the 1,4-a-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-a-D-glucan glucanohydrolase) hydrolyses single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner (Hema *et al.*, 2006).

Among various extracellular enzymes, alpha-amylase ranks first in terms of commercial exploitation (Babu *et al*., 1993). Spectrum of applications of alpha-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry (Ramachandran *et al*., 2004).

The amylase producing bacteria such as *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens, B. cereus*, and *B. megaterium* and fungi such as *Aspergillus niger, penicillium, Rhizopus, Cephalosporium and Nuerospora* are major amylase producing microorganisms (Pandey, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample collection

A total of fifty (50) soil samples were taken from 5 cm depth after removing 5 cm from the earth surface of refuse dump sites in Samaru, Basawa and Hanwa Zaria. The samples were collected into sterile plastic bags and transferred to labelled screw-capped bottles.

Stock culture of *Chlorella vulgaris* was obtained from National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna state and authenticated at the Biological Sciences Department, Ahmadu Bello University, Zaria.

3.2 Preparation of mannitol egg-Yolk polymyxin B agar (MYP)

The medium was prepared according to the manufacturer's instruction. Twenty one and a half (21.5) g of Mannitol egg-yolk polymycin B agar base was suspended in 450 ml of distilled water, mixed well and dissolved by heating. The medium was then boiled for one minute until complete dissolution. It was sterilized in an autoclave at 121ºC for 15 min. The medium was then cooled to 50ºC, after which one vial of polymycin-B supplement and egg-yolk suspensions were added, mixed well and dispensed into sterile Petri dishes.

3.3 Isolation of *Bacillus* sp

Ten (10) g of soil sample was dissolved in 90ml of sterile distilled water and diluted up to 10^{-4} times from which 0.1 ml was pipetted onto plates of Mannitol Egg-Yolk Polymycin B Agar (MYP) (Oxoid, Hampshire, England) using the spread plate technique. The inoculated agar plates were incubated at 37^{o}C for 24 h. Colonies that appeared pink and were surrounded by whitish zones of clearance on the *Bacillus cereus* selective agar plates were

picked and sub-cultured onto plates of nutrient agar. The isolates were further transferred to nutrient agar slants, incubated for 24 h and stored at 4^0C in the refrigerator until required for use.

3.4 Identification of presumptive *Bacillus* sp

Bacillus sp were identified based on the cultural, microscopic and biochemical characteristics and confirmed using the Microgen *Bacillus* Identification kit.

3.4.1 Cultural characterization of *Bacillus* sp

The colonial morphology of the isolates was noted with respect to shape, colour, size and nature of the colony.

3.4.2 Microscopic observation

The bacterial isolates were Gram stained and observed under ×100 oil immersion objective of the light microscope.

3.4.3 Endospore staining

Endospore staining was performed to observe the morphology of the cells as described by Sneath, 1984. A loop full of the suspension of each of the isolates was smeared on a clean microscopic slide. The smeared slide was heat fixed by passing it over the flame of a Bunsen burner. An absorbent paper was placed on the smeared heat fixed slide and flooded with malachite green stain which is a very strong stain that can penetrate endospores. It was flamed until steam was observed from the slide. After flooding with malachite green, the slide was washed free of the dye with water and counterstained with safranin, air dried and viewed under ×100 oil immersion objective of the light microscope.

3.4.4 Biochemical characterization of *Bacillus* sp

Biochemical tests for the preliminary identification of the isolates were as described below.

3.4.4.1 Catalase test

Fresh colonies of the isolates were immersed in test tubes containing 3ml of hydrogen peroxide solution using a wooden applicator and observed for gas bubbles which are an indication of a positive result.

3.4.4.2 Voges-Proskauer test

Colonies of the isolates were inoculated into methyl red Voges-Proskauer broth (MR-VP) and incubated until growth was observed. Alpha naphthol (0.6ml) and 0.2ml of 40% potassium hydroxide was added to 2.5ml of the culture broth and shaken well. A positive reaction was indicated by the formation of a pink to red product within 15min.

3.4.4.3 Nitrate reduction test

Cultures of the organisms were grown overnight in nitrate broth. Ten (10) drops of N, N-dimethyl-naphthalene and sufanilic acids in 30 % acetic acid were added to the culture. The development of red colour within 2 min is an indication of a positive result.

3.4.4.4 Lecithinase production test

The isolates were sub-cultured on egg-yolk agar and incubated for 24 h at 37^{o}C. A positive reaction was indicated by a white, opaque, and diffuse zone around the colonies that extended to the entire medium.

3.4.5 Confirmation of *Bacillus* sp isolates using the Microgen *Bacillus* identification kit

The presumptive *Bacillus sp* were confirmed using the Microgen *Bacillus* identification kit (Microgen Ltd, UK) (Sneath, 1984) as described below.

Suspensions of all the isolates were prepared in the suspending medium provided with the kit. It was carried out by picking fresh single 24 h colonies of the presumptive *Bacillus* sp isolates with a sterile inoculating loop and emulsified in the suspending medium. The adhesive sealing tape from each strip of the micro wells were peeled and the wells were inoculated with 3 drops of the bacteria suspension (with the aid of a sterile Pasteur pipette). The reagents for indole, nitrate and Voges-Proskauer tests were added and the Arginine wells were overlaid with Mineral oil and the entire wells were sealed and incubated at 30^{o}C for 18-24 h. The adhesive tape was removed and the results (coloured reactions) of the sugar fermentations including Citrate and Urease tests were read and recorded (in the forms provided) with the aid of colour chart and substrate reference table. The test strips were further Incubated at 30^{o}C for 18-24 h after which the results were also read and recorded.

The Octal Codes for all the isolates were calculated using the results of the coloured reactions. All tests which showed a positive reaction after either 18-24 h incubation or 48 h incubation were scored as positive. Only reactions negative at 24-48 h incubation were scored negative. Results were recorded on the report form provided and all the accumulated positive test results from both the 24 and 48 h readings were summed up. Results were analysed using the Microgen Identification System Software. The software provided the identification based on probability (%) with analysis of the quality of identification.

3.5 Screening of *Bacillus cereus* isolates for amylase production on nutrient agar plates supplemented with starch.

The isolates were spot inoculated on nutrient agar plates supplemented with 1% starch and incubated at 37^0C for 24 h. Clear zones of hydrolysis around the colonies were noted after staining with iodine solution (Ashwini *et al*., 2011)).

3.6 Measuring the diameter of the zone of hydrolysis

After incubation for 24 h on nutrient agar plates supplemented with 1% starch, the plates were flooded with iodine solution and the diameter of each zone of hydrolysis was measured in millimetre (mm) using a ruler.

3.7 Preparation of crude amylase

The production medium contained in g/l, Bacteriological Peptone 6, $MgSO_4.7H_2O$ 0.5, KCI 0.5, soluble starch1 % and the pH adjusted to 6.5. A loopful of bacterial culture was inoculated into 40 ml of the medium and cultivated by shaking flask at 200 rpm, for 24 h at 37^oC. Prior to inoculation, 200 ml of the amylase production medium was distributed into 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 min and allowed to cool to 30^oC (Abe *et al*., 1988).

3.8 Amylase assay

Cell-free culture filtrates were used as crude enzyme preparations to assay amylase activity. Amylase activity was determined by the method described by Okolo *et al*., (1995). The reaction mixture consisted 1.25 ml of 1 % soluble starch, 0.25 ml of 0.1M acetate buffer (PH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme extract. After 10 min incubation at 50^oC, the liberated reducing sugars (glucose equivalents) were estimated by dinitrosalicylic (DNS) method of Zambere, (2011). The blank contained 0.5 ml of 0.1M

acetate buffer (PH 5.0), 1.25 ml of 1 % starch solution and 0.25 ml of distilled water. Colour intensity was determined at 540 nm using a spectrophotometer. One International Unit (IU) of alpha amylase was defined as the amount of enzyme that releases one milligram of reducing-sugar (glucose equivalents) per minute under standard assay conditions. The isolate with the highest enzyme activity was selected for further studies.

3.9 Cultivation of *Chlorella vulgaris*

Cells of *Chlorella vulgaris* were maintained in inorganic WC medium containing in g per litre of distilled water $Cacl_{2.}$ $2H_20$ 36.8,$MgSO_47H_2O$ 37,$NaHCO_3$ 12.6,K_2HPO_4.3H2O 11.4,$NaNO_3$ 85,$NaSiO_3.9H_2O$ 21.2,TES buffer 0.115, micronutrient solution containing in g/l distilled water Na_2EDTA 4.36,$FeCL_3.6H_2O$ 3.15,$CuSO_4.5H_2O$ 0.01,$ZnSO_4.7H_2O$ 0.022,$CoCL_2.6H_2O$0.01, $MnCL_2.4H_2O$ 0.18,Na2$MoO_4.2H_2O$ 0.006,H_3BO_3 1.0 and vitamin solution in g/l distilled water containing thiamine HCL 0.1,biotin 0.0005 (Guillard *et al.*, 1972) at 21°C under 40W fluorescent light with 12 h of photoperiod and the pH adjusted to 8.2. Algae were kept in 250 ml Erlenmeyer with 50 ml of medium and shaken occasionally. In order to obtain larger quantities of biomass, cells were being grown in 2000 ml Erlenmeyer with 1500 ml of medium. Cells were collected and centrifuged at 10,400g for 10 min in a Sorval centrifuge, washed twice with chilled 95% ethanol, cold dried and ground with a pestle and mortar until a fine powder was obtained. The dried cell powder was kept frozen until required for use.

3.10 Pre-treatment of *Chlorella vulgaris* biomass

Prior to the hydrolysis experiments, five (5) g of the biomass was soaked in 100 ml dilute H_2S0_4 (7 %w/w) and autoclaved at 121°C for 45 min. It was then cooled and the liquor drained (Parviz *et al*., 2011).

3.11 Standardization of *Bacillus cereus* inocula

The concentration of the bacterial suspension was standardized using the 1.0 McFarland. Zero point one (0.1) ml of 1 % BaCl was added to 9.9 ml of 1 % H_2SO_4 in a test tube. The tube was stopped tightly and sealed. This served as a standard. Into a tube of the same diameter as the standard tube containing 10 ml of 0.85 % normal saline a measured aliquot of the bacterial suspension was added until the turbidity produced matched the turbidity of the standard tube corresponding to the concentration of $3.0x10^8$cfu/ml (Campbell *et al*., 1964)

3.12 Hydrolysis of pre-treated and un-treated *Chlorella vulgaris* biomass by *Bacillus cereus*

The investigation of *C. vulgaris* starch hydrolysis by *Bacillus cereus* was carried out in submerged cultivation (for both pre-treated and untreated *C. vulgaris* dry biomass and a control containing the cultivation medium and 1 % corn starch). The cultivation medium contained in g/l, Bacteriological Peptone 6, $MgSO_4 \cdot 7H_2O$ 0.5, KCI 0.5 and 1 % (w/v) dry weight *C. vulgaris* and the initial pH adjusted to 6.5. Five (5) ml of bacteria culture that was equivalent to 1.0 McFarland ($3.0x10^8$ CFU/ml) was inoculated into 250 ml of the medium and cultivated by shaking flask at 200 rpm, for 7 days at 37 ^oC . Supernatants of the fermented broth were collected daily, centrifuged at 5000 rpm for 15 min in a cooling centrifuge and the reducing sugar was determined using the dinitrosalicylic acid (DNS) method of Zambere, (2011).

3.13 Optimization of the conditions for *C. vulgaris* hydrolysis

3.13.1 Effect of incubation time on *C. vulgaris* hydrolysis

The effect of incubation time on *C. vulgaris* hydrolysis was carried out to determine the optimum incubation time for hydrolysis. The hydrolysis was carried out for 7 days at 37^{o}C and pH of 6.5. Samples were collected daily and analysed for reducing sugar (glucose equivalents).

3.13.2 The effect of pH on *C. vulgaris* hydrolysis

The effect of pH on amylase production and *C. vulgaris* hydrolysis was determined by culturing the bacterium at various pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9 in the hydrolysis reaction to obtain the optimum pH. Samples were collected after 24hrs for reducing sugar determination.

3.14.3 The effect of temperature on *C. vulgaris* hydrolysis

Temperature plays an important role in the production of amylase. The effect of temperature was studied by incubating the culture media at various temperatures 30, 35, 40, 45, and 50 ^{o}C in order to obtain the optimum temperature for *C. vulgaris* hydrolysis

3.15 Analysis of reducing sugar

The reducing sugar obtained from the conversion process was analyzed according to DNS method of Zambare, (2011). The reaction mixture consisted of 1.25 ml of 1 % soluble starch, 0.25 ml of 0.1M acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme (amylase) extract. After 10 min incubation at 50^{o}C, the liberated reducing sugars (glucose equivalents) were estimated by dinitrosalicylic (DNS) method of Zambere, (2011). The blank contained 0.5 ml of 0.1M acetate buffer (pH 5.0), 1.25 ml of 1 % starch solution and 0.25 ml of distilled water. Colour intensity was determined at 540 nm using a

spectrophotometer. One International Unit (IU) of alpha amylase is defined as the amount of enzyme that releases one milligram of reducing-sugar (glucose equivalents) per minute under standard assay conditions

3.16 Estimation of *C. vulgaris* total carbohydrate

The total carbohydrate estimation was done by the method described by Sadasivam *et al*., (2008). In this method, 200 mg of anthrone was dissolved in100ml of ice cold 95% sulphuric acid (it was prepared fresh before use). Standard glucose was prepared by dissolving 100 mg of glucose in 100 ml distilled water. One hundred milligram of dry *C. vulgaris* was weighed into a boiling tube. It was then hydrolysed in a boiling water bath for 3h with 5ml 2.5N hydrochloric acid. It was neutralised with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged. The supernatant was collected; 0.5 ml and 1.0 ml aliquots were used for analyses. Glucose standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working stock, 0 ml served as the blank. The volume was made up to 1 ml in all the tubes by adding distilled water, followed by the addition of 4ml of the anthrone reagent. It was then heated for 8min in a boiling water bath, cooled rapidly and the absorbance was read at 630 nm. A Standard curve was drawn by plotting concentration of the glucose concentration on x-axis versus absorbance on y-axis. From the graph the carbohydrate present in the sample was calculated by the following formula.

Amount of carbohydrate present in 100 mg of the sample

$$= \frac{mg\ of\ glucose \times 100}{volume\ of\ test\ sample}$$

3.17 Statistical analysis

The student t-test was used to analyze the data that were obtained with the aid of statistical package for social sciences (SPSS) version17.

CHAPTER FOUR

4.0 RESULTS

The Cultural and physiological properties of the *Bacillus sp* isolated from the soil showed that the isolates had colonies that were round in shape. The colonies were raised, large, flat, rough, dry and pink in colour with zones of egg yolk precipitate on MYP agar. The isolates were also rod shaped, Gram positive and spore forming indicating they belong to the genus *Bacillus*.

The biochemical characterization and identification of *Bacillus cereus* isolates using the Microgen Bacillus-ID System revealed that all the isolates fermented trehalose and most of them fermented sucrose. Also, all the isolates were positive to Voges Proskauer, arginine dihydrolase and nitrate reduction tests (Table 4.1).

The zone of hydrolysis of *Bacillus cereus* isolates on nutrient agar plates that were supplemented with starch showed that SB2 had the largest zone of hydrolysis of 12 mm while the reference strain and SB7 had the smallest zones of hydrolysis of 6.0 mm (Table 4.2).

The amylase activity of the *Bacillus cereus* isolates in amylase production media showed that SB2 had the highest amylase activity of 1.62 U/ml while the reference strain had the lowest amylase activity of 0.40 U/ml (Table 4.3). SB2 was therefore selected for *Chlorella vulgaris* hydrolysis.

Table 4.1: Biochemical characterization of *Bacillus sp* using Microgen *Bacillus* Identification Kit

Isolate	Arabinose	Cellobinse	Inositol	Mannitol	Mannose	Raffinose	Rhamnose	Salicin	Sorbitol	Succrose	Trehalose	Xylose	Adonitol	Galactose	Mdm	Mdg	Inulin	Melezitose	ONGP	Indole	Nitrate	Arg	Citrate	Voges Proskauer	Octal Code	Identity
SB1	_	+	_	_	_	_	+	+	_	_	+	_	_	_	_	_	_	+	_	_	+	+	_	+	20620113	*B. cereus*
SB2	_	+	_	_	+	+	_	+	_	+	+	_	_	_	_	_	_	_	_	_	+	+	+	+	23260017	*B. cereus*
SB3	_	+	_	_	_	_	_	+	_	_	+	+	_	_	_	+	_	+	_	_	+	+	_	+	20230513	*B. cereus*
SB4	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_	+	+	_	+	00020016	*B. cereus*
SB5	_	+	_	_	_	_	_	+	_	+	+	_	_	_	_	_	_	_	_	_	+	+	_	+	20260013	*B. cereus*
SB6	_	+	_	_	+	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_	+	+	+	+	22060017	*B. cereus*
SB7	_	+	_	_	+	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_	+	+	_	+	22060013	*B. cereus*

Key: Mdm=Methyl-D-Mannoside, Mdg=Methyl-D-Glucoside, Arg=ArginineDihydrolase, ONGP=O-nitrophenyl-β-D-galactopyranoside,SB=Soilbacillus.

Table 4.2: Isolates and their zone of hydrolysis on nutrient agar plates supplemented with starch.

Isolate	Zone of hydrolysis (mm)
SB1	10.0
SB2	12.0
SB3	8.0
SB4	9.0
SB5	8.0
SB6	8.0
SB7	6.0
SDB	6.0

SB= Soil *Bacillus*

SDB= Standard *Bacillus cereus* NCIB 6349

Table 4.3: Isolates and their amylase activity in amylase production media.

Isolate	Amylase activity (U/ml ± SD)
SB1	1.46 ± 0.01
SB2	1.62 ± 0.02
SB3	0.66 ± 0.02
SB4	1.25 ± 0.02
SB5	0.21 ± 0.01
SB6	1.30 ± 0.01
SB7	0.65 ± 0.03
SDB	0.40 ± 0.02

SB = Soil *Bacillus*

SDB = Standard *Bacillus* (*B. cereus* NCIB 6349)

SD = Standard deviation

The hydrolysis of pretreated and untreated *Chlorella vulgaris* dry biomass with corn starch serving as control showed that corn starch had the highest reducing sugar yields as compared to the pre-treated and untreated *C. vulgaris* biomass from day 1 to day 7 whereas the pre-treated *C. vulgaris* biomass had a slightly higher reducing sugar yields from day 1 to day 7 as compared to the untreated *C. vulgaris* biomass (Fig 4.1)

The student t-test for the mean reducing sugar values of pre-treated *C. vulgaris* biomass and the control (corn starch) showed that significant difference exists statistically between the reducing sugar yields of the two substrates at $p \leq 0.05$ from day 1 to day 5 although the observable difference was not much .

The effect of varying incubation time (days) on *Chlorella vulgaris* dry biomass hydrolysis showed that 24hrs was the optimum duration for maximum reducing sugar yield after which there was a decrease in amylase activities from day 2 to day 7 of hydrolysis (Fig 4.2)

The effect of various inoculum size on *C. vulgaris* hydrolysis showed that maximum reducing sugar yield was obtained at 4 % inoculum size. As the inoculum size increased beyond the optimum, reducing sugar yield decreased (Fig 4.3).

The effect of varying incubation temperature on *Chlorella vulgaris* dry biomass by *Bacillus cereus* (SB2) showed that maximum reducing sugar was obtained at 35^0C. Further increase in temperature resulted in a decrease in the enzyme activity (Fig 4.4).

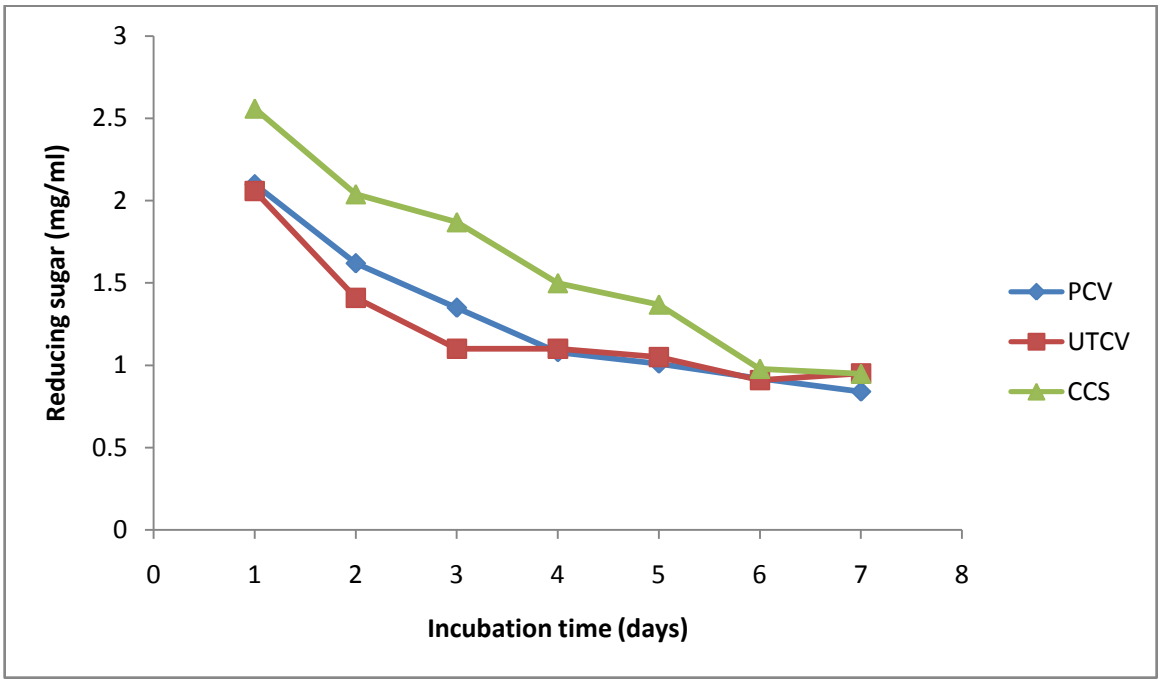

Figure 4.1: Hydrolysis of pre-treated *C. vulgaris*, untreated *C. vulgaris* and the control (corn starch).

Key:

Statistical significant difference exists between reducing sugar yields of PCV and CCS at $P \leq 0.05$

PCV = Pretreated *Chlorella vulgaris*

UTCV = Untreated *Chlorella vulgaris*

CSS = Control corn starch

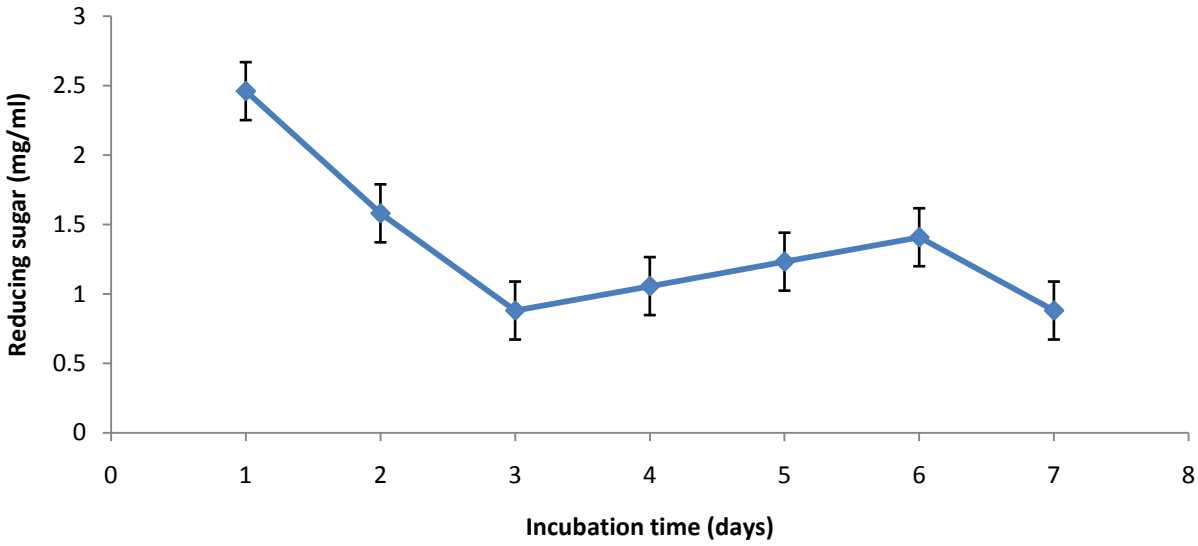

Figure 4.2: Effect of varying incubation periods on *Chlorella vulgaris* dry biomass hydrolysis

Key:

Y-error bars indicate the standard deviation (±SD) among the three parallel replicates

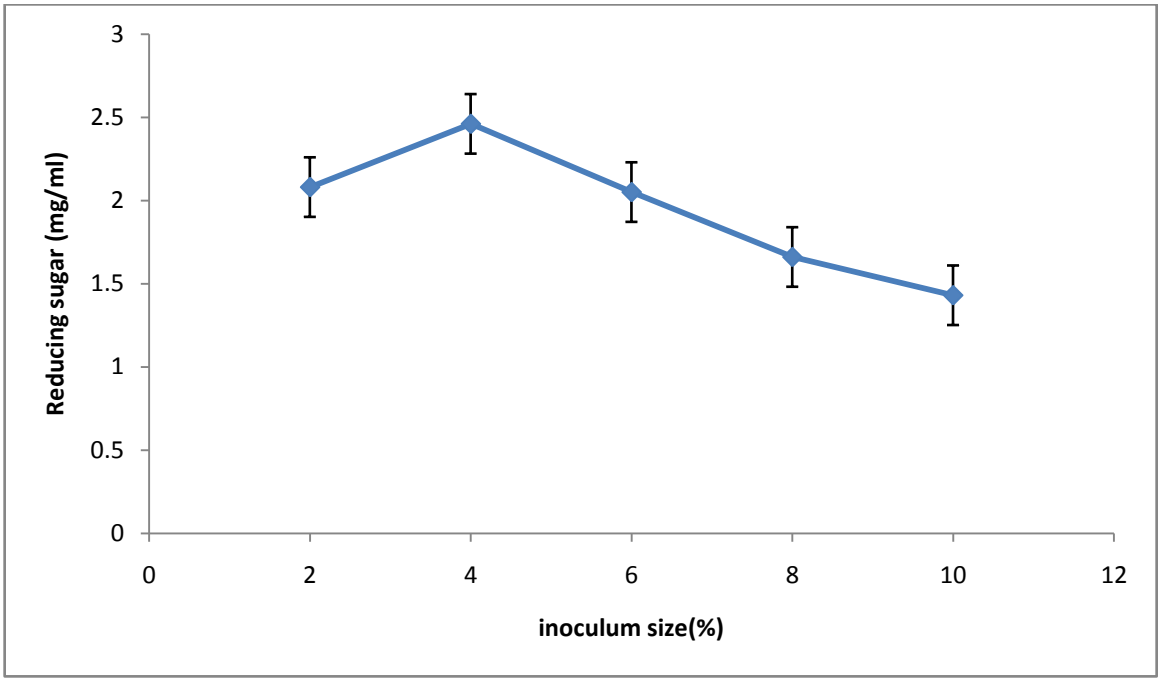

Figure 4.3: Effect of various inoculum size on *C. vulgaris* hydrolysis

Key:

Y-error bars indicate the standard deviation (±SD) among the three parallel replicates

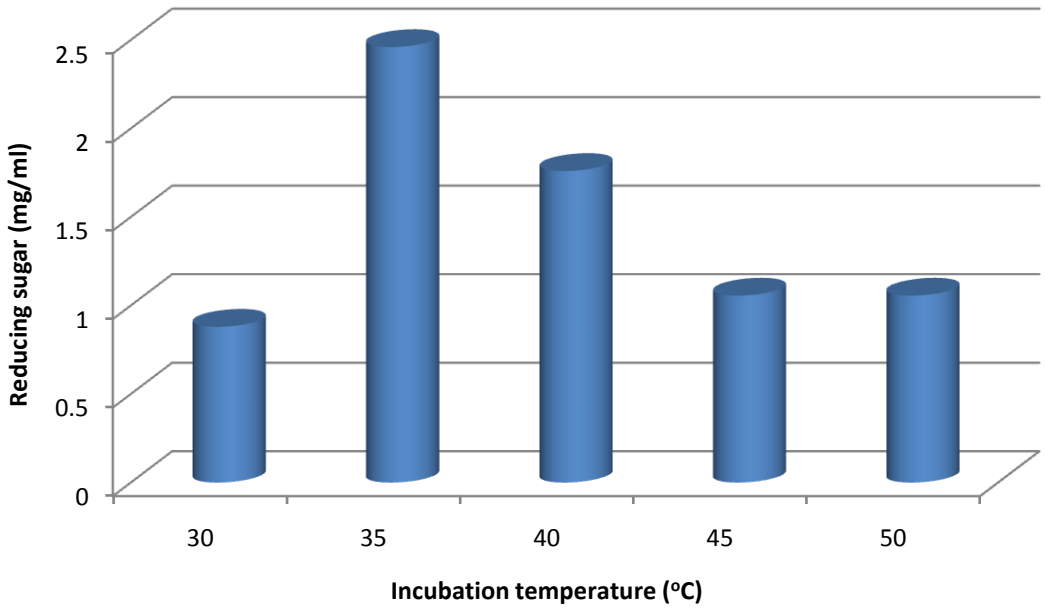

Figure 4.4: Effect of varying incubation Temperatures on *C. vulgaris* hydrolysis

The effect of varying pH on *C. vulgaris* dry biomass hydrolysis showed that reducing sugar production by *Bacillus cereus* was highest at pH 6.5. As the pH increased above 6.5 the reducing sugar yield diminished (Table 4.4).

The hydrolysis of *C. vulgaris* dry biomass under non-optimized and optimized conditions revealed that under non-optimized conditions, the maximum reducing sugar yield was 2.10 mg/ml whereas after optimization the maximum reducing sugar yield increased to 2.85 mg/ml after 24 h incubation (Fig 4.5).

The student t-test for the mean reducing sugar values of pre-treated *C. vulgaris* biomass under non-optimized and optimized culture conditions showed that significant difference exists at $P \leq 0.05$ from day 1 to day 4 between the reducing sugar produced under optimized and non-optimized conditions .

The carbohydrate content of *Chlorella vulgaris* was found to be 66 %.

Table 4.4: Effect of varying pH on *C. vulgaris* hydrolysis

pH	Reducing sugar (mg/ml ± SD)
6.0	1.06 ± 0.03
6.5	1.58 ± 0.01
7.0	0.85 ± 0.01
7.5	0.58 ± 0.03
8.0	0.88 ± 0.01
8.5	1.05 ± 0.02
9.0	0.89 ± 0.01

Key: SD = Standard deviation

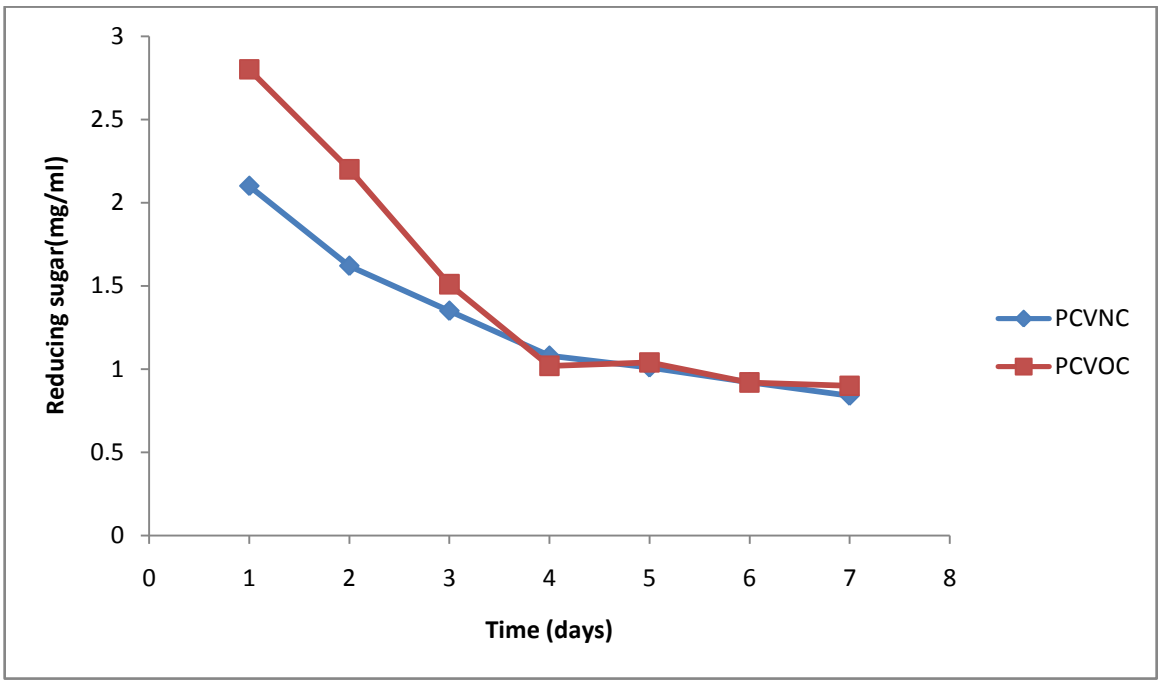

Fig.4.5 Pre-treated *Chlorella vulgaris* biomass hydrolysis under non-optimized and optimized conditions

KEY:

Statistical significant difference exists between reducing sugar yields of PCVNC and PCVOC at $P \leq 0.05$.

PCVNC = Pretreated *Chlorella vulgaris* under non-optimized conditions

PCVOC = Pretreated *Chlorella vulgaris* under optimized conditions

CHAPTER FIVE

5.0 DISCUSSION

In this research work, the seven (7) *B. cereus* strains were all outstanding in the growth and elaboration of the hydrolytic extracellular enzyme on nutrient agar plates supplemented with starch, detected by the appearance of clear zone of hydrolysis. The clear zone of hydrolysis around the bacterial colonies is due to the hydrolysis of starch by amylase enzyme secreted by the isolates.

Majority of the *Bacillus cereus* isolates (SB1-SB6) had zones of hydrolysis that were larger than the reference strain (*Bacillus cereus* NCIB 6349) while all the isolates (SB1-SB7) had amylase activities that were higher than the reference strain. The difference in zone of hydrolysis and amylase activities of the isolates and the reference strain could be due to the fact that they were isolated from different environments. It could also be due to different genetic makeup of the isolates which may have resulted in expression of varying amounts of amylase.

In the present research work, the hydrolysis of *C. vulgaris* dry biomass by *Bacillus cereus* (SB2) produced maximum reducing sugar yield that was comparable to that obtained for corn starch. Significant difference exists between the reducing sugar yields of pretreated *C. vulgaris* and corn starch. This could be due to the fact that corn starch is richer in starch as compared to *C. vulgaris* biomass. Although significant difference exists statistically between the reducing sugar yields of the two substrates, they are comparable in terms of the quantity of reducing sugar produced. Thus one of the substrates can be used as substitute for the other (*C. vulgaris* can be substituted for corn) for fermentable sugar production.

The result of this research work shows that the pretreated biomass produced a higher reducing sugar yield than the untreated biomass; however, it shows no observable significant difference between the two substrates. Although acid pretreatment helped the hydrolysis of cellulosic material, however, it has been reported that after acid treatment some degradation by-products like furfural and 5-hydroxyfurfural are produced which may have caused the low reducing sugar yield after pretreatment (Palmarola-Adrados *et al*., 2005).

The effect of incubation time on *C. vulgaris* hydrolysis showed that 24h was the optimum duration for maximum reducing sugar yield. Above this period, the reducing sugar yield started to decrease. This might be because; the cells may have reached the decline phase and displayed low amylase synthesis with low reducing sugar yield or the cells might have used up the available starch in the media and had low reducing sugar yield.

Inoculum size is an important factor that influences the production of metabolites. In the present study, the initial inoculum size has played an important role in *C. vulgaris* hydrolysis. Maximum reducing sugar yield was registered at 4% inoculums size. Above this level the reducing sugar yield decreased. This could be because inoculum size higher than the optimum value may produce a high amount of biomass which will rapidly deplete the nutrients necessary for growth and product synthesis. On the other hand lower inoculum size may give insufficient biomass and allow the growth of undesirable organisms known as contaminants in the production medium.

Physical factors are important in any fermentation for optimization of biochemical processes. The important physical factors that may determine the bioprocesses are pH, temperature among others (Ashokkumar *et al*., 2001).

The effect of varying incubation temperature on *Chlorella vulgaris* dry biomass by *Bacillus cereus* (SB2) revealed that maximum reducing sugar yield was obtained at 35^{o}C. Similar optimum temperature (35^{o}C) was observed for the production of amylase from banana stalk using *Bacillus subtilis* as reported by Krishna and Chandrasekaran (1996). The reducing sugar yield started reducing at 40°C. This might be due to the fact that at high temperatures, the growth of bacteria is usually inhibited and hence, enzyme formation is also reduced which might have affected the reducing sugar yield (Pandey *et al*., 2000 and Ramesh *et al*., 1991). It might also be due to enzyme inactivation by the high temperature or suppression of cell viability.

Reducing sugar yield was found to be maximum at pH 6.5 which is similar to the optimum pH obtained by Terui (1973) who reported 6.8 as optimum pH for the production of amylase by *B. subtilis.* Further increase in the pH resulted in a decrease in the amylase activity and reducing sugar yield. Ellaiah *et al* (2002) stated that at high pH, the metabolic activity of bacterium may be suppressed and thus it inhibits enzyme production.

The result of the student t-test carried out (using SPSS version 17) to compare the mean values of reducing sugar yields of pre-treated *C. vulgaris* biomass under non-optimized and optimized conditions revealed that significant difference exists between the reducing sugar produced from the hydrolysis of pretreated *C. vulgaris* biomass under non-optimized and optimized conditions from day 1 to day 4 at $P \leq 0.05$. Under non-optimized conditions (pH 6.5, temperature 37^{o}C, and inoculums size 5%) maximum reducing sugar yield was obtained after 24 h incubation whereas under optimized conditions (pH 6.5, temperature 35°C, inoculums size 4%), 1.3 fold reducing sugar yield was enhanced when compared with non-optimized conditions indicating the importance of optimization on *C. vulgaris* hydrolysis.

The result of this research work revealed that the carbohydrate content of *C. vulgaris* was comparable to the carbohydrate content of corn. This confirms that the *C. vulgaris* used in this study is rich in carbohydrates that can be hydrolysed into fermentable sugars.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Bacillus cereus SB2 is a potential strain for α-amylase production using submerged fermentation with *Chlorella vulgaris* as substrate.

The *C. vulgaris* strain with carbohydrate content 66 % used in this study produced maximum reducing sugar quantity (2.10 mg/ml) that was similar to the quantity produced by corn starch (2.56 mg/ml) with carbohydrate content of about 73 %. Therefore, it can be said that *Chlorella vulgaris* has potential as an alternative substrate for the production of fermentable sugar that can be converted to bioethanol.

6.2 Recommendation

Although many hydrolytic enzyme producing microorganisms have been isolated, studies to select the best hydrolytic enzymes producers, optimization of cultural parameters, purification of the enzyme as well as detailed physicochemical characterization of the enzymes, are recommended.

Biofuel production from microalgae has great potential for sustainable development. However, there have been difficulties in order to widely commercialize biofuel as an alternative to petroleum based fuels, and it needs more studies in the future to solve these troubles.

Studies to isolate, identify and characterize carbohydrate rich microalgae, including optimization of cultural conditions for bioethanol production are recommended.

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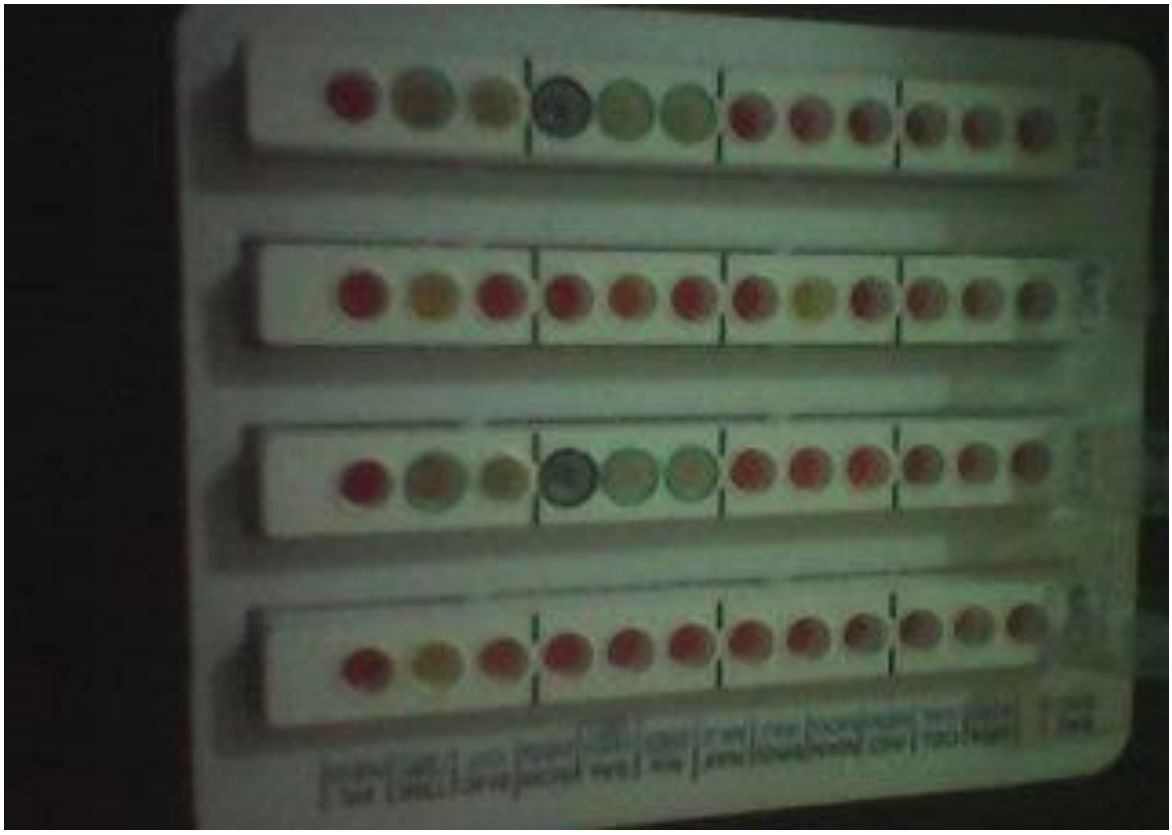
APPENDICES

Appendix 1: Colonial morphology of *Bacillus cereus* on nutrient agar plate

Appendix 2*: Bacillus cereus* (SB2) on nutrient agar plate supplemented with starch showing zone of hydrolysis after flooding with iodine solution.

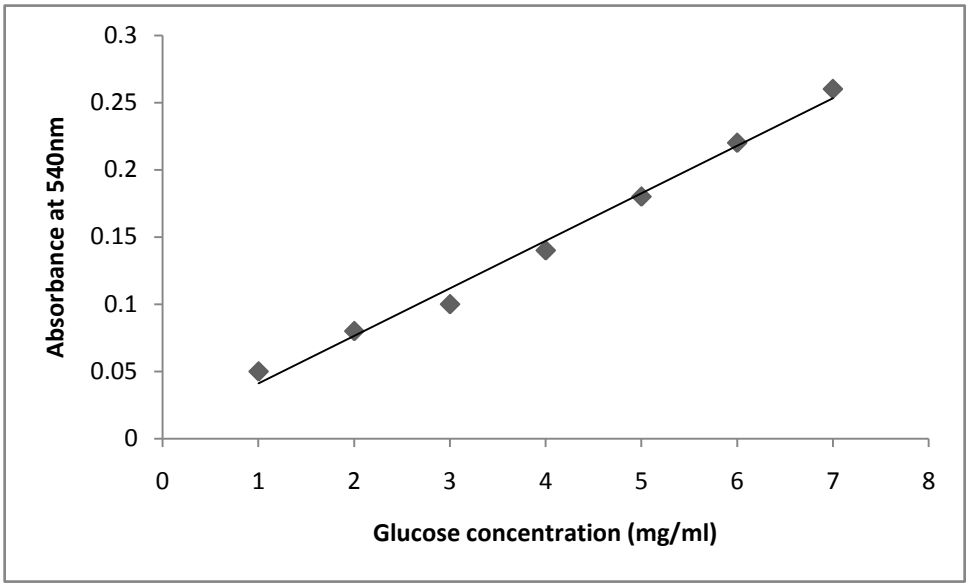


Appendix 3: Some Microgen Bacillus-ID system strip test showing colour change reactions.

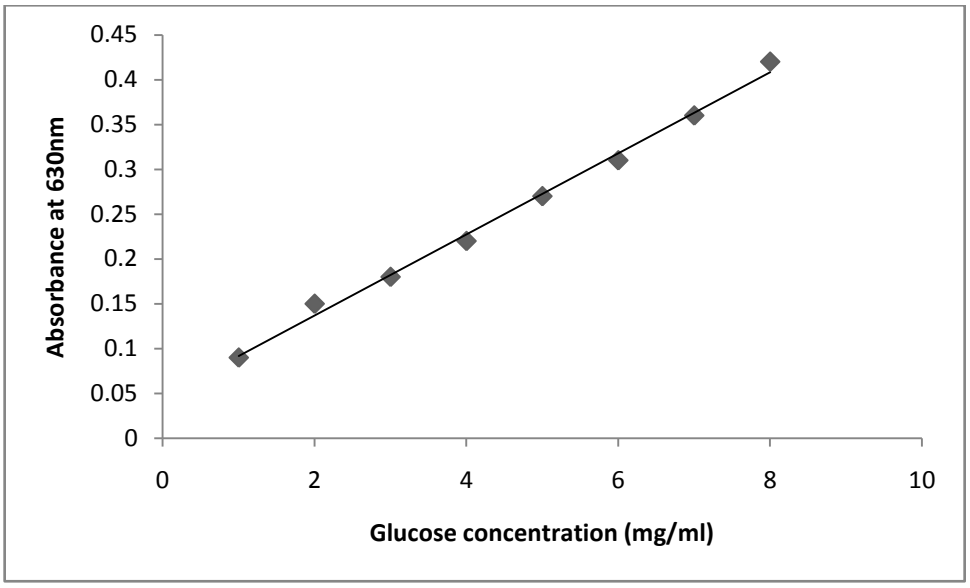
Appendix 4: *Chlorella vulgaris* growing in WC medium.

Appendix 5: Hydrolysis of pre-treated *Chlorella vulgaris* biomass (left), untreated *C. vulgaris*

biomass (right) and corn starch (middle) .



Appendix 6: Standard glucose curve for reducing sugar determination.



Appendix 7: Standard glucose curve for *C. vulgaris* carbohydrate content determination.

Appendix 8: Determination of *Chlorella vulgaris* carbohydrate content

Amount of carbohydrate present in 100mg of the sample.

$$= \frac{\text{mg of glucose x 100}}{\text{Volume of the test sample}}$$

$$= \frac{0.33 \times 100}{0.5}$$

= 66%

Appendix 9: Cultural and physiological properties of isolated *Bacillus* sp from soil sample in Zaria

Isolate	Colonial Morphology on MYP Agar	Vegetative Morphology	Gram Reaction	Motility	Starch Hydrolysis	Presence of Spore	Position of Spore	Lecithinase Production
SB1	Rough, dry, and pink	Rod Shape	+	+	+	+	Center	+
SB2	Rough, dry, and pink	Rod Shape	+	+	+	+	Terminal	+
SB3	Rough, dry, and pink	Rod Shape	+	+	+	+	Center	+
SB4	Rough, dry, and pink	Rod Shape	+	+	+	+	Center	+
SB5	Rough, dry, and pink	Rod Shape	+	+	+	+	Terminal	+
SB6	Rough, dry, and pink	Rod Shape	+	+	+	+	Center	+
SB7	Rough, dry, and pink	Rod Shape	+	+	+	+	Terminal	+

KEY: SB= Soil Bacillus,SB1,2 and 3 where obtained from Hanwa refuse dump sites, SB 4 and 5 from Basawa refuse dump sites, and SB 6 and 7 from Samaru refuse dump sites.

Appendix 10: Hydrolysis of Pre-treated *C. vulgaris*, Untreated *C. vulgaris* and the control (corn starch).

		Reducing sugar (mg/ml ± SD)	
Incubation time (days)	CCS	PCV	UTCV
1	2.56 ± 0.04	2.10 ± 0.14	2.06 ± 0.01
2	2.04 ± 0.04	1.62 ± 0.01	1.41 ± 0.01
3	1.87 ± 0.00	1.35 ± 0.00	1.10 ± 0.01
4	1.50 ± 0.02	1.08 ± 0.02	1.10 ± 0.02
5	1.37 ± 0.00	1.01 ± 0.01	1.05 ± 0.01
6	0.98 ± 0.02	0.92 ± 0.01	0.91 ± 0.01
7	0.95 ± 0.04	0.84 ± 0.00	0.95 ± 0.01

Key:

PCV = Pretreated *Chlorella vulgaris*

UTCV = Untreated *Chlorella vulgaris*

CSS = Control corn starch

SD = Standard deviation

Appendix 11: Effect of varying incubation periods on *Chlorella vulgaris* dry biomass hydrolysis.

Incubation time (days)	Reducing sugar (mg/ml ± SD)
1	2.46 ± 0.01
2	1.58 ± 0.01
3	0.88 ± 0.01
4	1.06 ± 0.01
5	1.23 ± 0.03
6	1.41 ± 0.01
7	0.88 ± 0.03

Appendix 12: Effect of various inoculums concentration on *C. vulgaris* hydrolysis

Inoculums concentration (%)	Reducing sugar (mg/ml ± SD)
2	2.08 ± 0.01
4	2.46 ± 0.01
6	2.05 ± 0.02
8	1.66 ± 0.01
10	1.43 ± 0.04

Appendix 13: Effect of varying incubation Temperatures on *C. vulgaris* hydrolysis

Incubation temperature (^{o}C)	Reducing sugar (mg/ml ± SD)
30	0.88 ± 0.01
35	2.46 ± 0.01
40	1.76 ± 0.02
45	1.06 ± 0.03
50	1.06 ± 0.03

Appendix 14: Independent Sample T-TEST for comparing means of pretreated C. vulgaris and corn starch reducing sugar yields (group statistics)

Sample	N	Mean	Std. Deviation	Std. Error Mean
1 PCV	2	2.1000	0.14142	0.10000

	CCS		2.5600	0.04243	0.03000
2	PCV	2	1.6200	0.01414	0.01000
	CCS		2.0500	0.04243	0.03000
3	PCV	2	1.3500	0.00000[a]	0.00000
	CCS		1.8700	0.00000[a]	0.00000
4	PCV	2	1.0800	0.00000	0.00000
	CCS		1.5000	0.02828	0.02000
5	PCV	2	1.0100	0.01414	0.01000
	CCS		1.3700	0.00000	0.00000
6	PCV	2	0.9200	0.01414	0.01000
	CCS		0.9800	0.02828	0.02000
7	PCV	2	0.8400	0.00000	0.00000
	CCS		0.9500	0.04243	0.03000

a= t cannot be computed because the standard deviations of both groups are zero.

Appendix 15: Independent sample T-test

	t-test for Equality of Means			
	t	df	Sig. (2-tailed)	Mean Difference
1 Equal variances assumed	-4.406	2	0.048*	-0.46000
Equal variances not assumed	-4.406	1.179	0.113	-0.46000

2	Equal variances assumed	-13.598	2	0.005*	-0.43000
	Equal variances not assumed	-13.598	1.220	0.028	-0.43000
4	Equal variances assumed	-21.000	2	0.002*	-0.42000
	Equal variances not assumed	-21.000	1.000	0.030	-0.42000
5	Equal variances assumed	-36.000	2	0.001*	-0.36000
	Equal variances not assumed	-36.000	1.000	0.018	-0.36000
6	Equal variances assumed	-2.683	2	0.115	-0.06000
	Equal variances not assumed	-2.683	1.471	0.158	-0.06000
7	Equal variances assumed	-3.667	2	0.067	-0.11000
	Equal variances not assumed	-3.667	1.000	0.170	-0.11000

At P≤ 0.05, significant difference exists.

Appendix 16: Independent sample T-Test for comparing means of reducing sugar yields of pretreated *C. vulgaris* under normal condition and pretreated biomass under optimum condition (group statistics).

Sample	N	Mean	Std. Deviation	Std. error Mean
1 PCVNC	2	2.1000	0.14142	0.10000
PCVOP		2.8500	0.14142	0.10000
2 PCVNC	2	1.6200	0.01414	0.01000
PCVOP		2.2000	0.14142	0.10000
3 PCVNC	2	1.3500	0.00000	0.00000
PCVOP		1.5100	0.02828	0.02000
4 PCVNC	2	1.0800	0.00000	0.00000
PCVOP		1.0200	0.01414	0.01000

5	PCVNC	2	1.0100	0.01414	0.01000
	PCVOP		1.0400	0.00000	0.00000
6	PCVNC	2	0.9200	0.01414	0.01000
	PCVOP		0.9200	0.01414	0.01000
7	PCVNC	2	0.8400	0.00000[a]	0.00000
	PCVOC		0.9000	0.00000[a]	0.00000

a= t cannot be computed because the standard deviations of both groups are zero.

Appendix 17: Independent sample T-test

	t-test for	Equality of	Means	
	t	df	Sig. (2-tailed)	Mean Difference
1 Equal variances assumed	-5.303	2	0.034*	-0.75000
Equal variances not assumed	-5.303	2.000	0.034	-0.75000
2 Equal variances assumed	-5.771	2	0.029*	-0.58000
Equal variances not assumed	-5.771	1.020	0.106	-0.58000
3 Equal variances assumed	-8.000	2	0.015*	-0.16000
Equal variances not assumed	-8.000	1.000	0.079	-0.16000
4 Equal variances assumed	6.000	2	0.027*	0.06000
Equal variances not assumed	6.000	1.000	0.105	0.06000
5 Equal variances assumed	-3.000	2	0.095	-0.03000

Equal variances not assumed	-3.000	1.000	0.205	-0.03000
6 Equal variances assumed	0.000	2	1.000	0.00000
Equal variances not assumed	0.000	2.000	1.000	0.00000

At P≤ 0.05, significant difference exists.