LIGNOCELLULOSIC BIOMASS PRETREATED SLUDGE MANAGEMENT: PHOSPHATE SOLUBILIZING BIO-FERTILIZER PRODUCTION AND NANO-HYDROXYAPATITE AS PHOSPHATE FERTILIZER

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ACRONYMS

P	Phosphorus
N	Nitrogen
K	Potassium
DM	Dry mass
ATP	Adenosine Tri Phosphate
ADP	Adenosine Di Phosphate
PSF	Phosphorus Solubilizing Fungi
PSB	Phosphorus Solubilizing Bacteria
HA	Hydroxyapatite
PSM	Phosphate Solubilizing Microorganism
MBM	Meat and Bone Meal
PCR	Polymerase Chain Reaction
Ppm	Parts per million
DAP	Diammonium phosphate
MAP	Monoammonium phosphate
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PGPR	Plant Growth Promoting Rhizobacteria
IAA	Indole Acetic Acid
PQQ	Pyrroloquinoline Quinine
HC	Hydrogen Cyanide
kDa	Kilo Dalton
HMF	Hydroxymethyl-furfural
DS	Dry Solid
g/l	gram per liter
CCR	Carbon Catabolite Repression
PEP	Phosphoenol pyruvate
PTS	Phosphotransferase System
Cre	Catabolite responsive elements
HPr	Histidine-containing protein
Tm	Melting temperature
OD	Optical Density
µg	Microgram
NA	Nutrient Agar
Bp	Base pair
Nm	Nanometer
DNS	Dinitrosalicylic acid
µg	Microgram
Ml	Milliliter
NADH	Nicotinamide Adenosine Diphosphate
mM	Millimolar
pH	Percentage of hydrogen
Hrs	Hours
Mins	Minutes
LB	Luria Bertania

EDTA	Ethylenediaminetetraacetic acid
TE	Tris EDTA
16s rRNA	Svedberg's unit Ribosomal Ribonucleic Acid
XRD	X-Ray Diffraction
BLAST	Basic Local Alignment Search Tool
GRAS	Generally recognized as safe
IAA	Indole Acetic Acid
HCN	Hydrogen cyanide
MR	Methyl Red
VP	Voges Proskauer
OF	Oxidative-Fermentative
HMF	HydroxyMethyl Furfural
nHA	Nano-Hydroxyapatite

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ABSTRACT

The globe is marred by myriads of environmental problems that are so intricate and complex and single solution could not be viable. The global warming mainly due to anthropogenic activities such as locomotion fuel and agricultural inputs has to be tamed and innovative approaches are required. Cereal based and energy crop dependent bio-ethanol production utilizes the land required for cereal production for food and has to be protected. Thus, utilization of agricultural litter for bio-ethanol production should be sought. Thus, utilization of lignocellulosic biomass pretreatment sludge to make bio-fertilizer could be an option for ecofriendly agricultural input and increase the revenue. This could partially substitute water soluble commercial fertilizers that cause ground water contamination. In addition, eutrophication is one of the problems of aquatic ecosystem arising from soluble phosphate fertilizer, so use of insoluble phosphate can minimize this risk. Moreover, having heterotrophic bacterial bio-fertilizer supplement that has ability to solubilize phosphate can also utilize ammonia and nitrate to prevent N_2O release. *Bacillus* species have these potentials as they are known to grow in several reduced carbon sources and can utilize different forms of N and are phosphate solubilizer. *Bacillus megaterium* and *Bacillus subtilis* were isolated from soil samples collected from different parts of Nepal based on selective reduced carbon source and respective protocol for isolation and screening have been developed. For the "Cyclic Economy" of phosphate, the bovine bones were used to synthesize insoluble nano-hydroxyapatite (nHA) that could serve as phosphate fertilizer. These isolated bacteria were able to grow in the media that contained nHA [$Ca_{10}(PO_4)_6(OH)_2$] as the sole phosphate source and they also released soluble phosphate in the media indicating that they are able to provide soluble phosphate to the soil microbes enhancing soil fertility and also to the plant root. Use of HA nanoparticles as fertilizer can provide both phosphate and calcium and upon solubilization these ions have pivotal roles in plant host pathogen interaction to modulate plant immunity. It is presumed that HA use in agriculture would create "Cyclic P Economy". Furthermore, the potential reserve of phosphorus, the phosphate rocks, are non-renewal so phosphate fertilizer synthesized from animal bones could be alternative for judicious use of depleting phosphate reserves with increasing intake of animal proteins. These bacteria were able to grow in acid pretreated lignocellulosic biomass sludge and were able to reduce the concentration of furfural or phenol in the sludge. Hence, having bio-ethanol and bio-fertilizer industry together could be economically viable and integration of diazotrophs for atmospheric N_2 fixation to substitute urea would be additional benefit and anthropogenic activities induced global warming could be mitigated to some extent.

Keywords: lignocellulose, pretreated sludge, bioethanol, *Bacillus*, eutrophication, nano-hydroxyapatite, phosphate solubilizing bacteria.

CHAPTER 1
INTRODUCTION

1.1 Background:

A fertilizer (also called plant food element) is a natural or synthetic, chemical-based substance that is added to the soil to enhance plant growth and fertility that also provides micronutrient for the plants growth on the order of parts-per-million (ppm), ranging from 0.15 to 400 ppm dry matter (DM; 0% moisture) or less than 0.04% DM (Benton *et al.*, 1996). Moreover, for the proper development and growth of plants, fertilizer furnishes one or more of the chemical elements necessary (Ifdc and Unido, 1998).

However, the globe is faced with myriads of problems due to the population growth which has increased anthropogenic activities resulting in environmental degradation (Galvani *et al.,* 2016). Population growth has been the most discussed demographic dimensions for the prospects of food crisis in near future because of its very direct impact on the growth in food demand (Chen and Kates, 1994; Valin *et al*., 2014). Population of the word at present is 7 plus billion that had been projected to cross 9 billion by 2050 (Keating *et al*., 2014) that would demand both fuel and food. But, the world is faced with the global warming problem that has been attributed to fossil fuel use and there is urgency of reducing fossil fuel use with more environmentally friendly alternative fuel.

The use of food crops such as grains, sugar beet and, oil seeds for ethanol/biodiesel (first-generation bio-fuels) production practice that has not only occupied the land (Putnam *et al.,* 1991) but also has resulted in deforestation with environmental impact (Varkkey *et al.,*2018). The plantation of the energy crops also use land and also require fertilizer to grow them. But are still limited in their ability to achieve targets of ethanol production (Kumar *et al*., 2009) and use of cereals in making bio-ethanol has been controversial (Limayem and Ricke, 2012).

Thus, bio-fuel has been conceived by using algal biomass for lipid production to make bio-diesel. Since this algal biomass is photoautotrophic (Murphy *et al*., 2015) they require horizontal land for mass production since bioreactors are inherently flawed because these cells hamper the transmission of light in deep inside vessel (Raj*et al.,*2005). This also causes use of land and the biomass productivity per unit land is far lower than the agricultural residue(Krausmann *et al.,*2008) questioning the rationale of algal biomass production(Slade and Bauen, 2013). Alternatively, lignocellulosic biomass has been used to produce bio-ethanol (Dragone *et al.,* 2010).

Lignocellulosic biomass is the most economical and highly renewable natural resource in the world. Lignocellulosic biomass includes corn stover, straw, wheat stover, algae and

others. Currently, such biomasses are the most important source of renewable energy and the only renewable source of carbon. About 13% of total energy consumption worldwide (IEA Statistics, 2008) is provided by the biomass. It constitutes a substantial renewable substrate for bioethanol production that does not compete with food and animal feed. Lignocellulosic biomass is widely abundant and offers a promising alternative to satisfy future energy. It is potentially carbon neutral source for producing a wide range of fuels and fuel additives (Demirbas, 2007). Lignocellulosic materials predominantly contain a mixture of carbohydrate polymers such as cellulose, hemicelluloses and lignin (Isikgor *et al.*, 2015).

Moreover, during the lignocellulosic biomass-based bio-ethanol production the biomass is treated with strong acid and/or alkali to degrade lignin (Limayem and Ricke, 2012) that is not fermentable to make bio-ethanol. The use of acid and/or alkali for releasing cellulose and hemicellulose from the biomass it generates different compounds that inhibit hydrolysis and has to be separated from the fermenting broth because cellulose is broken to glucose by cellulases during saccharification and subsequently is subjected to fermentation where monomeric sugars are metabolized to make ethanol by yeast (Ingram *et al*., 1999).

These separated hydrolysis inhibitors in the strong solvents are environmental hazard that needs to be properly managed for disposing adding cost to the lignocellulosic biomass-based bio-ethanol production(Goh *et al.,* 2010). Thus, alternative to manage this with value addition to generate additional revenue would lower the cost of bio-ethanol production and subsequently could compete with the price of fossil fuel.

With all these scenarios it is thought that demand for food is projected to double by 2030 (Cordell *et al.,* 2009b). In order to increase productivity and mitigate food scarcity extensive use of chemical fertilizer has been practiced (Cordell *et al*., 2009b). The most commonly used chemical is urea and use of urea releases carbon dioxide in the atmosphere and contributes to acid rain, and the soluble urea would enter groundwater that is crucial contaminant because of breakdown of urea to ammonia and nitrate (Singh and Verma, 2007). This is because these chemical fertilizers are water soluble and cause groundwater contamination (Boyd and Massaut, 1999).

In addition, it causes ozone depletion due to release of nitrous oxide (N_2O) by denitrification (Coldiron, 1992) during the nitrogen cycle. Nevertheless, nitrous oxide is a greenhouse gas which has around 300 times the heat-trapping capacity than CO_2 (Schneider and McCarl, 2003). Moreover, the second most used water soluble commercial phosphate fertilizer is the main causes of downstream eutrophication of aquatic ecosystem (Shaw *et al*., 2003) becoming additional environmental hazard.

Substitution of nitrogenous fertilizer could be thought by use of nitrogen fixing diazotrophs as bio-fertilizer. However, P that stands as the second limiting nutrient next to nitrogen and importantly is a key nutrient for higher and sustained agriculture productivity. P is necessary for growth of all the forms of life on the planet earth including plants, animals and microorganisms as a component of nucleic acids, phospholipids that compose cellular membrane, ATP and ADP molecules. Animals can take P in the form of plant products for herbivorous and animal sources for carnivorous. However, plants have to take P from the soil as P plays important role in all the metabolic process including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration.

However, because of its sparingly soluble nature, it is present in very less proportion in the soil for plant uptake(Ruttenberg, 2003). This deficiency is usually compensated by adding chemical fertilizers. However, the chemical fertilizers are expensive and are not eco-friendly. Non-judicious and irregular usage for a long time leads to decreased soil activity leading to imbalance in equilibrium(Ruttenberg, 2003). In addition, phosphate is not renewal(Ruttenberg, 2003) like nitrogenous fertilizers.

Improving phosphorus nutrition is an urgent priority to meet the increasing global demand for food (Sutton *et al*., 2013). Usage of microorganisms to augment the P availability could be better alternative (Rodríguez and Fraga, 1999). So, the focus is placed on the use of soil microorganisms (Zaidi *et al.,* 2009) which have very beneficial interaction with the plants. Such microorganisms are often termed as Biofertilizer. Biofertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. The biofertilizer contains microorganism able to increase soil fertility (Conditions, 2014). Biofertilizer helps in plant growth and development by performing the similar task as the fertilizers do. So, it is expected to reduce the use of chemical fertilizers and pesticides by the use of biofertilizer.

Since they play several roles, a preferred scientific term for such beneficial bacteria is plant- growth promoting rhizobacteria (PGPR). Phosphorus-solubilizing fungi (PSF) and bacteria are known as effective organisms for phosphorus solubilization. In soil, phosphorus solubilizing bacteria (PSB) constitute 1–50% and fungi 0.1–0.5% of the total respective population (Chen *et al*., 2006). The literatures reveal that a large number of autotrophic and heterotrophic soil micro-organisms demonstrate the *in-vitro* ability to solubilize mineral phosphorus like hydroxyapatite (HA) and play a key role in the mobilization of soil P in plant-available forms (Joshi *et al*., 2014).

Various phosphate solubilizing microorganisms (PSM) like fungi, actionmycetes and bacteria are reported to exhibit P solubilization and mineralization ability. Amongst the bacteria *Bacillus* species are also recognized as the strong P solubilizer (Babalola and Glick, 2012) and is one of the most important genera of phosphate solubilizer. It is a genus of gram-positive, rod-shaped sporulating, ubiquitous in nature and a member of the phylum Firmicutes. Thus, the use of *Bacillus* species could be a breakthrough in solubilizing the phosphate in soil that could be both in organic and in inorganic forms. Different mechanisms are involved in the solubilization of the both types. Organic acids like gluconic acids are more effective than inorganic acids in inorganic phosphate solubilization (Kim *et al*., 1997). The mechanism of organic P solubilization involves the Phosphatase (Feller *et al.,* 1994) and phytases (Sabu *et al.,* 2001) enzymes which are the main compounds that bring about the mineralization of most organic phosphorous. Because, now it has been validated that these PGPR are critical to plant growth because in the plant rhizosphere there are considerable population of beneficial bacteria that function as the soil amendment factor (Delgado-Baquerizo *et al.*, 2018).

Moreover, being recognized as a good P solubilizer *Bacillus* species is also involved in the degradation of the fermentation inhibitors formed during the production of second-generation bioethanol from lignocellulosic biomass. *Bacillus* are reported to degrade the inhibitors like furfural, hydroxymethyl furfural and phenolic compounds (Banerjee and Ghoshal, 2010). This clearly suggests that after neutralizing the solvents used to degrade lignin that solution could be used to grow *Bacillus* species which could be then developed as P fertilizer blended with phosphate rock dust of HA to supplement P fertilizer in the soil. In addition, a lot of cereals are used to make animal proteins as feed material. The animal bone contains huge amount of calcium and phosphate and if this could be reused in farming system it can give calcium that is critical for plant growth and immunity along with P source (Kerovuo *et al.,*1998). Moreover, direct use of bone could be religiously taboo, like bovine bones to Hindu community or swine bone to Muslim community, and mitigating this could be converting the bone in hydroxyapatite (HA) form and using *Bacillus* species that can solubilize HA to release phosphate to plants. This could be alternative to using easily washable commercial chemical fertilizer that causes eutrophication (Pearson *et al.,* 2003) in downstream water system as HA is insoluble (Hodge *et al*., 1943) and is not easily washed by irrigating or precipitating water. This would create a “Cyclic Economy” of phosphate that is non-renewal.

1.2 Current Studies:

Phosphorus being the non-renewable resource and must be used in judicious manner. The main raw materials in manufacturing of the phosphorus fertilizer are phosphorite and

apatite which are the main non-renewable sources of phosphorus (Pettersson *et al.,* 2008). It has been estimated that 70% of global production currently comes from reserves which will be depleted within 100 years (Cooper *et al.*, 2011). In May 2014, the European Commission published an updated list of raw materials (Tiess, 2010) that are crucial for the global economy. Among the six new materials attached to the 2011 list, phosphorus ores were mentioned. Therefore, it is the key measure to improve the balance use of phosphorus both globally and locally and the judicious use of phosphate must be implemented.

Promising alternatives of phosphorus sources are by-products of wastewater treatment, such as sewage sludge and ashes, as well as industrial side-streams. Various phosphorus rich residues such as meat and bone meal (MBM), municipal sewage sludge, phosphorus-rich ashes and agricultural residues can be used as an alternative to phosphate rocks as phosphorus can be recycled from these residues (Tan and Lagerkvist, 2011). The price of phosphate rock is increasing globally, together with the need to remove phosphorus (P) from wastewater (Elser, 2012) to control and avoid eutrophication and technology is required in making phosphorus recovery economically and environmentally important (Ge *et al.*, 2015) because only about one fourth of the P applied to agricultural fields is actually recycled (ParésViader *et al.,* 2015).

Different methods could be used to recycle these. The agricultural residues and municipal sewage sludge could be used for pyrolysis and co-combustion that not only generates electricity but the nutrients could be trapped in bio-char thus produced from which P solubilizing microbes can release phosphorous and the embedded carbon with nitrogen in biochar sequesters captured carbon in soil as bio-char is not easily degraded (Vassilev *et al.,* 2013) and is a good soil amendment to increase soil fertility as it eventually converts into humic substance (Dias *et al.,* 2010).

Moreover, the use of animal bones could be additional avenue and different approaches have been reported for the manufacture of natural hydroxapatite (secondary source of phosphate) from the bovine bones (Barakat *et al.*, 2009). This could be viable because the soil biotas play a significant role in utilization of P-bearing secondary raw materials as they have the ability to convert insoluble P to soluble P through the release of organic acids, chelation, and ion exchange. The action of organic acids is recognized as a major mechanism responsible for phosphorus release from hydroxyapatite structures (Saeid *et al.*, 2018).

In addition, one of the ways of securing food could be the use of economically viable, eco-friendly, renewable source for the production of biofuel. One of the alternatives could be lignocellulosic biomass as it is non-edible portion of the plant thus sustainable production

of liquid transportation fuel offers greater opportunity but the cost reduction of these industries in the longer term without impacting the nation's food supply is sought and potentially is of concern now (Michelin *et al.*, 2015). Thus, biofuel production could be intended more towards the sources that does not compete with food and is widely available as a low-cost feedstock like lignocellulosic biomass that is abundant on Earth (Taherzadeh and Karimi, 2008). However, during the ethanol production from the lignocellulosic biomass the release of fermentation inhibitors inhibit the growth of fermenting organisms hampering the reduction of fermentable sugars causing lower ethanol production rate (Larsson *et al.,* 1999). It is therefore the major concern to use the lignocellulosic biomass for the second generation bio-fuel production (Zheng *et al.,* 2014).

So, to overcome these problems use of organisms which can degrade such inhibitors can be the better options. One of the such organisms is *Bacillus* genera (Taylor *et al*., 2012) and they are also P solubilizer. Thus, an integrated approach of industry that is involved in both bio-fertilizer and bio-ethanol production could address these limitations. Thus, in addition to the reduction of cost of ethanol production it can increase the revenue by increasing additional income through bio-fertilizer sales. This could economically support bioethanol production facility that could be profitable even by using agricultural wastes as reduced carbon sources. This is because ethanol production halts at around 18% ethanol concentration and around 50% of reducing sugar, mainly glucose (Sun and Cheng, 2002) is converted to ethanol during fermentation. Moreover, the broken hydrolyzing enzyme inhibitors to acetate or other reducing sugars could directly be used for ethanol production because low amount of reducing sugar is required. This could increase the ethanol yield from the agricultural waste used (volume/weight) and decreasing significantly the cost of production. The fermented broth if distilled through vacuum or rotary evaporator then could be directly used to grow other nitrogen fixing bacteria in profit maximization and developing more environment friendly fertilization system.

1.3 Hypothesis:

Null Hypothesis H_0: *Bacillus* species cannot solubilize the nano-hydroxyapatite (nHA) as a P source (secondary phosphorus bearing raw materials) when used in media instead of other soluble source of P like dipotassium hydrogen phosphate and dihydrogen potassium phosphate.

Alternative Hypothesis H_1: *Bacillus* species can solubilize the nano-HA as a phosphorus (P) source (secondary phosphorus bearing raw materials) when used in media instead of other soluble source of P like dipotassium hydrogen phosphate and dihydrogen potassium phosphate.

1.4 Objective:

1.4.1 General objective:

1. To prepare nano-HA from bovine bone to develop as P fertilizer by integrating HA phosphate solubilizing *Bacillus* species bacteria upon isolation and characterization from different soil samples.

1.4.2 Specific Objectives:

1. To synthesize the nano-HA from the raw buffalo bone and to use HA as an insoluble P source.
2. To isolate putative *Bacillus megaterium* as P solubilizing bacteria based on utilization of different reduced carbon sources (CCR mechanism).
3. To isolate putative *Bacillus subtilis* as P solubilizing bacteria based on CCR mechanism.
4. To extract the genomic DNA, perform the PCR and Sequence analysis for the molecular identification of the isolates.
5. To quantify the soluble phosphate after HA, solubilize by the bacteria.
6. To determine the amount of reducing sugar, furfural and phenol in the acid treated lignocellulosic biomass (*Saccharum spontaneum)* after incubating the bacteria.

1.5 Rationale of the study: -

The study provides an approach to overcome the problem derived by the use of water-soluble phosphate fertilizer and lignocellulosic biomass-based bio-ethanol production to reduce the cost of alternative ecofriendly green industries. It provides a clear vision for the management of the waste and utilizes them as a phosphate source with the view of reducing the burden towards already choked limited phosphate sources (naturally occurring rocks and primary apatite). The study is focused towards the use of alternative phosphate source as Hydroxyapatite because of its main characteristics of insolubility that has the runoff almost to completely zero and the applied phosphate solubilizing microorganisms (PSM) can liberate phosphorus from the HA in the form that can be easily taken by the plants for sustained release of P fertilizer. Moreover, the study is intended also towards the feasibility of profitability of ethanol production from ecofriendly agricultural litter lignocellulosic biomass by complete utilization of entire biomass for ethanol production. Thus, an integrated bio-fertilizer and bio-ethanol production industry could be developed that can address both the issue of fossil fuel in transportation and reduce the use of chemical fertilizers with “Cyclic Economy” of phosphate. The study is primarily to develop “One Window System” for bio-ethanol and bio-fertilizer production to tame the global warming and also address the alarming concern of food security for burgeoning food demand to global population growth.

CHAPTER 2
LITERATURE REVIEW

2.1 Population growth and food demand:

The current population of the word is around 7 plus billon and is projected to grow over to 9 billion which clearly indicates that the consumption growth where the global demand for food will increase for at least another 40 years (Keating *et al.,* 2014). To satisfy the expected food and feed demand, it is important to increase the global food production by 70% within 2050 to meet the demand of an additional quantity of nearly 1 billion tons of cereals and 200 million tons of meat. However, for the sustained crop yield and food production it requires application of fertilizers containing phosphorus, nitrogen and potassium. Without the application of the fertilizer world's escalating food needs cannot be achieved. Efficient and effective use of fertilizer must be kept into consideration. For this, The 4Rs — right source, right rate, right time, and right place are the underpinning principles of fertilizer management (Roberts, 2009).

On the other hand, inappropriate application of fertilizers is the main sources of water bodies' pollution. When high fertilizer rates are applied, nutrients get lost by surface run-off creating negative impacts on land-based and aquatic ecosystem. Oxygen depletion conditions of the water bodies cause algal biomass to bloom creating eutrophication problems (Kremser and Schnug, 2002). Growing competition for land, water and energy causes the human to extract and deplete Earth's resources at growing rates without considering for the natural balance of the environment (Tester and Langridge, 2010). Thus, judicious use of fertilizer and new technologies are required. Keeping all these in mind use of biofertilizer can be appropriate solutions of above-mentioned problems. So it can set up as a promising alternative for the agricultural production (Eustacio Ramirez, 2015).

2.2 Eutrophication:

When the water bodies become over enriched with the nutrients like nitrogen and phosphorus it results in excessive growth of plants and algal biomass. Such nutrients originate from agriculture or sewage treatment. The oxygen depletion condition of the aquatic ecosystem arises and the aquatic life forms face the verse of extinction. This condition is termed as eutrophication. It is widely recognized as the anthropogenic problems and the growing environmental problem which creates negative impact in the environment (Serediak *et al.,* 2013). With phosphorus in particular, released from sediments can be a major source of this nutrient in water (Shaw *et al.*, 2003). According to Ullmann's Encyclopedia, phosphate is the primary limiting factor for eutrophication.

Some cyanobacteria have the capacity to produce the toxins dangerous to the human beings. The toxins may be found either free in the water or bound to the algal or cyanobacterial cell. There are various types of toxins that targets the various organ in mammals, like Microcystins, Nodularin (Abraxis, 2015) and cylindrospermopsins (Solter and Beasley, 2013a) target the mammalian liver. Similarly Anatoxin-a, Anatoxin-a(S) (Solter and Beasley, 2013b) target Nerve synapse and Aplysiatoxins, Lyngbyatoxin-a target the skin (Taylor *et al.*, 2014).

2.3 Fertilizer:

A fertilizer (also called plant food element) is a natural or synthetic, chemical-based substance that is added to the soil to enhance plant growth and fertility. For the proper development and growth of plants these fertilizers furnishes one or more of the chemical elements necessary (Ifdc and Unido, 1998). It may also enhance water retention, aeration and filter any excess liquid thus enhancing soil effectiveness. Fertilizer typically offers the three major macronutrients in the soil, Nitrogen(N), Phosphorus(P) and Potassium(K) (El-kramany *et al.,* 2010). Being the macronutrient, these are consumed in large quantities and are present in plant tissue in quantities ranging from 0.15% -6% on a dry matter (DM; 0% moisture) basis. Fertilizers also add other three secondary macronutrients apart from NPK which include Sulfur(S), Magnesium (Mg) and Calcium (Ca). Fertilizer also provide micronutrient for the plants growth on the order of parts-per million (ppm), ranging from 0.15 to 400 ppm DM, or less than 0.04%DM (Benton *et al.,* 1996).

Fertilizer may be Organic (Natural) or Synthetic (Inorganic). Organic fertilizer is made from naturally occurring mineral deposits and organic material. Generally, use of alfalfa meal, cottonseed meal or fish emulsion to provide nitrogen, bone meal or rock phosphate to provide phosphorus and kelp meal or granite meal to provide potassium. Soil microbes play an important role in converting organic fertilizer into soluble nutrients that can be absorbed by plants (Fontaine *et al.,* 2003).The drawback of organic fertilizer is that they work comparatively much slowly. First, they need to be broken up into the form that the root of the plants can easily absorb and then making their way up to the plants from root because the nutrients in organic fertilizer are not water-soluble and are released to the plants slowly over a period of months or even years (Eppinger *et al*., 2011).

Whereas synthetic fertilizers are made by chemically processing raw materials (Benton Jones Jr., 2012). Synthetic fertilizers are water-soluble and can be taken up by the plant almost immediately. Synthetic fertilizers give plants a quick boost but do little to improve soil texture, stimulate soil life or improve the soil's long-term fertility. However, when large quantities are used over and over again, its byproduct will actually build up in the soil and in time they can hinder plant growth (Freney, 1995). In addition, synthetic

fertilizers are highly soluble thus can also leach out into streams and ponds. Another drawback of using synthetic fertilizer is that they can over time; even destroy the beneficial organisms needed for healthy soil (Hazra, 2016).

2.4 Phosphorus and its importance:

Phosphorus (P) is the second most limiting nutrient after nitrogen. P is one of the most essential plant nutrients which profoundly affect the overall growth of plants by influencing various key metabolic process such as cell division and development, energy transport, signal transduction, macromolecular (DNA, RNA etc.) biosynthesis (Paytan and McLaughlin, 2007), photosynthesis and respiration of plants (Ahemed *et al.,* 2009). P is an important constituent of Adenosine triphosphate (ATP) hence is indispensable in the energy transfer process in living cells and important in bone and teeth formation (Soetan *et al.,*2010). Animals get their phosphorus from eating plants whereas plants take up phosphorus in the form of phosphate from soil or water and phosphate is the most abundant source for elemental phosphorus.

2.5 Phosphorus cycle:

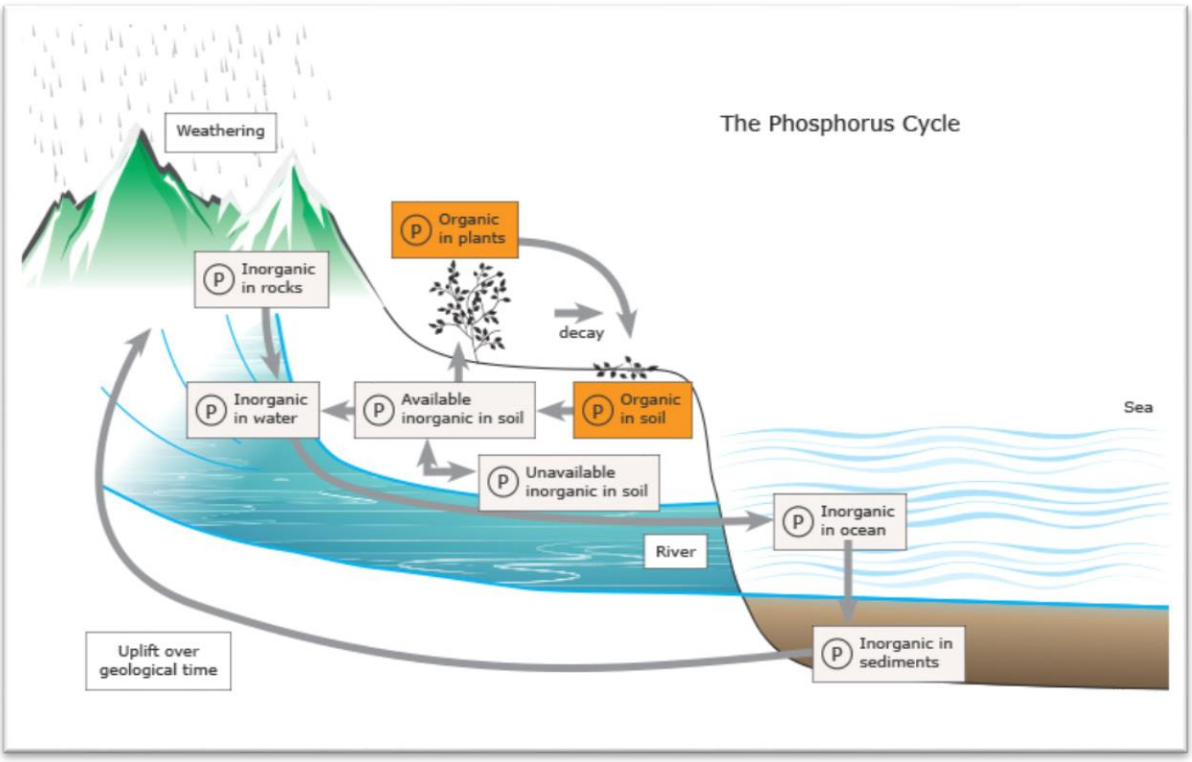


Figure: 1 Phosphorus Cycle

- The rainfall and weathering of phosphate rocks cause the release of phosphate ions and other minerals. This inorganic phosphate is then distributed in soils and water.
- Thus, released inorganic phosphate is taken up by the plants. Animal fulfills their phosphate need by consuming the plants. Once in the plant or animal, the phosphate is incorporated into organic molecules such as DNA. Animal wastes and decayed products of dead animals return organic phosphate into the soil.
- Within the soil, organic forms of phosphate can be made available to plants by bacteria by mineralization.
- Phosphorus from soil is passed into the water stream and eventually to the ocean bottom as deposits of slightly soluble phosphate rock.

2.6 Phosphorus in the soil-plant system:

In the plants P is absorbed mainly during the vegetative growth and, thereafter, most of the absorbed P is re-translocated into fruits and seeds during reproductive stages. There are various problems seen in P-deficient plants which includes retarded growth (reduced cell and leaf expansion, respiration and photosynthesis), and often a dark green color (higher chlorophyll concentration) and reddish coloration (enhanced anthocyanin formation) (Grant *et al.,* 2001). The total P concentration in agricultural crops generally varies from 0.1 to 0.5 percent (IPNI (International Plant Nutrition Institute), 1999).

Water-soluble P (WSP) fertilizer is applied to the soil, it reacts rapidly with the soil compounds. Generally, commercially available P fertilizers are used which are easily dissolved in the soil solution and available for plant uptake. These includes MAP [monoammonium phosphate, ($NH_3H_2PO_4$)], DAP [diammonium phosphate, ($(NH_3)_2HPO_4$)] or TSP [Triple Superphosphate, $Ca(H_2PO_4)_2$] are water soluble phosphate salts, and thus, are regarded as high quality fertilizers (Fageria, 1998). These soluble phosphate salts are very mobile in the soil and the large portions are often end in surface waterbodies through erosion, runoff or seepage, causing eutrophication.

2.7 Phosphorus availability in soil:

P is found in numerous compound forms, in the form of phosphate ion (PO_4^{3-}). The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less (10 M $H_2PO_4^-$). In comparison to other nutrients, P concentration in soil solution is much lower. The total P concentration in soils is generally in the range from 200 to 5000 mg P per kg soil with an average of 600 mg P per kg soil (Kuo, 1996). There are two forms of phosphorus found in the soil. One is the organic form and another is the inorganic form.

The main inorganic form of phosphorus in the soil is $H_2PO_4^-$ and HPO_4^{2-}. The phosphorus in this ionic form is used by the plants. However, these ions are adsorbed into the soil matter in the soil and become unavailable to the plants (Cornforth, 1983). Organic form of phosphorus is present at the 50-80% in the soil. These come from plant residues, manures and microbial tissue. Soil organic P is largely found in the form of inositol phosphate (soil phytate), that is also insoluble and plants cannot take it unless broken to phosphate ions. Other organic P compounds that have been reported are: phosphomonoesters, phosphodiesters, phospholipids, nucleic acids, and phosphotriesters (H Rodríguez and Fraga, 1999).

The natural and the biggest reserves of inorganic phosphorus are rocks and other deposits such as primary apatite (Hughes and Rakovan, 2002) and other primary minerals formed during the geological age. The apatites are the most common source of the P required for the plants. The primary apatites include chlorapatite ($Ca_5(PO_4)_3Cl$), fluorapatite ($Ca_5(PO_4)_3F$) and hydroxyl apatite ($Ca_5(PO_4)_3OH$). The principal characteristics of these apatites are their insolubility but under an appropriate condition, they can be solubilized and become available for plants and microorganisms. Apart from their importance as a fertilizer they are also very important in the chemical and pharmaceutical industries. A large amount of phosphorus fertilizer applied in the agricultural field, plant can't take up the applied phosphorus because these enter into the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+}in acidic soil and Ca^{2+}in calcareous or normal soils (Badawi, 2010).

2.8 Mineralization:

The increasing number in population means higher demand for food. The animal derived food like meat, dairy products and eggs have relatively higher bioavailability phosphorus compared to the plants derived food like whole grains, legumes, peas, nuts, and seeds (McCarty, 2014). Thus, mineralization of these animal residues could alternatively be a sustained source of P fertilization.

Unlike nitrate, that other major ingredient of fertilizer, phosphate cannot be synthesized from abundant elements like oxygen and nitrogen. There is no exchange of phosphorus in the atmosphere so its cycle in the biosphere is considered as 'open' or 'sedimentary' (Begon *et al.,* 1990). It needs either to be recycled (for instance through use of manure) or mined from phosphate rocks. Many investigations extending over last 80 years have shown that only 10-20% of applied phosphorus is utilized by the plants and rest is fixed in a form which is not readily available to the plant (Sashidhar and Podile, 2009).

Inorder to minimize these problems it is reported that apatite also been attempted as P fertilizers where the phosphate is locked in a solid form and is less easily available to the

alga and also less easily being transported by runoff or soil erosion. However, these apatites are less effective in nutriting the plants because of hindering by the particle size. So, the nanotechnology is effective way to cope such problems, with the potential to improve fertilizer formulations (Ghormade *et al.,* 2011); (Gogos *et al.,* 2012). To improve P efficiency, hydroxyapatite nanoparticles (nHA) were evaluated as a potential fertilizer based on the hypothesis that nano-sized particles can potentially move in the soil and reach the plant roots. The use of sparingly soluble calcium phosphates such as hydroxyapatite has been studied as an alternative to conventional water-soluble P fertilizers for acidic and strongly P sorbing soils (Bertran *et al.,* 2015).The figure down clearly signifies the advantage of using nanosized solid P fertilizer over the regular solid P fertilizer counterpart.

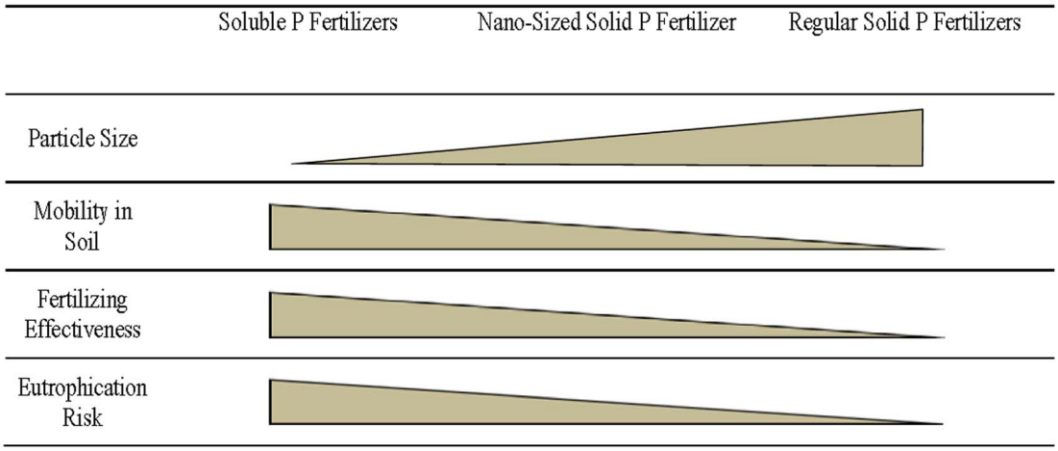


Figure 2: A schematic comparison of soluble P, Nano-sized solid P, and solid P on their environmental properties (note: some of nanoparticle properties are hypothetical).

2.9 Hydroxyapatite (HA):

Hydroxyapatite (HA) is a class of calcium phosphate based bioceramics, the main mineral component of human and animal bones (Pattanayak *et al.,* 2011). HA shows strong biocompatibility with the bone and teeth so it is frequently used in biomedicine (Ragu *et al.,* 2014). It is reported that HA exhibit the properties like osteoconductive, non-toxic non-immunogenicbehavior (Szcześ *et al.,* 2017). HA derived either from the natural or synthetic source can form a strong chemical bond with the host tissue so is recognized as a goodbone substitute material (Nazarpak *et al.,* 2009. The stoichiometric HA has the chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ with Ca/P molar ratio of 1.67 (Y. Zhao *et al.,* 2007).

Apart from the use of HA in biomedicine, it is also used in the agriculture as phosphate fertilizer. It is reported that nHA particle has increased the growth of Glycine max (soybean), in which the growth rate and seed yield has increased by 32.6% and 20.4% respectively, compared to those of soybeans treated with a regular P Biomass productions

were enhanced by 18.2% (above-ground) and 41.2% (below-ground) (Liu and Lal, 2014) . HA-nanorod was observed beneficial on the seed germination and growth of Cicer arietinum (Chickpea) plants. The maximum increase was observed in the presence of 1 mg/ml HA-nanorod where the plant growth rate was more than two times over the control (Bala *et al.,* 2014).

2.10 Phosphorus flow and the Current Situation:

The only large mines are located in Morocco, Russia, China and the US (Rosmarin, 2004). Furthermore, the extraction process phosphate rock is difficult and economically not viable. As much as four-fifths of phosphorus is wasted during production, from the moment it is mined to the final moments of processing. efficiency of P in animal production is very low, a large amount of undigested feed P excreted with manure, and the P enrichment in the environment resulted in serious P pollution, such as eutrophication (Guo *et al.,* 2018).

In addition, there is the problem of economic scarcity, where farmers with buying power get access to fertilizer market, instead of it been accessible to all farmers who need it for crop productivity. So the Governmental structure must be alert enough to monitor the equal distribution of the Phosphate throughout the world. It is predicted that demand for phosphorus will increase by 50–100% by 2050 with increased global demand for food and changing diets (Cordell *et al.,* 2009a). With depleting resources alternative technologies are required because, after all, one can live without cars or unusual species, but if phosphorus ran out human have to live without sufficient food.

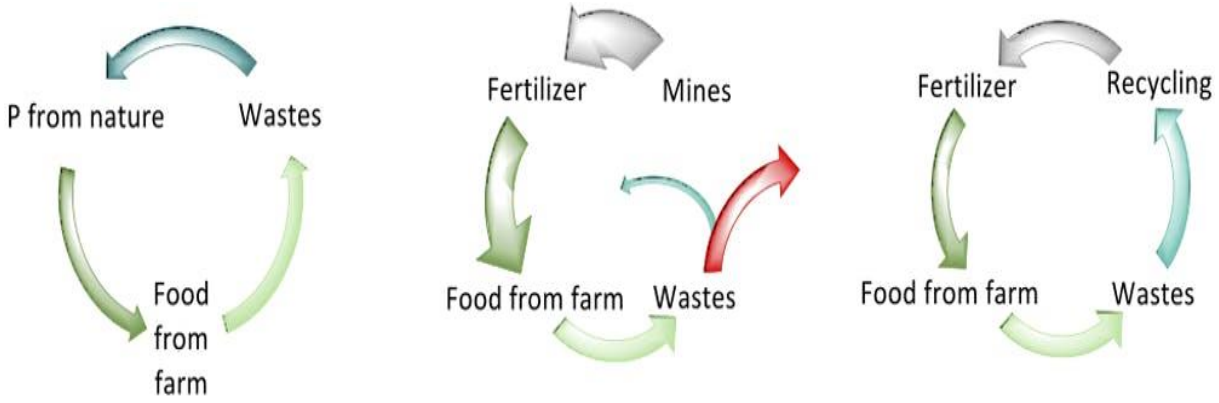


Figure a)Original phosphorus cycle Figure b)The broken cycle Figure c) An optimized cycle

Figure 3: Phosphorus Flow.

2.11 Biofertilizer:

In comparison to nonrhizospheric soil the rhizospheric soil contain considerably higher concentration of phosphate solubilizing bacteria (Raghu and MacRae, 1966). Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. Such microorganisms are often termed as Biofertilizer. Biofertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. The biofertilizer contains microorganism able to increase soil fertility (Conditions, 2014). Biofertililizer add nutrients through the natural process of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-Promoting substances. Biofertilizer helps in plant growth and development and perform the similar task as the fertilizers do. So, it is expected to reduce the use of chemical fertilizers and pesticides by the use of biofertilizer. Since they play several roles, a preferred scientific term for such beneficial bacteria is plant- growth promoting rhizobacteria (PGPR). Now it has been validated that these PGPR are critical to plant growth because in the plant rhizosphere there are considerable population of beneficial bacteria that function as the soil amendment factor (Delgado-Baquerizo *et al.*, 2018).

PGPR are free-living bacteria of beneficial agricultural importance. Some PGPR may have more than one mechanism to improve soil fertility and crop yield by reducing the negative impacts accomplishing plant growth (Ahmad *et al.,* 2008). Biofertilizer such as *Rhizobium, Azatobacter, Azospirillium* and Blue Green Algae (BGA) have been in use a long time (Bhat *et al.,* 2009). Moreover, the PGPR presence encourage beneficial effects on plant health and growth, suppress disease-causing microbes and accelerate nutrient availability and assimilation (Gupta *et al.,* 2014). PGPR shows various direct or indirect mechanisms including antagonism to pathogenic fungi, siderophore production, nitrogen fixation, phosphate solubilization, the production of organic acid and Indole Acetic Acid (IAA), Ammonia (NH3), Hydrogen Cyanide (HCN), the release of enzymes (soil dehydrogenase, phosphatase, nitrogenase, etc.) and the induction of systemic disease resistance (ISR) (A. Kumar *et al*., 2015).

2.11.1 Other advantages of using biofertilizer:

- They help to get high quality of crops by making the soil rich with nutrients and useful microorganisms necessary for the growth of plants

- They have replaced the chemical fertilizers ass chemical fertilizers are not beneficial for the plants. They make the environment pollution by releasing harmful chemicals.
- Plant growth can be increased if biofertilizers are used because they contain natural components which do not harm the plant.
- They destroy those harmful components from the soil which cause diseases in the plants.
- They are not costly and even poor farmers can make use of them.

They are environment-friendly and protect the environment against pollutants (OJHA1, 2016).

2.12 Phosphate solubilizing microorganisms (PSM):

PSM includes a large number of microbial organisms that exhibit P solubilization and mineralization ability. It includes bacteria, fungi, actinomycetes and algae. Strains from the bacterial genera includes *Pseudomonas*, *Bacillus* and *Rhizobium* are recognized as the strong P solubilizer (Babalola and Glick, 2012). Soil fungi have been reported to solubilize inorganic phosphate in soil more easily than the bacteria (Sharma *et al.,* 2013). Similarly,the microbial fungi includes the strain of fungi such as *Pencillium, Aspergillus, Fusarium, Helminthosparium, Alternaria,* etc. are the most powerful phosphate solubilizersand P solubilization is a complex phenomenon and it is dependent on nutritional, physiological and growth condition of the culture (Behera *et al.,* 2014).

2.12.1 Mechanisms of inorganic phosphate solubilization by PSM:

Several theories have been proposed that explains the mechanism of inorganic phosphate solubilization. As observed in many experiments, the principal mechanism is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions and CO_2 (H Rodríguez and Fraga, 1999). Organic acids produced by the PSM in the periplasmic space by the direct oxidation pathway (Zhao *et al.,* 2014) with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release P. Among the various organic acids, produced by the microorganisms; gluconic acid is the most frequent agent of mineral phosphate solubilization. Gluconic acid chelates the cations (Ca, Al, Fe) bound to phosphate, thus making the phosphate available to plants. Gram-negative bacteria solubilize mineral phosphate by direct oxidation of glucose to gluconic acid (Sashidhar and Podile, 2010). Pyrroloquinoline Quinone (PQQ) acts as a redox cofactor in glucose dehydrogenases (GDH) resulting in phosphate solubilization (Rodríguez *et al.*, 2000).

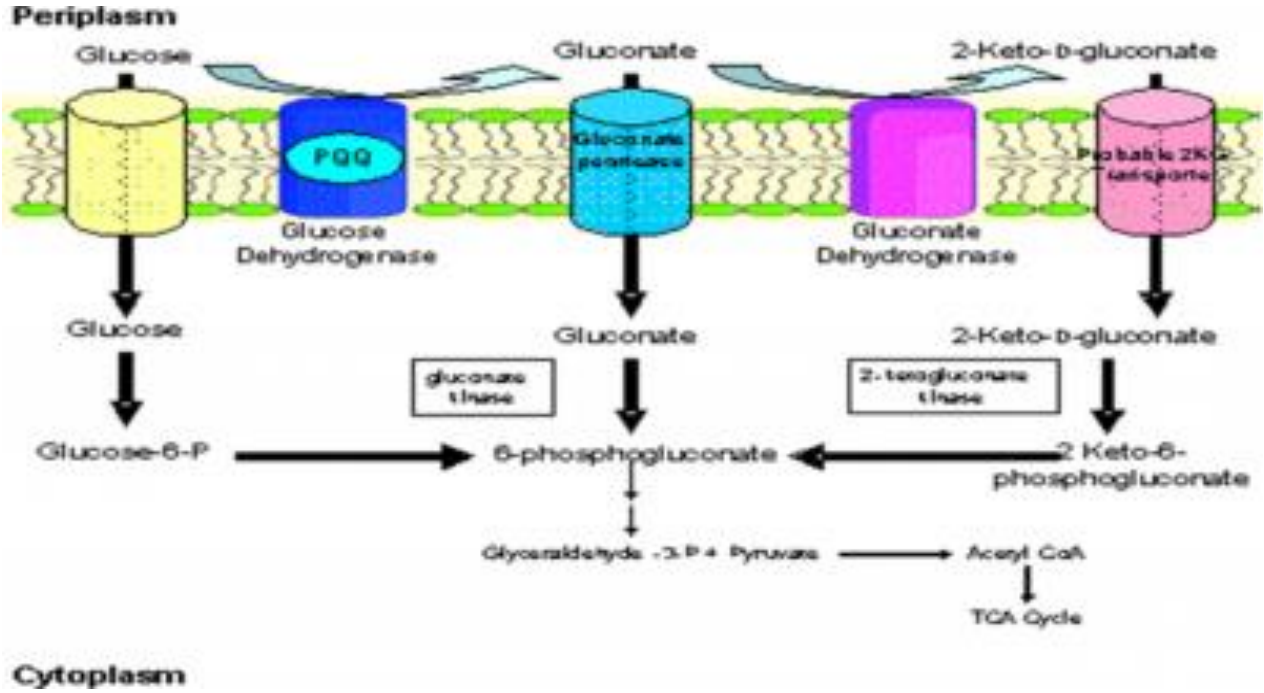


Figure 4: Production of gluconic acid via the alternative extracellular oxidation pathway of glucose metabolism.

2.12.2 Mechanisms of organic phosphorus mineralization:

Phosphate mineralization refers to the solubilization of organic phosphorus. Various types of organic matter are found in the soil which contains the phosphorus compound. Phosphatase enzymes are the main enzyme involved in bringing about the mineralization of most organic phosphorous. It has been reported that the significant amount of phosphatase activity is present in soil (Feller *et al.,* 1994). These enzymes can either be acidic or alkaline phosphatase (Acuña and Jorquera, 2011). Thus, the organic phosphorus sources must be converted (hydrolyzed) first into inorganic form that can then only be available to the organisms including animals and can meet their phosphate need (Zhao and Liu, 2013).

In addition, another type of enzyme involved in phosphate solubilization is phytases. Phytases catalyze the hydrolysis of phytate (Myo-inositol hexa-phosphate, IP6), into Myo-inositol and phosphoric acid and is an important metabolic process in many biological systems (Sabu *et al.*, 2001). Moreover, the most common organic form of phosphorus found in the soil is inositol phosphate (soil phytate).

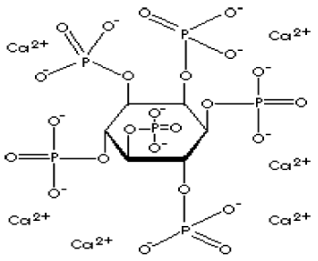


Figure 5: Molecular structure of insoluble phytate.

Phytic acid (PA) is found principally in food derived from plants, in cereals and legumes. The term phytic acid, phytate, phytin refer to the free acid, salt and, calcium/magnesium salt, respectively. The salts form of phytic is referred to as phytates (Neevel *et al.,* 2007). Phytic acid has a unique structure and it is unstable in free acid form and occurs mainly as a complex with nutritionally important metal cations such as calcium (Ca^{++}), iron (Fe^{++}), zinc (Zn^{++}), magnesium (Mg^{++}), potassium (K^{+}) and manganese (Mn^{++}). The complex forming nature of phytic acid gives it the ability to bind minerals, proteins, and starch, and the resulting detrimental effects. It is the main inhibitor of Fe and Zn absorption (Sandberg, 2002). It lowers the bioavailability of essential minerals and protein in human and non-ruminant. So it is referred to as Antinutrient (Addo *et al.,*2018).

Phytic acid has also been attributed to the phosphorous mediated environmental problems because high amount of phosphorus cause pollution of water and soil (Oatway *et al.,* 2001). Since the phytase enzyme is absent in monogastric animals the cereals consumed by such animals is sparingly used as phosphorous source if the gut microbiome is not have been involved for digestion of it.

Phosphate solubilizing bacteria produce the phytase which is active within the pH of 4.5-8.5 and temperature ranges 25-70°C, the molecular mass of 37-55 kDa. These are resistant to the action of proteases found in the gastrointestinal tract of monogastric animals (Jain *et al.,* 2016). Thus, phosphate solubilizing microbes are not only important and P solubilizing bio-fertilizer but as supplement to feed as probiotic formulation. Some of *Bacillus* species are known as probiotics (Shahcheraghi, 2015) and they are also known as P solubilize (Rodríguez and Fraga, 1999). Hence, incorporation of them in feed could be one of the mechanisms to supply phosphate to the animals.

2.13 Factors influencing microbial phosphate solubilization:

Significant populations of microorganisms present in the soil have the capacity to dissolve poorly soluble mineral phosphates. Studies indicate that the physiology and biochemistry of Carbon, Nitrogen, and Phosphorus play a role in the phosphate solubilization process (Nahas, 2007) and also on the growth status of the organism. PSM found in the extreme environmental conditions have the better tendency to solubilize more phosphate than

PSM from soils from more moderate (Zhu *et al.,* 2011). Extreme environmental conditions include saline-alkaline soils, soil with a high level of nutrient deficiency, or soil from extreme temperature environments. Different researchers have different views regarding the influence of temperature on phosphorus solubilization by microbes.

2.14 Phosphate solubilizing Bacteria:

Bacillus is one of the most important genera of phosphate solubilizer (Rodríguez and Fraga, 1999). It is a genus of gram-positive, rod-shaped sporulating, ubiquitous in nature and a member of the phylum Firmicutes. Bacillus includes both free-living (nonparasitic) and parasitic pathogenic species. Most of the species of *Bacillus* are harmless saprophytes with some exception. *Bacillus anthracis*, is the agent of anthrax, it shows some activity outside of its vertebrate hosts, transmission of anthrax spore to grazing hosts (Ganz *et al.,* 2014). Some of the species of *Bacillus* are the pathogen of the specific group of insects which include *Bacillu*s larvae, *B. lentimorbus, B. popilliae, B. sphaericus, and B. thuringiensis, B. cereus,* are occasional pathogens of humans and livestock (Turnbull, 1996).

Scientific classification of *Bacillus*

Domain- Bacteria

Division- Firmicutes

Class- Bacilli

Order - Bacillales

Family - Bacillaceae

Genus - *Bacillus*

Species -*acidiceler, acidicola, aerius, agri, anthracis, cibi, flexus, halophilus, subtilis, thuringiensis, megaterium* etc.

2.14.1 *Bacillus megaterium*:

Bacillus megaterium is one of the important phosphates solubilizer and is known to produce vitamins like Riboflavin, Biotin, folic acid and cobalamin (Perkins and Pero, 1993). It has recently been accepted as a perfect model for anaerobic vitamin B12 biosynthesis. Since the chemical synthesis of Vitamin B12 is uneconomic and requires 60 steps (Mohammed *et al.,* 2014) and unique among the vitamins as it appears to be solely produced by prokaryotes (Biedendieck *et al*., 2010). So, *Bacillus megaterium* mediated vitamin B12 biosynthesis is in practice for industrial production and recognized as GRAS organism (Barg *et al.,* 2005). It is reported that Riboflavin (vitamin B2) treatment protect

plants from infections (Roje, 2007). Folic acid (vitamin B9) enables plant to regulate their DNA function and also to metabolize carbohydrates, proteins and lipids in plants (Scott *et al*., 2000). The presence of tryptophan in the media induce the synthesis of IAA in *Bacillus megaterium* (Glick, 2014).

Bacillus megaterium has been industrially employed for more than 50 years, as it possesses some very useful and unusual enzymes and a high capacity for the production of exoenzymes, variety of proteins and is a source of bioremediation. Since it doesn't possess external alkaline proteases and can stably maintain a variety of plasmid vector. It is considered as a desirable cloning host for the production of intact proteins (Vary *et al.,* 2007). Furthermore the endophytic nature of *Bacillus megaterium* exert several beneficial effects on host plants it is also involved in the IAA production, nitrogen fixation, siderophores production, phosphate solubilization and induction of resistance to plant pathogens, HCN, ammonia, cellulolytic and pectinolytic activity and antifungal metabolite. These make rhizospheric environment more friendly for other beneficial microbes.

2.14.2 *Bacillus subtilis*:

Bacillus subtilis is also known as the hay bacillus or grass bacillus. It is rod shaped, Gram positive, catalase positive and endospore forming (Wei *et al.*, 2003) found in the soil and gastrointestional tract of ruminants and human. This bacterium is widely used on an industrial scale by biotechnology companies, for the production of enzymes, pharmaceutical components and GMOs. These are identified as a probiotic organism, since the last 15 years (Shahcheraghi, 2015).

This bacterium does not produce endotoxins (Harwood, 1992). *Bacillus subtilis* has the ability to take up and recombine extracellular DNA into its genome, which makes it naturally qualified for genetical transformation (Westers *et al.,* 2004). They have beneficial effects on their host, which depends on their potency to endure oxygen stressors, heat, and osmotic stress during storage and processing (Rudrappa *et al*., 2007). *Bacillus* subtilis is among the important biofertilizer that is used to enhance the crop yield and alternatives to chemical fertilizers and pesticides. These are among the strong phosphate solubilizer. Some of the *Bacillus* species release ammonia from nitrogenous organic matter (Hayat *et al*., 2010). It is reported that some of the *Bacillus* spp. have the nifH gene and produce nitrogenase (EC 1.18.6.1), to fix atmospheric N2 and provide it to plants to enhance plant growth and yield by delaying senescence (Kuan *et al.,* 2016) so it is used as a biological tool for crop improvement (Radhakrishnan *et al*., 2017). These are involved in the synthesis of indole-3-acetic acid, gibberellic acid and ACC deminase which regulate the intracellular phytohormone metabolism and increase plant stress tolerance. It shows competitive inhibition by producing antibiotics which suppress the

pathogenic microorganisms in supporting better plant growth. They form preemptive biofilm colonizing on the plant rhizome that potentially blocks invasion by pathogenic microorganisms, showing the fungicidal properties (Beauregard *et al*., 2013).

2.15 Lignocellulosic biomass:

Taking into the consideration of increasing demand for energy, the hunt for alternative sources of energy generation that is economically viable, eco-friendly, renewable, and can replace the non-renewal fossil fuels is of interest and obviously that should not compete with food sources but is widely available as a low-cost feedstock. One of the approaches for this is the utilization of plant residues into bio-fuels by using the lignocellulosic biomass (Kang *et al.,* 2014). The lignocellulosic biomass conversion into sustainable chemical and fuel is one of the alternatives to reduce the burden on fossil fuel and one of the ways of sustainable development. The lignocellulosic biomass is an abundant organic material that is frequently used in the sustainable production of bioenergy and bio-fuels such as biogas (about 50–75% CH_4 and 25–50% CO_2) (Zheng *et al.,* 2014). Lignocellulosic biomass is the three-dimensional polymeric material, primarily composed of cellulose, hemicellulose, and lignin (Figure 6) with smaller amounts of other components like, acetyl groups, minerals, extractives, phenolic substituents (Isikgor *et al.*, 2015).

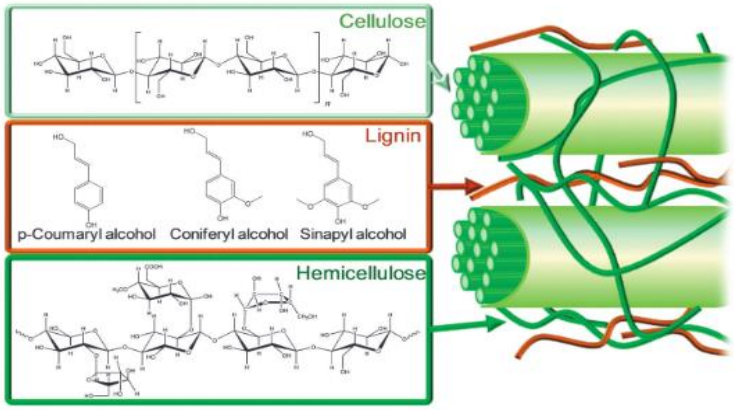


Figure 6: Structure of lignocellulosic biomass (Alonso *et al.,* 2012).

Cellulose and Hemicellulose portions in the lignocellulosic biomass are carbohydrates, that can be converted into fuels and chemicals by fermentation (Hyun, 2013). The lignocellulosic biomass includes cereal straw, bagasse, forest residues, and purpose-grown energy crops such as vegetative grasses and short rotation forests. It is an important component of the major food crops that is non-edible portion of the plant, but used for biofuel production These are considered as the second generation bio-fuel (Sims *et al.,* 2010).

Apart from lignocellulosic biomass the first-generation bio-fuels are also used. First generation bio-fuels are produced primarily from food crops such as grains, sugar beet and, oil seeds but these are limited in their ability to achieve targets of mitigating global warming as it competes with food and feed (Kumar *et al.,* 2009). Thus, alternatively lignocellulosic biomass has been the choice of making bio-fuel. Economically, the lignocellulosic biomass has an advantage over other agriculturally important bio-fuels feedstock. It plays a great role in a sustainable production of liquid transportation fuels that offers greater cost reduction potential in the longer term without impacting the food supply (Michelin *et al.,* 2015) and some of potential feedstock are listed (Table 1).

Table 1: Composition of some common sources of biomass (Sun and Cheng, 2002).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Coastal bermudagrass	25	35.7	6.4
Corn Cobs	45	35	15
Cotton seed hairs	80-95	5-20	0
Grasses	25-40	35-50	10-30
Hardwoods steam	40-55	24-40	18-25
Leaves	15-20	80-85	0
Newspaper	40-55	25-40	18-30
Nut shells	25-30	25-30	30-40
Paper	85-99	0	0-15
Primary wastewater solids	8-15	NA	24-29
Softwoods stems	45-50	25-35	25-35
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Sorted refuse	60	20	20
Swine waste	6.0	28	NA
Switchgrass	45	31.4	12.0
Waste papers from chemical pulps	60-70	10-20	5-10
Wheat straw	30	50	15

2.15.1 Cellulose:

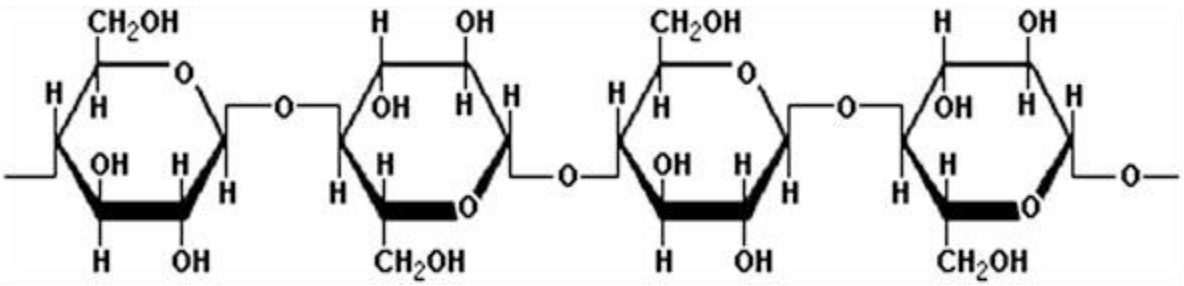


Figure 7: Structure of cellulose.

Cellulose is the most abundant material on the earth which comprise 40–50% of the lignocellulosic biomass feedstock. It is the main constituent of plants cell wall serving to maintain their structure, and also present in bacteria, fungi, algae and even in animals (O'Sullivan, 1997). It is a high molecular weight linear homopolymer of repeated units of cellobiose (two anhydrous glucose rings joined by β-(1, 4)-glycosidicbonds. The degree of

polymerization can range approximately from 4000-8000 glucose residues giving the estimated size of 200-2000 kDa (Aristidou and Penttilä, 2000). The long-chain of cellulose polymers are linked together by hydrogen and Van Der Wall's bond, which cause the cellulose to be packed into microfibrils. By forming these hydrogen bonds, the chains tend to arrange in parallel and form a crystalline structure. Cellulose in biomass is present in both crystalline and amorphous forms. The cellulose in its amorphous form is more susceptible to enzymatic degradation. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose (Thygesen *et al.,* 2005).

2.15.2 Hemicellulose:

Hemicellulose is the second most abundant polymer containing about 20–50 % of lignocellulose biomass. Like cellulose, hemicelluloses function as supporting material in plant cell wall (Ebringerova *et al.,* 2005). Hemicellulose is short lateral chain branched, heteropolysaccharide. The monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (4-O-methylglucuronic, D glucuronic, and D-galactouronic acids) (Ebringerová *et al., 2*005). The backbone of Hemicellulose is with short branches linked by β-(1, 4)-glycosidic bonds and occasionally β-(1, 3)-glycosidic bonds and may be either a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars) (D fengel and Wegener, 1984). In comparison to cellulose; hemicelluloses have lower molecular weight and are easily hydrolyzed. Hemicelluloses is more reactive than cellulose, makes it attractive for heterogeneous catalytic processing which allows hemicellulose to be removed under milder reaction conditions (Saha, 2003). Hemicellulose is typically a polymer of C5 sugars, mainly xylose although its composition depends on the source of the lignocellulosic biomass. Hemicellulose differ from cellulose by the composition of sugar units, presence of shorter chains, branching of main chain molecules, and being amorphous (D fengel and Wegener, 1984), that makes it more hydrolysable than cellulose.

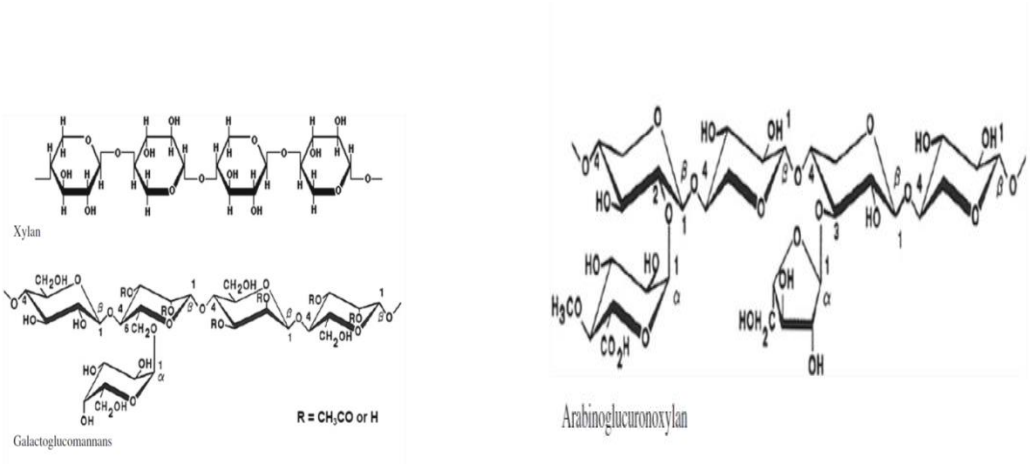


Figure 8: Structure of hemicellulose.

2.15.3 Lignin:

Lignin is a complex, large molecular structure is an amorphous polymer of phenolic monomer that varies in structure depending on the biomass source. Lignin makes up 15–30% of the biomass weight, is rich in aromatic functionality, and consists of three primary monomers. It is present in the primary cell wall, imparting structural support, impermeability, oxidative stress and resistance against microbial attack (Patil, Tanguy, & Yan, 2015). Lignin has a significant role in the coating industry since it is used to prepare phenolic, polyester, polyurethane, and epoxy resins. In general, herbaceous plants (like grasses) have the lowest contents of lignin, whereas the highest lignin content is present in softwoods (Hendriks and Zeeman, 2009). Lignin binds the cellulose and hemicelluloses together in the lignocellulosic biomass so it is generally accepted as the 'glue'. Three phenyl propionic alcohols that exist as monomers of lignin are as follows-

– Coniferyl alcohol (guaiacyl propanol)

– Coumaryl alcohol (p-hydroxyphenyl propanol)

– Sinapyl alcohol (syringyl alcohol) (Patil *et al.*, 2015)

Lignin present in the lignocellulosic biomass not only acts as a physical barrier for processing also has another harmful effect which includes:

– Nonspecific adsorption of hydrolytic enzymes to "sticky" lignin.

– Interference with and non-productive binding of cellulolytic enzymes to lignin-carbohydrates complexes.

– Toxicity of lignin derivatives to microorganisms (Chang and Holtzapple, 2000).

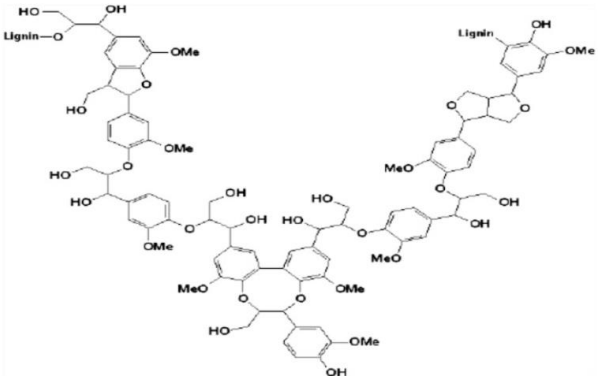


Figure 9: Structure of lignin.

2.16 Pretreatment:

The lignocellulosic biomass is subjected to the pretreatment for the conversion into fermentable sugars and biofuels (D. McMillan, 1994). Pretreatment is a crucial step in biofuel and bio-energy production. The goal of pretreatment is to make the cellulose accessible to hydrolysis for conversion to fuels. Thus, lignocellulosic biomass must be first subjected to the pretreatment before enzymatic hydrolysis because pretreatment step makes biomass more susceptible to saccharification and generate high yields of fermentable sugars (Scullin *et al.*, 2015).

Without any pretreatment, the conversion of native cellulose into sugar is extremely slow since cellulose is well protected by the matrix of lignin and hemicelluloses in microfibrils. The cellulose present in the lignocellulosic biomass is the main component that is hydrolyzed to the fermentable sugar but the hydrolysis of the cellulose is hindered by the presence of many physicochemical, structural and compositional factors in biomass because of hemicelluloses and lignin. These make accessibility of cellulose for enzyme difficult. Pretreatment fractionates the carbohydrate into monomeric sugar rapidly and with greater yield (Sha *et al.*, 2015).

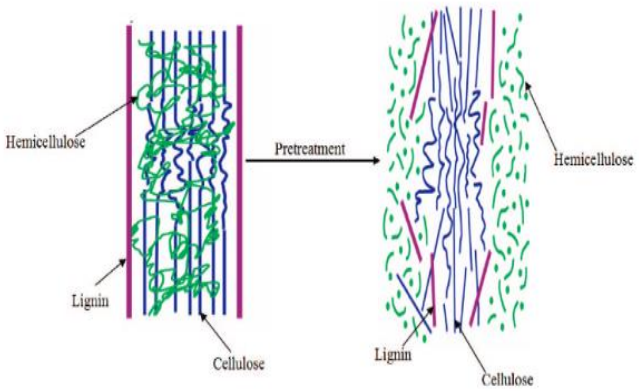


Figure 10: Schematic diagram of the effect of pretreatment on the lignocellulosic biomass (Ladisch *et al.,* 1979).

- **The lists of different pretreatment methods along with their operating conditions, advantages and disadvantages are shown in the table below:**

Table 2: Methods for the lignocellulosic biomass pretreatment (Kumar *et al*., 2009).

		Operating conditions	Advantages	Disadvantages
Physical	Chipping Grinding Milling	Room temperature Energy input < 30Kw per ton biomass	Reduces cellulose critallinity	Power consumption higher than inherent biomass energy
Physio-chemical	Steam pretreatment	160-260°C (0. 69-4.83MPa) for 5-15 min	Causes hemicellulose auto hydrolysis and lignin transformation; cost-effective for hardwoods and agricultural residues	Destruction of a portion of the xylan fraction; incomplete distruption of the lignin-carboydrate matrix; generation of inhibitory compounds; less effective for softwoods
	AFEX (Ammonia fiber explosion method)	90°C for 30 min.1-2kg ammonia /kg dry biomass	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose;
	ARP (Ammonia recycle percolation method)	150-170°C for 14 min Fluid velocity 1cm/min	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose;
	CO_2 explosion	4kg CO_2/kg fiber at 5.62 Mpa 160 bar for 90 min at 50 °C under supercritical carbon dioxide	Do not produce inhibitors for downstream processes. Increases accessible surface area, does not cause formation of inhibitory compounds	It is not suitable for biomass with high lignin content (such as woods and nut shells) Does not modify lignin neither hydrolyze hemicelluloses
	Ozonolysis	Room temperature	Reduce lignin content; does not produce toxic residues	Expensive for the ozone required;
	Wet oxidation	148-200°C for 30 min	Efficient removal of lignin; low formation of inhibitors; low energy demand	High cost of oxygen and alkaline catalyst
Chemical	Acid hydrolysis: dilute-acid pretreatment	Type I: T>160°, continuous-flow process for low solid loading 5-10%,)-Type II: T<160°C, batch process for high solid loadings	Hydrolyzes hemicellulose to xylose and other sugar; alters lignin structure	Equipment corrosion; formation of toxic substances

	Alkaline hydrolysis	Low temperature; Long time high. Concentration of the base; For soybean straw: ammonia liquor (10%) for 24 h at room temperature	Removes hemicelluloses and lignin; increases accessible surface area	Residual salts in biomass
	Organosolv	150-200 °C with or without addition of catalysts (oxalic, salicylic, acetylsalicylic acid)	Hydrolyzes lignin and hemicelluloses	High costs due to the solvents recovery
Biological		Several fungi (brown-, white- and soft-rot fungi	Degrades lignin and hemicelluloses; low energy requirements	Slow hydrolysis rates
Electrical	Pulsed electrical field in the range of 5-20 kV/cm,	~2000 pulses of 8 kV/cm	Ambient conditions; disrupts plant cells; simple equipment	Process needs more research

For the production of ethanol from lignocellulosic materials, first of all the bundle of complex structure should be opened so that the individual components are released from the process called pretreatment. It reduces the lignin content and allows the cellulose and hemicelluloses content for enzymatic hydrolysis. The second step is to break down the thus separated cellulose and hemicelluloses by enzymatic hydrolysis into the simpler sugar. The third step is the conversion of monomeric sugar (hexoses and pentoses) for ethanol solution (mash) production through microorganisms. The last step is the ethanol production and purification. Purification of the ethanol from mash is done by distillation and dehydration. By-product recovery, utilities (steam and electricity generation and cooling water), wastewater treatment, and eventually enzyme production are the other units which are demanded in ethanol production from lignocellulosic materials.

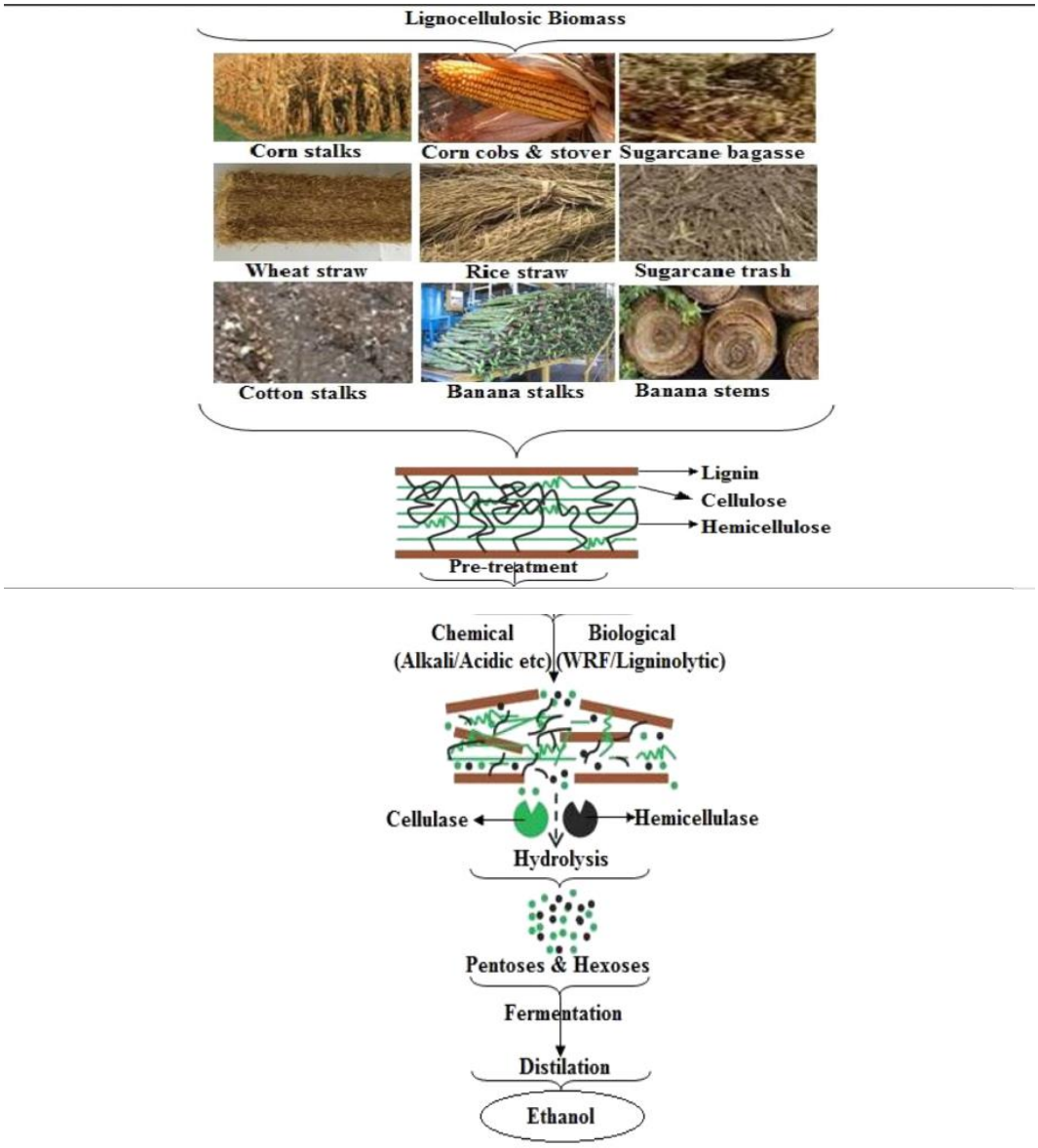


Figure 11: Generalized schematic representation of lignocellulosic materials bio-conversion into ethanol (Anwar *et al.,* 2014).

2.17 Enzymatic hydrolysis of lignocellulosic biomass:

During the production of ethanol from the lignocellulosic biomass, hydrolysis of the lignocellulosic biomass is the most important because the amount of glucose that is offered for the fermentation is dependent in it. Only the partial degradation of the cellulose, hemicelluloses and lignin is done by pretreatment; which expose the cellulose for further hydrolysis by the enzyme cellulose (F. and Shastri, 2016).

The process of converting of the biomass biopolymers to fermentable sugars is called hydrolysis. Mainly the two major categories of hydrolysis method are employed which are: acid hydrolysis (acid as a catalyst) and the second is enzymatic hydrolysis (cellulases enzyme is used). Cellulases are the enzyme which are highly specific catalysts. The

hydrolysis is performed under mild conditions (e.g. pH 4.5-5.0 and temperature 40-50°C). The cellulases are proteins and are conventionally divided into three major groups:

I. **Endoglucanase-** attacks low crystallinity regions in the cellulose fibers by endoaction, creating free chain-ends.

II. **Exoglucanases or cellobiohydrolases-** hydrolyze the 1, 4-glycocidyl linkages to form cellobiose (dimer of glucose) from the free chain-ends.

III. β- **glucosidase-** converts cellooligosaccharidesand disaccharide cellobiose into glucose residues (Verardi *et al*., 2012).

In addition to these major groups of cellulase enzymes, there are also a number of other enzymes that attack hemicelluloses, such as glucoronide, acetylesterase, xylanase, β-xylosidase, galactomannase and glucomannase. The use of enzymes in the hydrolysis of cellulose has advantages over the use of inorganic catalysts. This is because enzymes are highly specific and can work at mild process conditions. In spite of these advantages, enzymatic hydrolysis for the industrial process is still limited by several factors: most enzymes are relatively unstable at high temperatures, enzyme isolation and purification costs are high and it is quite difficult to recover them from the reaction mixtures (Walker and Wilson, 1991).

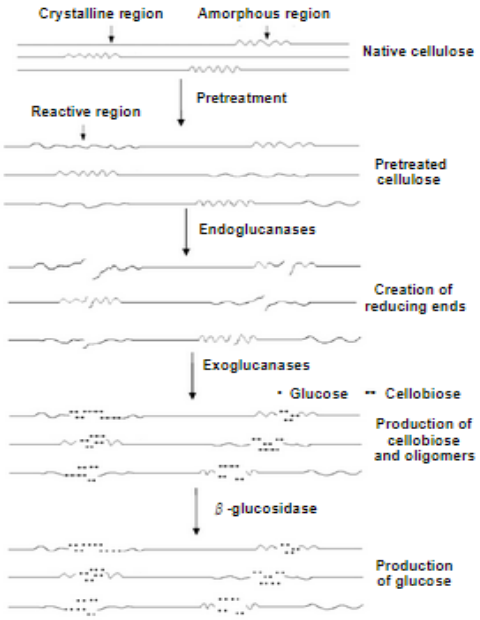


Figure 12: Schematic presentation of hydrolysis of cellulose to glucose by cellulolytic enzymes (Taherzadeh and karimi, 2007).

2.18 Acidic hydrolysis of lignocellulosic biomass:

One of the methods of hydrolysis of cellulose and hemicellulose is by using either concentrated or dilute acid. The sulphuric and the hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic residues. Apart from these acids phosphoric acid is also used for the process of hydrolysis. But in comparison to other acid, this acid is less aggressive so there is the high chance of production of the growth inhibitors of the microorganism such as furfural or acetic acids (Lenihan *et al*., 2010).

- **The advantages of the acid hydrolysis**
  - **I.** The acid can penetrate lignin without pretreatment.
  - **II.** The rate of acid hydrolysis is faster than enzyme hydrolysis
  - **III.** The acid hydrolyzes hemicellulose to xylose and other sugar and alters the structure of the lignin.
- **The disadvantage of the acid hydrolysis**
  - **I.** Equipment corrosion and formation of toxic substances (Jones and Semrau, 1984).
  - **II.** Requires large amount of the acids.

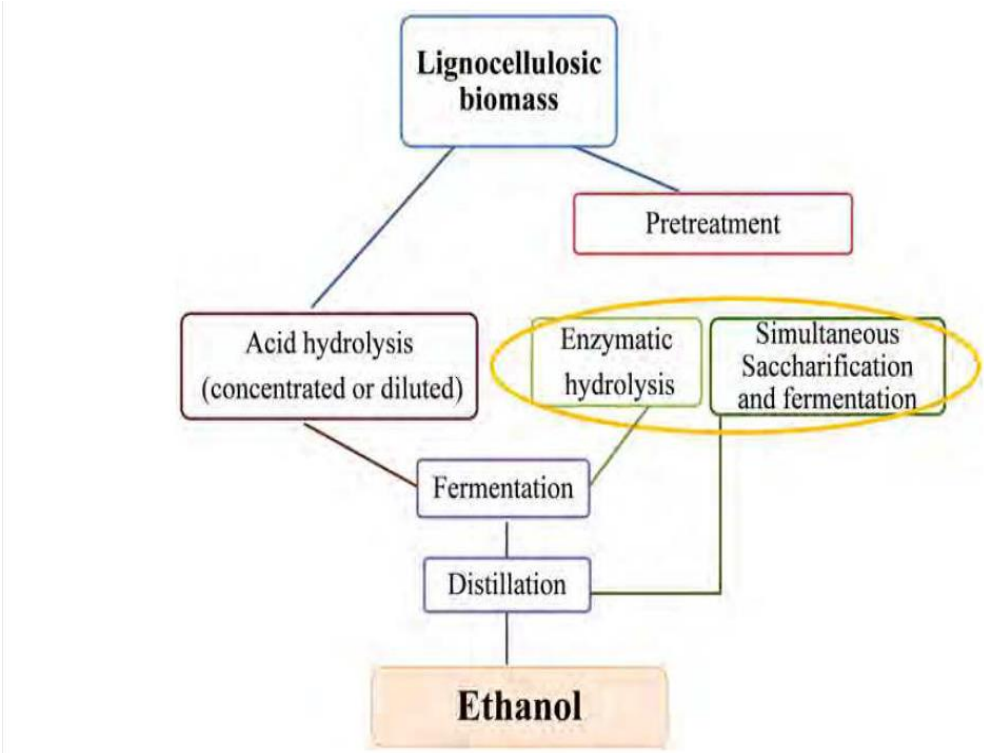


Figure 13: Process for production ethanol from lignocellulosic biomass.

The main advantage of the dilute hydrolysis process is the requirement of low amount of acid (2-5%). In order to achieve acceptable rates of cellulose conversion this process is

needed to be carried out at high temperatures. But the problem of performing at a high temperature is to increase in the rates of the hemicellulose sugars decomposition thus causing the formation of toxic compounds such as Furfural and 5-hydroxymethyl-furfural (HMF).

Although the hydrolysis by using dilute-acid is fast and easy to perform, it is hampered by non-selectivity and by-product formation (Fan *et al.*, 1982). Normally, dilute-acid hydrolysis is carried out using mineral acids such as H_2SO_4 and HCl, at temperatures between 120°C and 200°C (Torget and Teh-An, 1994). The ethanol yields obtained using dilute-acid hydrolysisand the fermentation is only 50±60% of the theoretical values (Wyman, 1994). Hydrolysis procedures which involve treatment of the lignocellulose at high temperature under acidic conditionslead to the formation and liberation of a range of compounds. The main degradation pathways are schematically presented in figure below.

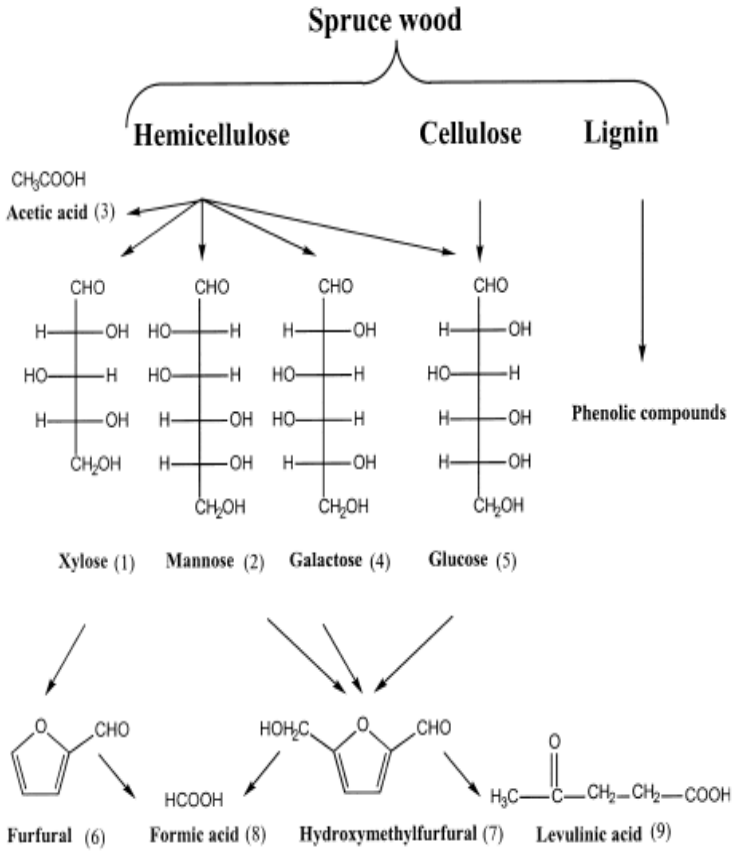


Figure 14: Reactions occurring during hydrolysis of the lignocellulosic materials.

The partial breakdown of the lignin gives the phenolic compound reported to be formed. Furfural, HMF and phenolic compounds are the inhibitor of the fermentation. These compounds inhibit yeast cells and the subsequent fermentation stage, reduction of

fermentable sugars causing a lower ethanol production rate (Larsson *et al.*, 1999). The decrease in the concentration of fermentable sugars coincided with the formation of furfural and HMF. Hydrolysis temperature, time and acid concentration are the factor that influence the generation of fermentation inhibitor. The low molecular weight phenolic compounds are the most toxic and have been suggested to exert a considerable inhibitory effect in the fermentation of the lignocellulosic hydrolysates (Palmqvistand Hahn-Hägerdal, 2000). The loss in integrity is caused by the partition of phenolic compounds into the biological membrane, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper *et al.*, 1994).

2.19 Saccharum spontaneum:

Saccharum spontaneum also known as wild sugarcane, or kans grass is a native to the Indian subcontinent. It is a perennial grass, free-tillering, often with aggressive rhizomes. It grows up to 8 meters in height, but normally within the range 2-3.5 meters.

Since it is very uneconomic and unsuitable alternative to produce fuel ethanol from value-added food and feedstock. So, the selection of cheap and carbohydrate rich raw material rather than food/feed value-based grains or juices is one of the sustainable ways for ethanol production. It is reported to produce the ethanol using cheap weed material-*Saccharum spontaneum* (wild sugarcane). It has ability to grow fast without aiding any economic input. It consists of 45.10 ± 0.35% cellulose and 22.75 ± 0.28% of hemicellulose on dry solid (DS) basis. The reducing sugar yield of aqueous ammonia pretreated delignified *Saccharum spontaneum* has been found to be 53.91 ± 0.44 g/L (539.10 ± 0.55 mg/g of substrate) (Chandel *et al*., 2009). Because of its extensive rhizome network, it is a very efficient binder of soils, and hence, particularly useful for soil erosion controller and preserver. It provides a good thatching material and also used in ropes, mats and broom production. Pulp is suitable for wrapping, writing, and printing and for production of grease-proof paper. It is extensively used in breeding the sugarcane to increase the sugar content.

Figure 15: *Saccharum spontaneum.*

Scientific classification

Kingdom: Plantae

Clade: Angiosperms

Clade: Monocots

Clade: Commelinids

Order: Poales

Family: Poaceae

Genus: *Saccharum*

Species: *spontaneum*

2.20 Carbon Catabolite Repression (CCR):

Every organism has their quest towards their preferred nutrient source. As for any organism, the supply of the nutrient is of prime importance for bacteria, too. The most common decision bacteria have to make is the choice between alternative carbon sources, each sustaining a specific, maximal growth rates. Generally, for many heterotrophic bacteria, glucose is the preferred (primary) source of carbon (Singh *et al*., 2008). In the presence of glucose source in the media the genes required for the utilization of secondary carbon sources are not expressed and preexisting enzymes are often inactivated so as to prevent the waste of resources. This phenomenon is referred to as carbon catabolite repression (CCR) (Kremling *et al*., 2015). So, CCR is a regulatory mechanism by which the expression of genes required for the utilization of secondary sources of carbon is prevented by the presence of a preferred substrate. Carbon catabolite control is specific to carbon source-mediated regulation (Brückner and Titgemeyer, 2002). A different mechanism of CCR is operative in Enterobacteriaceae (Escherichia, Salmonella) and Firmicutes (*Bacilli*, *Staphylococci*, and *Lactobacilli*). But in

both, the CCR is mediated via components of the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS), which transports and phosphorylates carbohydrates (Deutscher, 2008).

2.20.1 Carbon catabolite repression in *Bacillus subtilis*:

The global mechanism of CCR is mediated by the pleiotropic transcription factor CcpA (catabolite control protein A) (Fujita, 2009). The target site of CcpA to bind is the catabolite responsive elements (cre). The ability of CcpA to bind the cre is in turn controlled by the presence of its cofactors, HPr (histidine-containing protein) (Ser-P) and Crh (Ser-P) (Schumacher *et al*., 2006). In the presence of glucose, CcpA represses several hundred catabolic genes and activates the transcription of some genes of overflow. The glucose mediated catabolite repression is caused by binding of CcpA to the promoter regions of catabolic region. When glucose is present in the media, HPr, and presumably also Crh, is phosphorylated by the bifunctional HPr kinase/phosphorylase (HPrK/P) on Ser46, whereas in the cells growing in absence of sugar this site is less phosphorylated. The dephosphorylation of HPr (Ser-P) is catalyzed by the phosphorylase activity of HPrK. The HPrK is a bifunctional enzyme; its kinase activity is allosterically activated by ATP and fructose 1,6-bisphosphate (FBP), i.e., under conditions of good nutrient supply (Jault *et al*., 2000). The Only form of HPr (SerP) is able to productively interact with CcpA and to exert CCR, but none of the other HPr forms (Schumacher *et al*., 2004). Recently malate is considered as the second preferred carbon in *Bacillus subtilis* after glucose. The repression by malate is also exerted by the CcpA-HPr (Ser-P) complex. Similar mechanism as that in presence of glucose, the complex malate metabolism allows the accumulation of the regulatory metabolites ATP and FBP, these are sufficient to trigger the HPrK kinase activity and thus carbon catabolite repression (Meyer *et al*., 2011).

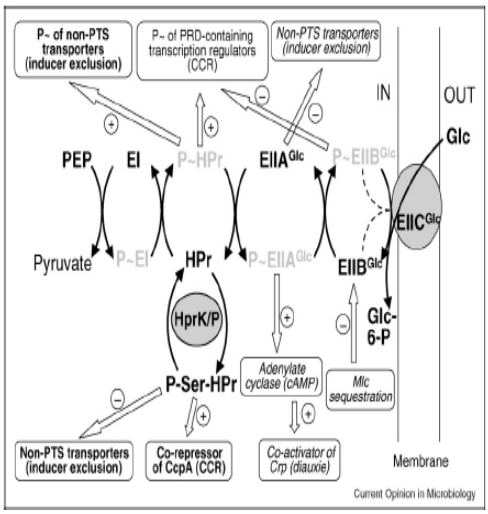


Figure 16: General overview of CCR in Enterobacteriaceae and Firmicutes (Deutscher, 2008)

CHAPTER 3
METHODS

3.1 Isolation of bacteria from the collected soil sample:

Soil samples were collected from the Panchase region of the Pokhara valley and stored at Central Department of Biotechnology, Tribhuvan University, Kirtipur. Soil samples from five different parts of Panchase region were taken for the isolation of the bacteria. Isolation procedure for the *Bacillus megaterium* and *Bacillus subtilis* were slightly different.

3.1.1 Isolation of *Bacillus megaterium*:

One gram of each soil sample were taken which were from 5 different parts of Panchase region. Soil samples were enriched in 5 ml of sodium acetate base media (in test-tube) to initiate the spore germination which utilize the acetate as a sole carbon source. The test-tubes were heat shocked at 80°C in water bath for 10 minutes to kill any vegetative cells present and 100 µl of above media containing soil was taken and mixed into 10 ml of soft agar (top agar). Top agar was poured immediately into the plate with basal acetate agar media (bottom agar) and incubated at 28°C for 48 hours. The single colonies were further screened to grow in to the L-tryptophan, catechol and phenol as a carbon source in the media. L-tryptophan is reported as a sole carbon source for *Bacillus megaterium*. The bacterial isolates survived in the phenol media were considered as putative *Bacillus megaterium*.

3.1.2 Isolation of *Bacillus Subtilis*:

One gram of each soil samples was taken from 5 different parts of parts of Panchase region. Soil sample was dissolved in 10 ml of water (in test-tube) and heat shocked at 80°C for 10 minutes in order to kill vegetative cells and non-spore forming bacteria. The samples were serially diluted up to 10^{-2} times and 100 µl of the solution was taken and spread with the help of spreader on Nutrient Agar (NA) plate and the plates were incubated at 28°C for 24 hours. The single colonies were further screened to grow in maleic acid, catechol and phenol as a carbon source in the media. Maleic acid is the sole carbon source for *Bacillus subtilis*. The bacterial isolates survived in the phenol media were considered as putative *Bacillus subtilis.*

3.2 Biochemical identification of bacteria:

3.2.1 Gram's staining:

For the Gram's staining, microorganisms were first inoculated and smeared on the sterile glass slides with the help of sterile inoculating loop. Smear was made in circular motion to about 1 cm in diameter, excess spreading may result in the disruption of cellular arrangement. The smear was allowed to air dry and then heat-fixed quickly. Primary stain i.e. Crystal violet was flooded over the smear on each slide and let stand for 1 minute. The slides were tilted slightly and gently washed with the distilled water. Dilute solution of iodine, a mordant i.e. Gram's iodine was flooded gently over the smear and let stand for 1 minute. The slides were gently washed with the distilled water and decolorization was done with an organic solvent, ethyl alcohol (95%) for 5-10 seconds. After washing with the distilled water, the slides were flooded with safranin to counter-stain and let stand for 45 seconds. Then the slides were washed with distilled water, allowed to dry and microscopic observations of the slides were performed.

3.2.2 Starch hydrolysis test:

NA plates were supplemented with 1% starch and the microbial cultures were inoculated by streaking on the agar plate using a sterile inoculating loop. The plates were incubated at 28°c for 24 hrs. Then the plates were flooded with Gram's iodine solution and a clear zone around the growth of bacterial colony indicated the starch utilization.

3.2.3 MR-VP test:

The microorganisms were aseptically inoculated into the 2 test tubes labeled as the MR and VP medium. The tubes were incubated at 28°c for 48 hrs. After that, 5-6 drops of MR reagent were added to the test tube labeled as MR and color change of the media was observed. Similarly, for VP, Barritt's reagent A and B in the ratio of 3:1 (α-Naphthol: KOH) were added in the tubes and shaken to provide the oxygen. The changes in color were noticed after 20-30 mins of incubation.

3.2.4 Citrate utilization test:

The names of microorganisms were labeled in the slant of Simmon's citrate agar tube. Then the microorganisms were aseptically inoculated on the surface of the medium in a zig-zag way and incubated at 28°c for 24-48 hrs and the color change in the medium were observed.

3.2.5 Catalase test:

Small inoculums of the microorganisms were mixed with a drop of 3% H_2O_2 using a sterile wooden stick (toothpick) and the effervescence marked by bubbles were observed.

3.2.6 Nitrate reduction test:

Nitrate broth was prepared and the microorganisms were inoculated into the respective labeled tubes. The tubes were incubated at 28°c for 24-48 hrs. After incubation, reagent A i.e. Sulfanilic acid and reagent B i.e. Naphthylamine; 5 drops each were added. Color changed were observed which indicated that the nitrate was reduced resulting in the pink-red color. To the tubes which were colorless even after the addition of the reagents, a small amount of Zinc powder was added and shaken vigorously and allowed to stand at room temperature for 10-15 mins. And for the medium which remained colorless even after the addition of Zinc powder the result was positive and the media which turned pink after the addition of the Zinc powder, the result was negative.

3.2.7 Gelatin hydrolysis test:

The media used was the nutrient broth to which 12% gelatin was added. The microorganisms were inoculated aseptically into the tubes containing the media. Then the tubes were incubated 28°C for 24 hrs. Liquefaction even after keeping the tubes at 4°c indicated the hydrolysis of gelatin.

3.2.8 Urease test:

For this test the media used was urea broth media. The broth media was inoculated with a loopful of test organisms in the test tubes. The tubes were incubated at 28°C for 24 hrs. A positive urease test was indicated by the change in media color from yellow to pink.

3.2.9 Oxidative-fermentative test (OF):

For this test the media was hugh-leifson media. The media was prepared in separated in different test tubes and labelled as “O” for oxidative and “F” for fermentative. Microorganisms were inoculated using sterile wire by stabbing straight. In order to maintain the anaerobic condition in the tube labeled as “F” small drop of heavy paraffin oil was added after the inoculation of microorganisms. Tubes were incubated at 28°C and change in media color from green to yellow was interpreted as positive test.

3.2.10 Sulphur Indole Motility test (SIM):

For this test the SIM media was prepared in the test tube and isolated colonies were inoculated with the help of sterile inoculating loop. Inoculation was done by stabbing the colonies at the center of the media at the depth of 1-2 inches. After the inoculation of the organisms the tube was incubated at 28°C for 24 hrs. After incubation the tubes were observed for the production of hydrogen sulfide (H_2S) gas, formation of indole and the motility. After the observation of H_2S production and motility; formation of indole was tested by adding the Kovacs regent (3 drops) at the surface of the media and development of pink to red color was interpreted as positive indole test.

- **Interpretation of the results:**

A positive H_2S test was denoted by a blackening of the media along the line of inoculation and a negative H_2S test was denoted by the absence of blackening.

- A positive motility test was indicated by a diffuse zone of growth flaring from the line of inoculation.
- A negative motility test was indicated by growth confined to the stab line.
- A positive test for indole was denoted when a pink to red color band was formed at the top of the media after addition of Kovacs reagent.
- A negative test for indole was denoted by the yellow color after the addition of the Kovacs reagent.

3.3 Molecular characterization of the Bacteria:

The screened and the identified bacteria based on their morphology and the biochemical characterization were further subjected to the molecular characterization to confirm the specific isolates. So, the genomic DNA extraction and PCR amplification were performed to confirm the specific bacteria.

3.3.1 Genomic DNA (gDNA) extraction:

The DNA of the useful bacterial colonies was extracted using various chemicals and enzymes. Cells were streaked on the LB agar plate and incubated overnight at 37°c.The single colony was isolated and inoculated in 2 ml of LB medium for 12 hours. From the overnight culture (1.5 ml) was transferred into the sterile eppendorf (EP) tube. Tube was centrifuged at 5000 rpm for 5 minutes at 4°c. Immediately, the supernatant was discarded by aspiration and remaining overnight culture (0.5 ml) was also added to the same tube containing the cell pellet and centrifuged again at 5,000 rpm for 5 minutes 4°c. Then supernatant was removed as much as possible without disturbing the pellet. Cell pellet was re-suspended in 450 µl of TE1 buffer (pH 7.5) by gentle pipetting. The solution was spitted into 2 fresh sterilized E.P tubes by transferring 225 µl of the above suspension to each tube. To each tube 180 µl of lysozyme (1mg/ml) was added. Both the tubes were incubated at 37°C for 30 minutes gently mixing the solution by inverting the tube every 5 minutes for proper cell lysis. Then, 45 µl of STEP solution was added in both tubes. Tubes were incubated for 45 minutes or until the solution became clean due to the cell lysis with gentle inversion in between the ice incubation period. Equal volume of chilled phenol (450 µl) was added and mixed by votexing. The mixture was centrifuged at 13,000 rpm for 10 minutes. The upper aqueous layer containing DNA was transferred to the fresh sterilized E.P tubes without carrying of lower organic phase. Again, equal volume of chilled phenol: chloroform: Isoamyl alcohol (25:24:1) was added to the above aqueous solution mixed by vortexing. The tubes were centrifuged at 13,000 rpm for 10 minutes at 4°C. After

collection of aqueous layers in a fresh tube, equal volume of chloroform was added and vortexed. The mixture was then centrifuged at 13,000 rpm for 2 minutes and aqueous phase was collected in a fresh E.P tube. To the aqueous solution (450 µl) containing genomic DNA, 100 µl of 3 M chilled sodium acetate (pH 5.2) and double volume of 95% ethanol (i.e. 1,100 µl) was added. Tubes were incubated at -20°C for 30 minutes. The mixture was centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was poured off and pellet was washed with 250 µl of 70% ethanol without disturbing the pellet. The tubes were centrifuged at 13,000 rpm for 10 minutes at 4°C. After draining the supernatant, remaining ethanol was removed by keeping the tubes open in room temperature for 5-10 minutes. Care was taken so as not to over-dry the DNA pellet. The genomic DNA was re-suspended in 100 µl of autoclaved MilliQ water or TE buffer (pH 8.0) and stored at -20°C until use or gel run.

3.3.2 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction (PCR) is a method of exponential amplification of single copy of specific segment of DNA to generate thousands to millions of more copies of that particular DNA segment. In PCR, the reaction is repeatedly cycled through a series of temperature changes, which allow many copies of target region to be produced. The thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions- specifically DNA melting and enzyme- driven DNA replication. PCR relies on a thermostable DNA polymerase, and requires DNA primers which serve as the starting point for DNA synthesis.

The first step is the DNA denaturation. During this step the hydrogen bond holding the two stands of DNA is denatured. This provides single-stranded template for the next step i.e. annealing. In the second step (annealing) the reaction temperature is lowered and the primers bind to the complementary sequences on the single-stranded DNA template for thermo-stable DNA polymerase to amplify from the 3'-end of the primer. Since two primers are used; one is forward and another is the reverse primer which binds the sense strand and antisense strand respectively. The two strands of DNA become template for DNA polymerase to enzymatically assemble a new DNA strand. The final step is the extension where the temperature is raised to optimize extension by the polymerase. And the continuous cycle of heating and cooling take place until sufficient amount of DNA is synthesized.

3.4 Acid-treatment of lignocellulosic biomass (*Saccharum spontaneum*):

The crude extract of *Saccharum spontaneum* was provided, 6 grams was weighed and dissolved in 6 ml 72% H_2SO_4 in a tube which was neutral towards the acid. Then the tube

was incubated in the water bath at 40°C with continuous stirring in an interval of 5 minutes for the fast action of acid towards the biomass. After an hour the acid hydrolyzed product was transferred from tube to the reagent bottle and volume was diluted and maintained 100 ml and autoclaved. After autoclave, it was filtered using filter paper and filtrate (sludge) was collected and used for the media as carbon source to grow the bacteria and to perform the DNS (reducing sugar test), Phenol and Furfural test.

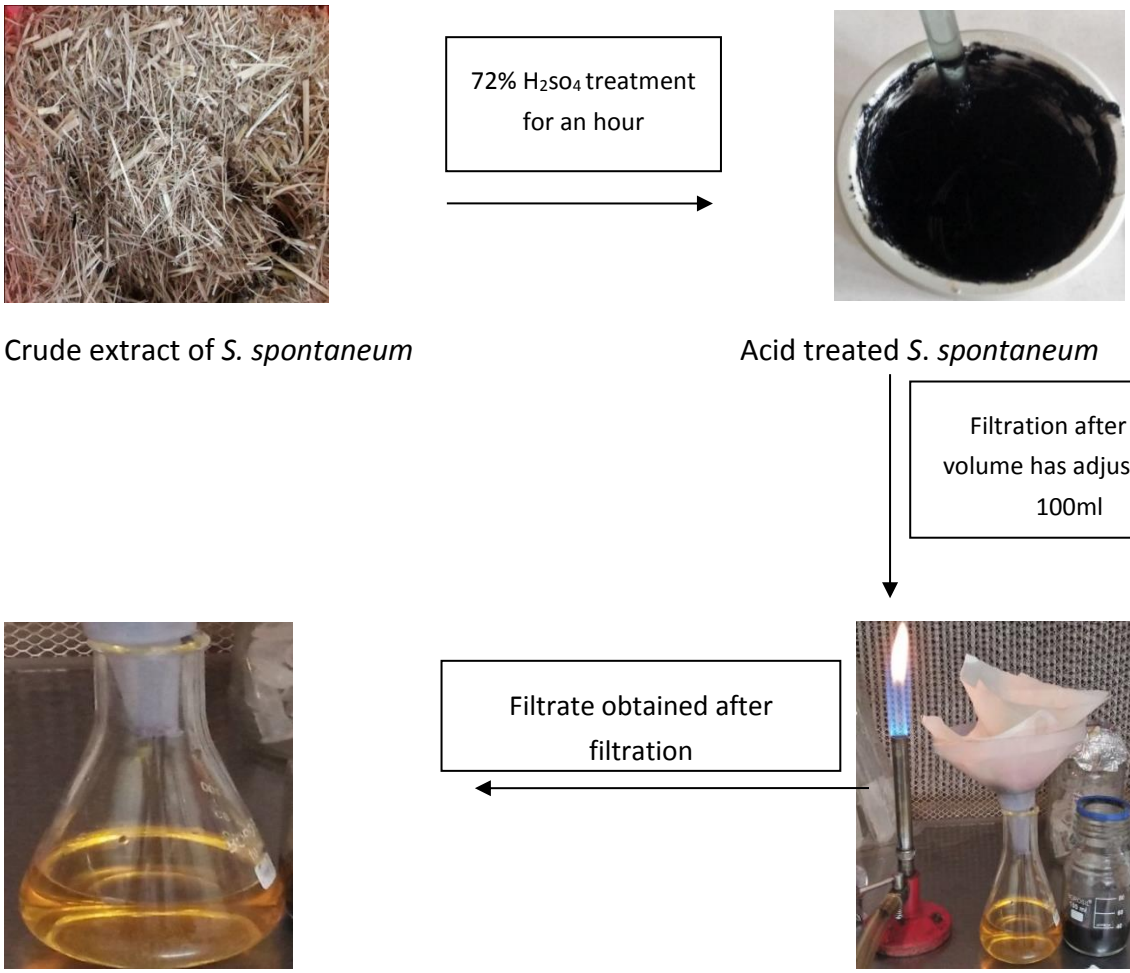


Figure 17: Different steps involved in acidic pretreatment of *Saccharum spontaneum.*

3.4.1 Reducing sugar test:

In this test to each 1 ml of sample, 1 ml of Dinitrosalicylic acid (DNS) reagent was added in the test tube. Test tubes were allowed to boil in beaker for about 5 minutes. After the tubes were cooled to the room temperature, 300 µl of 40% Rochellels salt was added and mixed by vortexing for few seconds and absorbance was taken at 540 nm.

3.4.2 Phenol test:

The phenolic compounds present in the lignocellulosic biomass were determined. For this test to each 0.1 ml of samples, 1 ml of FolinCiocalteu (FC) reagent (1/10-fold dilution, should be freshly prepared) and 0.8 ml of Sodium carbonate (1 M) was added. The mixture in the test tube was incubated for 15 minutes at the room temperature and absorbance was taken at 765 nm.

3.4.3 Furfural test:

For the determination of the furfural present in the lignocellulosic biomass this test was performed. For this test, 0.1 ml of sample was taken to which 0.4 ml of 50% ethanol, 20 µl aniline, 5 µl 37% HCl and again 800 µl 50% ethanol was added. Finally, absorbance was taken at 530 nm. Presence of pink color indicated the positive furfural test.

3.5 Nanohydroxyapatite (nHA) preparation from bovine bone:

Nanohydroxyapatite particle was prepared from the raw buffalo bone. For this the femur part was taken. The raw bone was first boiled in water for 6 hrs. to remove the bone marrow and tendons. After boiling it was washed with acetone. Then it was dried in oven for 48 hrs. at 160°C for the complete removal of water present. The dried bone was broken into smaller fragments mechanically by hitting. Further smaller powdery form was obtained by subjecting into the grinding mill. Sieving was done by using sieve and the powder sized less than 450 µm was taken. The powder was washed several times by distilled water in the beaker. The bone powder was subjected to the oven for dry at 80°C (overnight). Then the dried bone powder was treated with 4 M NaOH (1:40 wt./vol of bone powder and NaOH) at 250°C for 5 hrs. The alkali treated bone powder was filtered several times to bring toward the neutral pH. Filtration was done and dried at 100°C to remove the water particles. The deproteinized and defatted bone powder was then subjected to the calcination at 650°C and 900°C. Thus, the HA nanoparticle was synthesized. The characterization of thus synthesized HA was done by XRD.

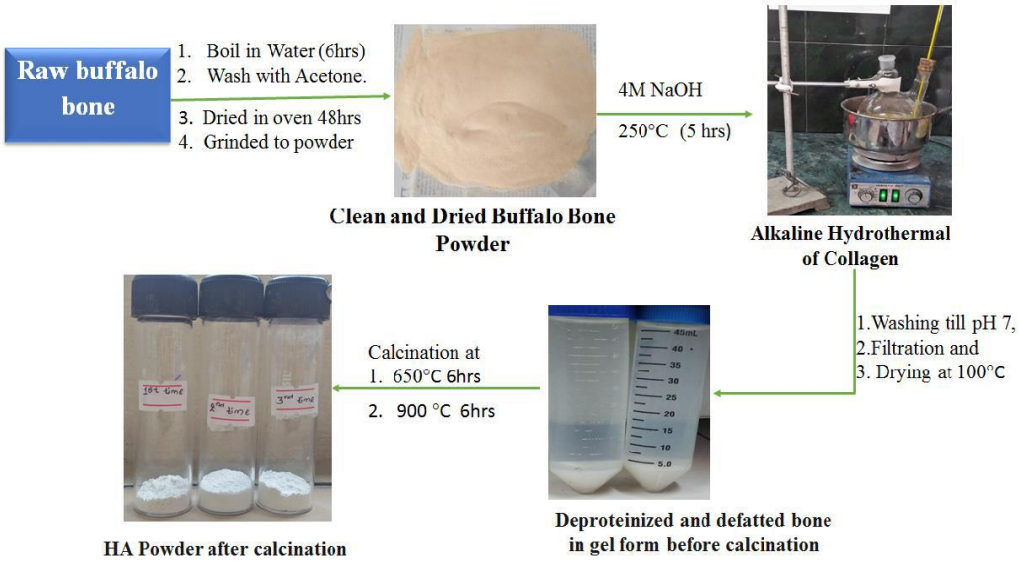


Figure 18: Synthesis of nano-hydroxyapatite (nHA) from buffalo bone by alkaline hydrothermal hydrolysis method.

3.6 Quantification of soluble phosphate in the media:

Soluble phosphate was quantified in the bacterial growth media by 'A single solution reagent method'.

Reagents used were:

- Sulfuric acid (5 N)
- Ammonium molybdate (4%)
- Ascorbic acid (0.1M)

Mixed reagent was prepared an hour before use and absorbance measured at 827 nm.

Soluble phosphate solubilized by thus isolated bacteria was determined:

For this, to each ml of samples, 1.25 ml of reagent mix was added in the test tube and the let them stand for 24 hrs. at room temperature after 24 hrs. spectrophotometric analysis was done at 827 nm.

CHAPTER 4
RESULT AND DISCUSSION

4.1 Isolation, screening and identification of *Bacillus megaterium:*

Soil samples collected earlier from Panchase region of Pokhara valley and stored at Central Department of Biotechnology, Tribhuvan University, Kirtipur were used to isolate the bacteria. Soil samples were mainly collected from the rhizospheric part because considerably higher amounts of P solubilizing bacteria are found commonly in rhizosphere in comparison with nonrhizosphere soil (Raghu and MacRae, 1966). Five different soil samples were taken for the isolation of the *Bacillus megaterium*. Putative strain of *Bacillus megaterium* were isolated screened and identified based on morphology, biochemical assay, and PCR and Sequence analysis.

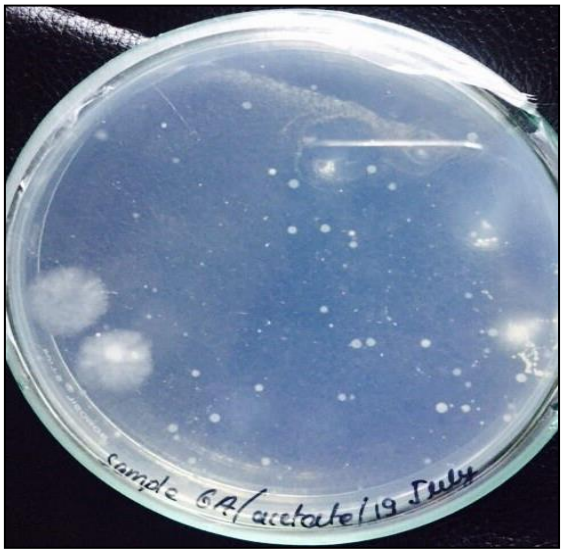


Figure 19: Culture of bacteria on Sodium acetate agar media.

The soil sample was serially diluted in sterilized water and as *Bacillus megaterium* is spore forming, (Scholle *et al.,* 2003) the soil samples were first exposed to pasteurization to kill any vegetative cells. In addition, *Bacillus megaterium* is known to utilize different carbon sources. *Bacillus megaterium* has been reported to harbor gene that can utilize acetate (Williams *et al*., 2012), thus the pasteurized soil sample was mixed in top agar containing acetate as sole reduced carbon sources. This was done to prevent growth of other spore forming organisms which utilize other reduced carbon sources but find acetate toxic. The top agar was then poured in basal agar media in the Petri disc that also contained acetate as sole reduced carbon source to screen for the colonies.

Among the colonies arising in the agar medium, it was presumed that within the fast-

growing colonies there would be *Bacillus megaterium* in sodium acetate agar media (Figure 19) since *Bacillus megaterium* is fast grower (Bergey *et al*., 2009) and the size of the bacteria is bigger thus giving bigger colonies. Acetate as sole reduced carbon source was chosen for the initial screening process because acetate acts as a stress for certain group of bacteria (Shiloach and Rinas, 2009) and also those which can't use acetate as a carbon source will die and make the screening process much easier. Because, it has been reported that *Escherichia coli* exhibits decreased growth rate and specific productivities at acetate concentrations lower than 5 g/L. Moreover, acetic acid tolerant bacteria remain active at acetate levels well over 40 g/L (Lasko *et al.,* 1997) because these are naturally resistant to the detrimental effects of acetate in their surroundings. *B*acillus *megaterium* utilize the acetate as a reduced carbon source and induces spore germination (Greene' and Slepecky, 2019). Moreover, *Bacillus megaterium* spores excrete acetate during germination (Setlow, 2006) thus it was presumed that providing acetate in the medium would facilitate spore germination and allow the cells to grow earlier than other organisms. In addition, some of the *Bacillus megaterium* are known to produce acetate in certain growth conditions (Freedman *et al*., 2018).

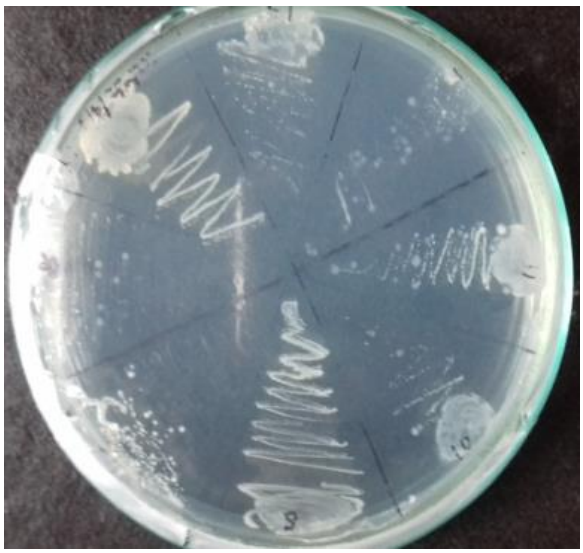


Figure 20: Culture of bacteria from sodium acetate agar media on L-tryptophan media.

The isolates selected from acetate media were then further cultured in the L-tryptophan based media because it has been reported that *Bacillus* megaterium could grow in L-tryptophan as sole source of reduced carbon (Bouknight and Sadoff, 1975). Among 40 colonies sub-cultured in L-tryptophan containing media 33 isolates survived (Figure 20). Thus, it is presumed that those colonies that did not survive in L-tryptophan containing media could have been other spore formers that utilize acetate as sole carbon source. Thus, the surviving isolates in L-tryptophan were presumed to be putative *Bacillus megaterium* strains.

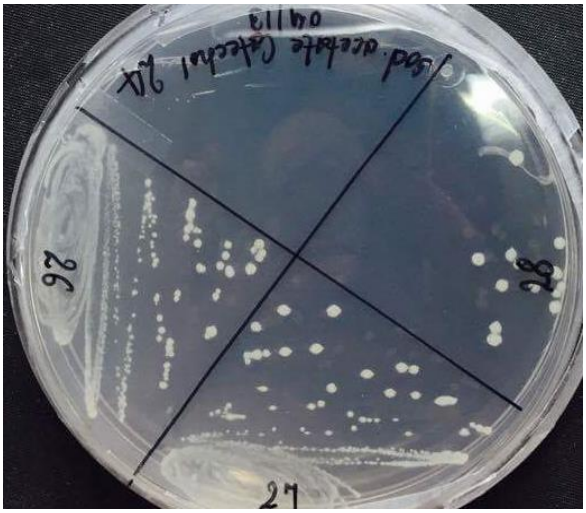


Figure 21: Culture of bacteria from tryptophan media on catechol media.

As mentioned above, *Bacillus megaterium* can utilize different reduced carbon sources including catechol and phenol, the isolated colonies from tryptophan media were cultured further in the media which contained catechol as a reduced carbon source. Those isolates that survived in catechol containing media were subjected further screening in phenol containing media. Thus, the isolated colonies were cultured in the media containing phenol as a reduced carbon source (Briggs, 1966). The isolates able to survive in these media were presumed as *Bacillus megaterium* (Figure 22). From different 5 soil samples in total of 33 isolates were primarily screened to be putative *Bacillus megaterium* strains which were then further subjected to other screening processes.

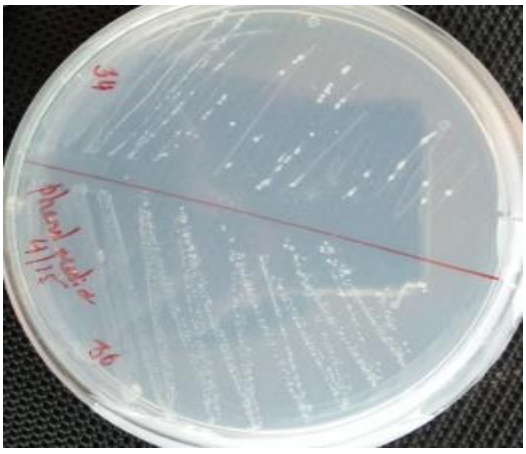


Figure 22: Culture of bacteria from catechol media on phenol media.

4.1.1 Morphological analysis:

Morphological analysis was done through Gram's staining and microscopic analysis. Gram staining is one of the most common, important, and most used differential staining techniques mostly used in microbiology. It allows the differentiation of bacterial species into two large groups: Gram's-positive and Gram's-negative bacteria on the basis of differential staining with a crystal violet-iodine complex and a safranin counter stain (Coico, 2005). The 33 isolates were subjected to Gram's staining and 9 isolates were found to be Gram's positive and were rods (Figure 23). Remaining 24 isolates were discarded as *Bacillus megaterium* is known to be Gram's positive and rods (Brown and Hodges, 1974).

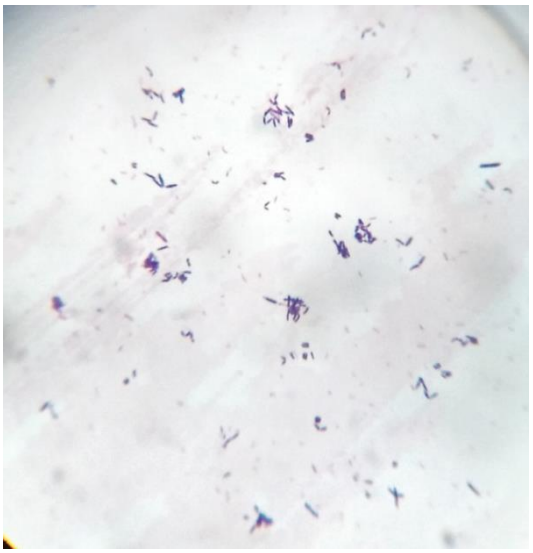


Figure 23: Gram-positive rod-shaped *Bacillus megaterium.*

For those bacteria that exhibited Gram's positive nature in staining were selected for further screening through different biochemical tests and those which were found to be gram negative were discarded. Thus, all together 12 different biochemical tests were performed and the isolates having the same biochemical tests to the preferred bacteria were further subjected to molecular tests.

Table 3: Results of different biochemical tests for putative *Bacillus megaterium.*

SN	Tests	S13	S15	S19	S20	S23	S27	S28	S31	S32
1.	Gram's Staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Morphology	Rod	rod	Rod	rod	rod	rod	rod	Rod	Rod
3.	Starch hydrolysis	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
4.	MR	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve
5.	VP	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6.	Citrate Utilization	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
7.	Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
8.	Nitrate Reduction	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve
9.	Gelatin hydrolysis	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve
10.	Urease	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
11.	OF	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve
12.	Sulphur	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
13.	Indole	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve
14.	Motility	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve

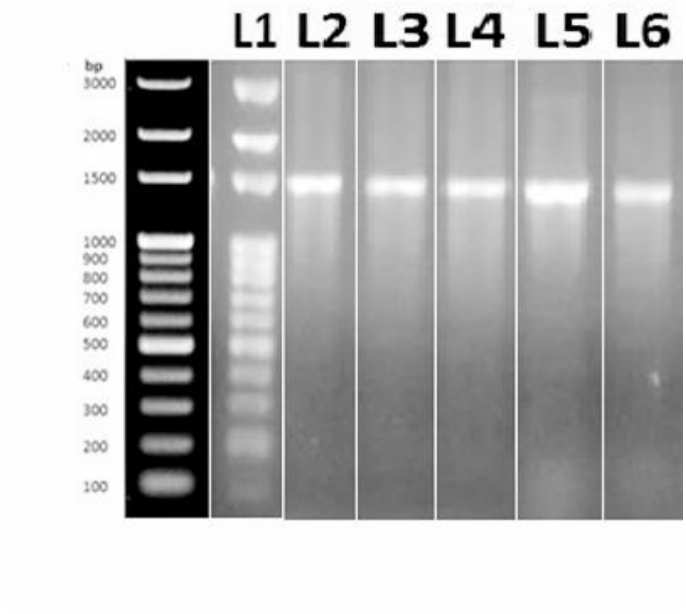
Bacillus megaterium is reported to be Gram's positive, rod shaped and spore former. It shows positive test for catalase, citrate and negative test for indole, MR. Thus, those colonies that exhibited these characteristics (Table 3) were taken as putative *Bacillus megaterium*. Mainly isolates 13, 15, 19, 20, 23, 27, 28, 31 and 32 were subjected to molecular characterization.

4.1.2 Molecular characterization of isolated *Bacillus megaterium:*

For the molecular characterization of the putative *Bacillus megaterium*; genomic DNA (gDNA) was extracted and subjected to PCR.

4.1.3 PCR amplification of *Bacillus megaterium* genomic DNA by universal 16srRNA primer:

The genomic DNA of all the positive gram stained bacteria were extracted. The PCR amplification of genomic DNA was performed using universal 16s rRNA genomic region amplifying primers to confirm whether the isolates were bacteria or not. PCR was performed using the 16s primers on those 9 isolates and were found to be positive for 5 samples only. A ladder of 100 bp was used and the amplified product had the size of 1500 bp (Figure 24). This proved that these isolates were bacteria and putative *Bacillus megaterium*.



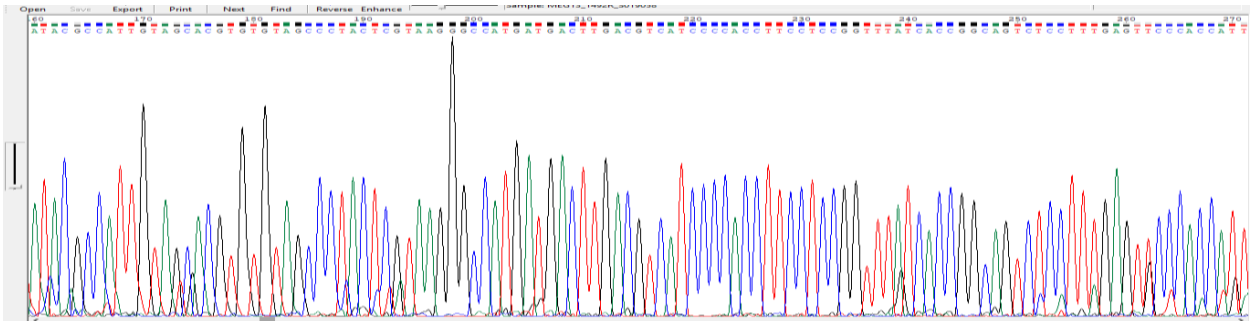
L1=100 bp ladder;
L2= Amplicon from S13;
L3= Amplicon from S15;
L4= Amplicon from S23;
L5= Amplicon from S28;
L6= Amplicon from S32.

Figure 24: PCR amplification of *Bacillus megaterium* genomic DNA by universal 16s rRNA primer. Ladder used is of GeneRuler^{Tm} 100 bp which is supplied with 6X loading Dye on 1% agarose gel electrophoresis.

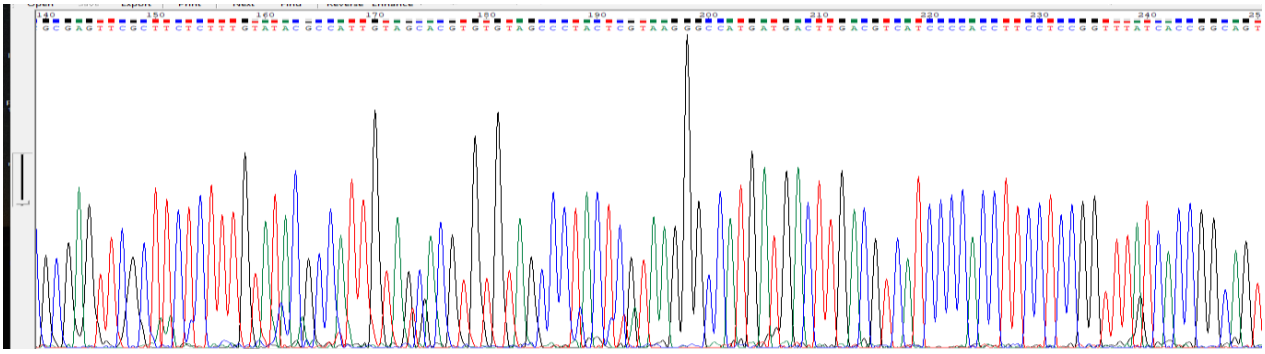
4.1.4 DNA sequencing analysis:

DNA sequence data answers many types of question since DNA sequences differ considerably between species and between individuals within a species. DNA sequences are extensively used for identification and was used for the same purpose here as well.

The PCR product of the isolates were further characterized by DNA sequence analysis to confirm the bacterial species. The sequence read obtained showed maximum resemblance with the presumed respective bacteria. The sample S13 showed 95% with the gene cluster sequence of *Bacillus megaterium* available from gene bank database. The chromatogram obtained from sequencing are shown in the figure below:



(A)



(B)

Figure 25: Chromatogram of sample S13 (A) for forward primer sequence (B) for reverse primer sequence.

4.1.5 Multiple sequence alignment:

The sequence obtained by DNA sequencing was further characterized by multiple sequence alignment. The highly similar sequence obtained in BLAST search indicate a significant alignment of the putative DNA sequence to 16s rRNA genes of *Bacillus megaterium.* The cluster approach called neighbor joining (NJ) method was used for the reconstruction of phylogenetic trees that yielded unrooted tree with branch lengths as follows:

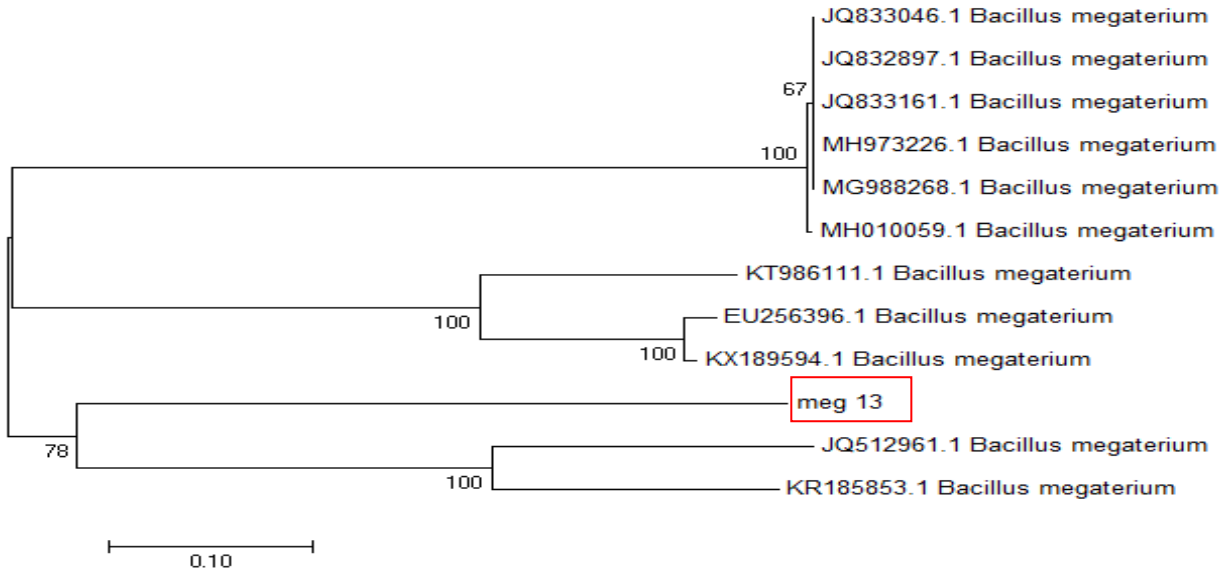


Figure 26: 16s rRNA sequence tree generated with the neighbor joining method with 1000 bootstrap resampling for *Bacillus megaterium* 13.

According to the 16s rRNA phylogenetic tree (Figure 26), S13 has a close relationship with *Bacillus megaterium*. The tree shows that the 16s rRNA sequence of this organism is very similar to *Bacillus megaterium*16s rRNA sequences.

The bootstrap results in common interprets the probability that phylogenetic estimation represents the true phylogeny. Explicitly, under various conditions such as equal rates of change, symmetric phylogenies, and internodal change of <20% of the characters, bootstrap proportions of >70% usually correspond to a probability of >95% that the corresponding clade is real. Therefore, S13 showed the identical similarities with *Bacillus megaterium.*

4.2 Isolation, screening and identification of *Bacillus subtilis*:

The phosphate solubilizing *Bacillus subtilis* were also isolated and screened from soil samples collected from Panchase region of near Pokhara valley that were stored at Central Department of Biotechnology, Tribhuvan University, Kirtipur. Five different soil samples were taken for the isolaton of the *Bacillus subtilis*. Putative strain of *Bacillus subtilis* were isolated screened and identified based on morphology, biochemical assay, and PCR and DNA sequence analysis based molecular techniques.

Figure 27 (a) NA plate

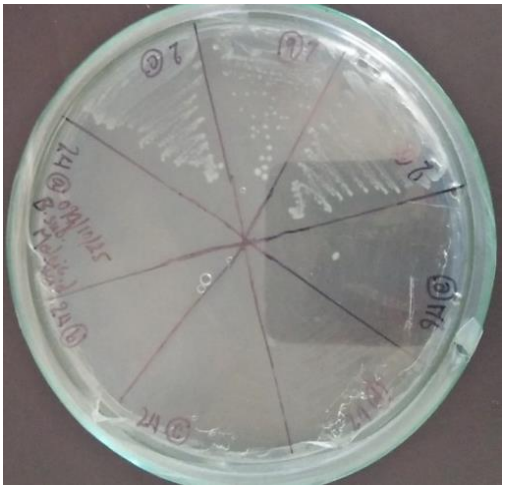


Figure 27 (b) Maleic acid plate

Figure 27: Culture of bacteria on Nutrient Agar (NA) plate and subsequent survival in maleic acid plates.

Soil sample was dissolved in 10 ml distilled water in the test tubes and tubes were subjected to pasteurization as described for *Bacillus megaterium*. The soil sample was serially diluted up to 2-fold. After that the soil solution (100 µl) was spread in the Petri plate containing Nutrient agar for faster bacterial growth.

The colonies surviving in the NA media [Figure 27 (a)] were then subsequently cultured into the media containing maleic acid as a sole source of carbon. It is reported that maleic acid can be used as the sole carbon source by *Bacillus subtilis.* Thus, it is presumed that those colonies that did not survive in maleic acid could have been other spore former that cannot utilize this as the sole carbon source. Among the randomly picked 50 colonies that were sub-cultured in the maleic acid media, only 30 isolates survived [Figure 27 (b)]. Pasteurization is common technique to kill vegetative cells thus based on this it was presumed that all the colonies arising in NA media plates were spore formers and maleic acid utilization is specific to *Bacillus subtilis* among different *Bacillus* group it was presumed that these 30 colonies were putative *Bacillus subtilis* and subjected to morphological analysis.

4.2.1 Morphological analysis:

Morphological analysis of putative strain of *Bacillus subtilis* was done through Gram's staining and microscopic analysis. Among the 30 isolates subjected to Gram's staining only 17 isolates were found to be Gram's positive and rods (Figure 28). Remaining 13 isolates were discarded as *Bacillus subtilis* is known to be Gram's positive and rods (Borriss *et al*., 2017). This clearly indicated that there could be spore forming organism that could utilize

maleic acid as sole carbon source and grow. Thus, it was assumed that further screening should be done to screen *Bacillus subtilis.*

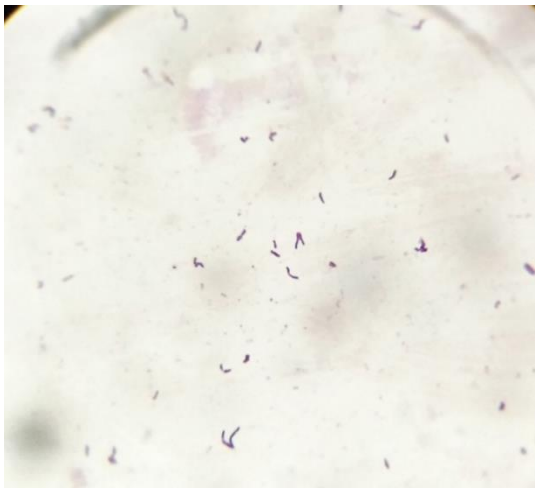


Figure 28: Gram-positive rod-shaped *Bacillus subtilis.*

4.2.2 Screening of *Bacillus subtilis* based on reduced carbon source utilization:

As mentioned above for *Bacillus megaterium*, *Bacillus subtilis* can also utilize different reduced carbon sources including catechol and phenol (Tam *et al.,* 2006). The isolated colonies from maleic acid media were cultured further in the media which contained catechol as a reduced carbon source. Those isolates that survived in catechol containing media were subjected further screening in phenol containing media. Thus, the isolated colonies were cultured in the media containing phenol as a reduced carbon source. The isolates able to survive in these media were presumed as *Bacillus subtilis* [Figure 29(b)]

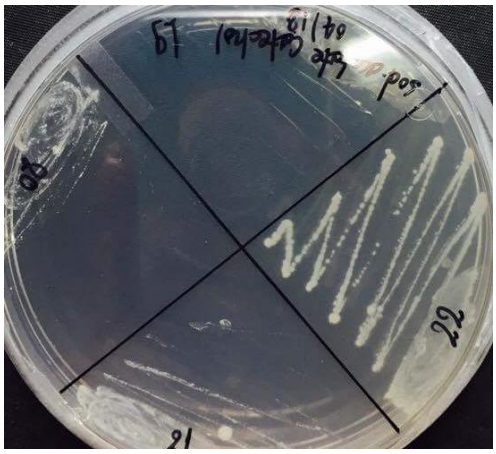


Figure 29 (a) Catechol plate

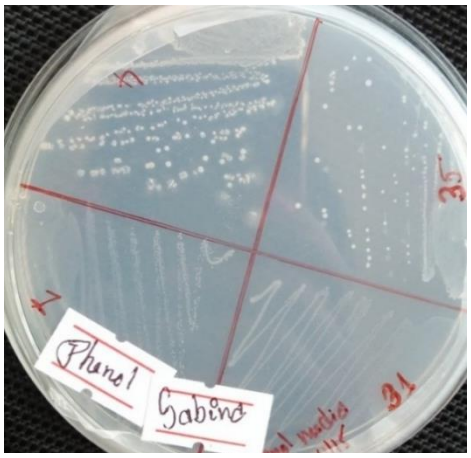


Figure 29 (b) Phenol plate

Figure 29: Culture of bacteria surviving in maleic acid on catechol containing media and subsequently in phenol containing media.

From the 5 soil samples in total of 30 isolates were primarily screened to be putative *Bacillus subtilis* based on maleic acid utilization and they were then further subjected to other screening processes. As screened from Gram staining the reduced carbon source selection process also gave total of 11 colonies that survived in all the screening media. Thus, it was presumed that these colonies were to be putative *Bacillus subtilis* isolates. The basic difference between the screening process among *Bacillus megaterium* and *Bacillus subtilis* has been selection of primary screening reduced carbon source, L-tryptophan and maleic acid, respectively for the respective organism. Thus, it is suggested that use of different reduced carbon source to differentiate between the bacteria could be handy and cost-effective process when screening for new organism from samples that could harbor different organisms.

4.2.3 Screening based on biochemical tests for putative screened *Bacillus subtilis:*

Those bacteria that exhibited Gram's positive nature in staining and survived in different screening media were then subjected for further screening through different biochemical tests and those exhibiting Gram's negative nature were discarded. All together 12 different biochemical tests were performed.

Table 4: Results of different biochemical tests for putative *Bacillus subtilis.*

SN	Tests	S1	S2	S4	S8	S9	S11	S12	S16	S21	S24	S30
1.	Gram's staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Morphology	rod	Rod	rod	rod	rod	rod	Rod	rod	rod	rod	Rod
3.	Starch hydrolysis	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4.	MR	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
5.	VP	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6.	Citrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
7.	Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
8.	Nitrate reduction	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
9.	Gelatin hydrolysis	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve
10.	Urease	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
11.	OF	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
12.	Sulphur	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
13.	Indole	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
14.	Motility	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve

Bacillus subtilis is reported to be rod shaped, Gram's positive and spore former. It gives positive test for catalase, citrate and negative test for indole, MR. Although difference was found specially in citrate reduction test (Table 4) but other characteristics were similar thus they were taken as putative *Bacillus subtilis*. These isolates were subjected to molecular characterization.

4.2.4 Molecular characterization of isolated *Bacillus* subtilis:

For the molecular characterization of the putative *Bacillus subtilis*; genomic DNA (gDNA) was extracted and subjected to PCR. The genomic DNA of all the Gram-positive stained bacteria were extracted. The PCR amplification of genomic DNA was performed using universal 16s rRNA genomic region amplifying primers to confirm whether the isolates were bacteria or not.

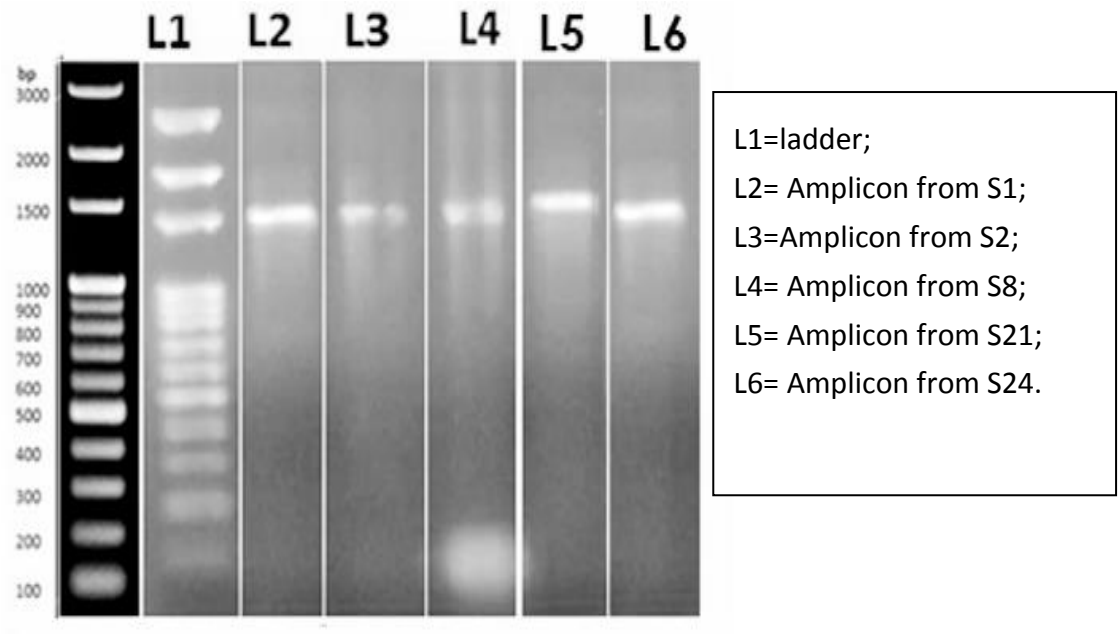


Figure 30: PCR amplification of *Bacillus subtilis* by 16s rRNA universal primer. Ladder used is of GeneRuler^{Tm} 100 bp which is supplied with 6X loading Dye.

PCR was performed using the 16s rRNA genomic DNA amplification universal primers on those isolates. A ladder of 100 bp was used and the amplified product had the size of 1500 bp (Figure 30). This suggested that these isolates were bacteria and putative *Bacillus subtilis*. The main objective of the research was to isolate *Bacillus subtilis* strain that could solubilize insoluble phosphate, mainly HA, as organic manure when applied in soil converts into HA (Li *et al*., 2015) also these isolates were subjected to HA solubilization test and also to observe their role in degradation of fermentation inhibitors.

4.2.5 Multiple sequence alignment:

The sequence obtained by DNA sequencing was further characterized by multiple sequence alignment. The highly similar sequence obtained in BLAST search indicate a significant alignment of the putative DNA sequence to 16s rRNA genes of *Bacillus subtilis.* The cluster approach called neighbor joining (NJ) method was used for the reconstruction of phylogenetic trees that yielded unrooted tree with branch lengths as follows:

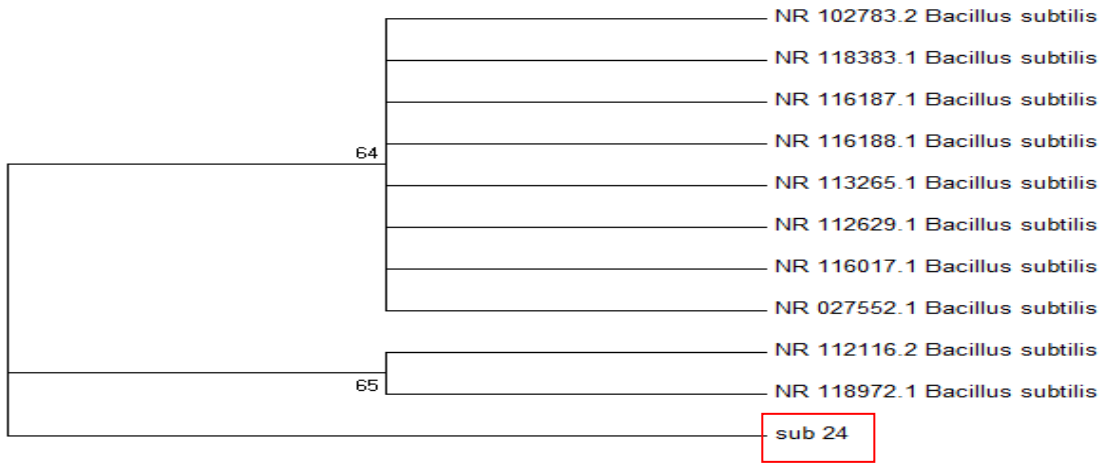


Figure 31: 16s rRNA sequence tree generated with the neighbor joining method with 1000 bootstrap resampling for *Bacillus subtilis 24.*

According to the 16s rRNA phylogenetic tree (Figure 31), S24 has a close relationship with *Bacillus subtilis*. The tree shows that the 16s rRNA sequence of this organism is very similar to *Bacillus subtilis* 16s rRNA sequences.

The bootstrap results in common interprets the probability that phylogenetic estimation represents the true phylogeny. Explicitly, under various conditions such as equal rates of change, symmetric phylogenies, and internodal change of <20% of the characters, bootstrap proportions of >70% usually correspond to a probability of >95% that the corresponding clade is real. Therefore, S24 showed the identical similarities with *Bacillus subtilis*

4.3 Salt tolerance assay:

The 2 utmost threat affecting agricultural areas are the soil degradation and salinization. The problem associated to the of low productivity of saline soils may be ascribed not only to their salt toxicity or damage caused by excess amounts of soluble salts but also arising from the lack of organic matter (Lakhdar *et al*., 2009). Nitrogenous fertilizers are highly soluble in water this result in the addition of total salt in the soil (Shrivastava and Kumar, 2015). In order to substitute chemical fertilizers with biological fertilizers, it is prudent to have the salt tolerance in these isolates. The screened isolates of putative *Bacillus* species

were subjected to salt tolerance tests by growing in broth media with different concentration of salt. Since it was tedious to check for all 10 isolates, so only each of *Bacillus megaterium* and *Bacillus subtilis* were taken. *Bacillus megaterium* 13 and *Bacillus* subtilis *24* grew up to the 5% salt (Table 5) indicating that they were moderately salt tolerant.

Table 5: Growth of bacterial isolates at varied sodium chloride concentration

Samples	Salt concentration (NaCl) in percentage					
	1%	2%	3%	4%	5%	6%
*B. megaterium*13	+	+	+	+	+	-
B. subtilis 24	+	+	+	+	+	-

Symbol: + (growth), - (no growth)

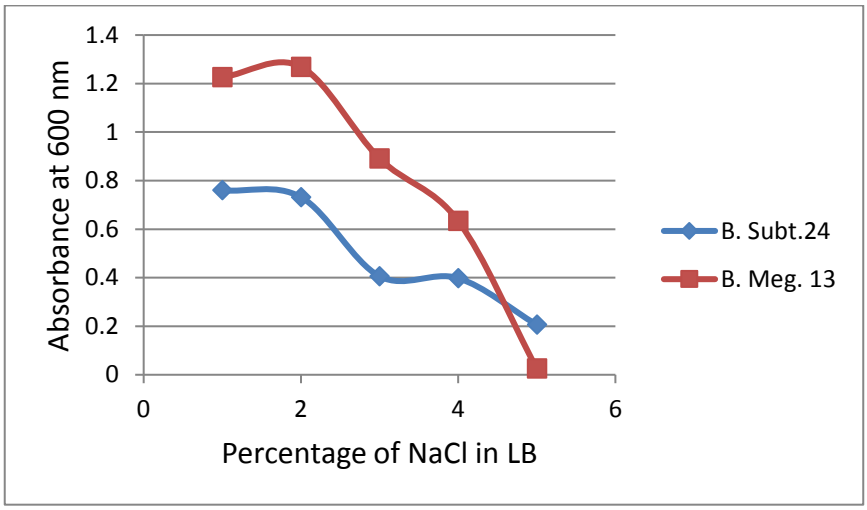


Figure 32: Determination of optimal salt tolerance of *Bacillus megaterium* 13 and *Bacillus subtilis* 24.

Salinity test was performed at different concentration (percentage) of NaCl ranging from 1%-6% in LB media. The spectrophotometric analysis of growth at 600 nm found the cell growth was impacted by the increasing amount of salt. The growth almost halved at 4% NaCl concentration from which the growth dramatically decreased (Figure 32). This showed that the bacteria flourish well at the NaCl concentration of 1 and 2% and completely zero at 6% and above. This suggests that the bacteria could be used as bio-fertilizer in slightly saline soil that might have arose due to the application of chemical fertilizers that react with the metals in the soil and increase the salinity.

These cells also grew at concentration of 2%. The sea water is presumed to be around 3.5% saline, thus the rivers that enter Terai could have some salinity and use of these bacteria could manage the salinity by integrating the metal ions in respective cell growth and cations for other functions. Therefore, these isolates could be developed as biofertilizer to supply in field where soil salinity is high due to prolong use of chemical fertilizer.

4.4 Growth at various pH concentration:

Each organism shows an optimum pH where growth proceeds most rapidly, and the growth slows down as the pH values either exceed, or fall below that optimum. Since pH refers the concentration of hydrogen ions [H^+], at the high pH the hydrogen bonds holding DNA strands together break up. Large proteins, such as enzymes, are also affected by pH.

Continuous use of N and P fertilizer could result in significant changes in soil pH. It is demonstrated that the application of N-based fertilizer can severely reduce the pH of surface soil. And this is because the oxidation of NH_4^+ to NO_3^-(nitrification) produce two moles of H^+ per moles of NH_4^+ (Savci, 2012). Similarly, basic fertilizers application in the field lead to basic soil. Therefore, it is necessary to detect pH tolerance capability of isolates because it is better if isolates have the ability to survive in low and high pH range of soil to use as alternative of chemical fertilizer.

For this test, screened isolated were introduced to varying pH range from 4.0 to 11.0 in LB media. Observed result suggested that optimum pH for the growth of these bacteria was from 6.0 to 8.0 for *Bacillus megaterium* 13 and 7.0 to 9.0 for *Bacillus subtilis* 24*.* But they were also able to grow at low pH 5 and high pH 10 (Table 6) indicating these isolates could also perform as bio-fertilizer in little bit acidic and basic soil condition.

Table 6: The growth of isolates at different pH concentration

Samples	pH range							
	4	5	6	7	8	9	10	11
Bacillus megaterium 13	-	+	++	++	++	+	+	-
Bacillus subtilis 24	-	+	+	++	++	++	+	-

Symbol: ++ (better growth), + growth), - (no growth).

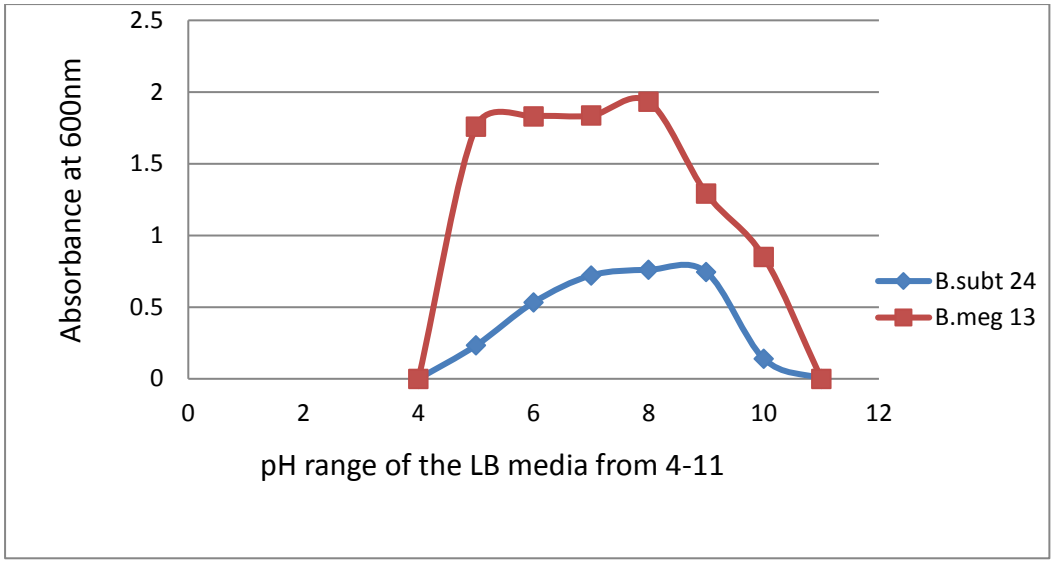


Figure 33: Determination of optimal pH for growth of *Bacillus megaterium* 13 and *Bacillus subtilis* 24.

Thus, the bacteria could be used in soils having pH between 5-10, which is also harsh condition for plants. Thus, use of this organism could balance the pH by consuming either proton for low pH soil and hydroxyl group of high pH soil. Thus, it is suggested that this organism can be developed as bio-fertilizer

4.5 Growth of bacteria in acid treated lignocellulosic biomass:

Lignin, hemicellulose, and cellulose are the 3 primary structure components of lignocellulosic biomass. Lignocellulosic biomass is subjected to various pre-treatment conditions to produce reducing sugars and then fermentation of the sugars to ethanol (Galbe and Zacchi, 2002). The chemical hydrolysis of lignocellulose give rise to the generation of toxic compounds in parallel with the release of sugars. These compounds, collectively termed pre-treatment inhibitors, impair metabolic functionality and growth. These include furfural, hydroxymethyl furfural (HMF) and phenol. This adds the cost for bio-ethanol producing industries. Thus, different approaches are being sought but with limited success. Firmicutes have been engineered using various mechanisms for development of ethanol production processes. Species of particular interest include *Geobacillus thermoglucosidasius*, *Thermoanaerobacter mathranii*, various *Clostridium* species, *Thermoanaerobacterium* species, *Bacillus subtilis* and *Bacillus megaterium*. Properties such as the degradation of phenolic compounds via the meta cleavage pathways have been reported in related species such as *Bacillus stearothermophilus*, *Bacillus brevis* and *Bacillus cereus*. *Ureibacillus thermosphaericus* has been shown to detoxify a variety of inhibitors including HMF and furfural (M. P. Taylor *et al*., 2012).

The *Bacillus megterium* and *Bacillus subtilis* isolates were chosen to see whether they have the potential to degrade such fermentation inhibitors. Those isolates were incubated

in the sludge of acid treated lignocellulosic biomass (*Saccharum spontaneum*) that had been neutralized with the view of if they can grow in that sludge and can survive by utilizing the carbon source for their cellular component making. In addition, it was thought that whether they have the ability to degrade the furfural and phenol (fermentation inhibitors) during the incubation period. Five isolate each of *Bacillus megaterium* and *Bacillus subtilis* were chosen in such a way that their biochemical assay has shown more similarity to the *Bacillus megaterium* and *Bacillus subtilis* and were also confirmed by the molecular characterization. So, the 5 isolates for *Bacillus megaterium* included strain number 13, 15, 23, 28 and 32. Similarly for *Bacillus* subtilis they were 1, 2, 8, 21 and 24.

Figure 34: Growth of *Bacillus megaterium* in acid treated lignocellulosic biomass.

The media for growth was designed in such a way that it did not contain any form of reduced carbon sources except the sludge. The *Bacillus megaterium* isolates were incubated into the modified media. These were incubated for the successive period of 16 days with the regular measure of growth at 600nm, DNS test at 540nm, furfural test at 530 nm and phenol test at 765 nm at 2-day interval. The cells showed growth in the media (Figure 34) indicated by the visible turbidity of the media except for the control. This indicated that the bacteria were able to use the reduced carbon source present in the pretreatment sludge.

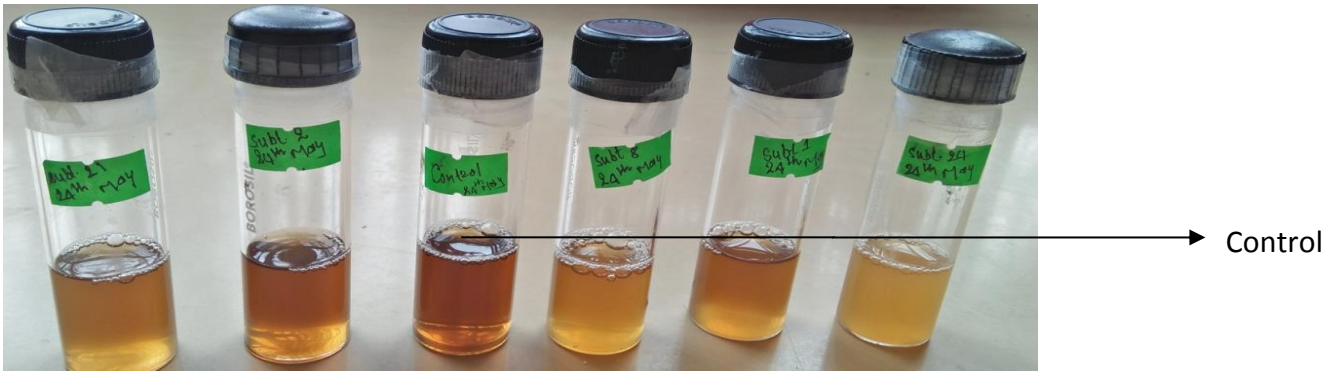


Figure 35: Growth of *Bacillus subtilis* in acid treated lignocellulosic biomass.

The 5 isolates of *Bacillus subtilis* were also incubated in the acid pretreated sludge and were checked for the DNS test, furfural test and phenol test for the successive period of 16 days at an interval of 2 day. The cells showed growth in the media (Figure 35) which was indicated by the visible turbidity of the media except for the control. This indicated that the bacteria were able to use the reduced carbon source present in the pretreatment sludge.

4.5.1 Quantification of reducing sugar in the bacterial inoculated broth:

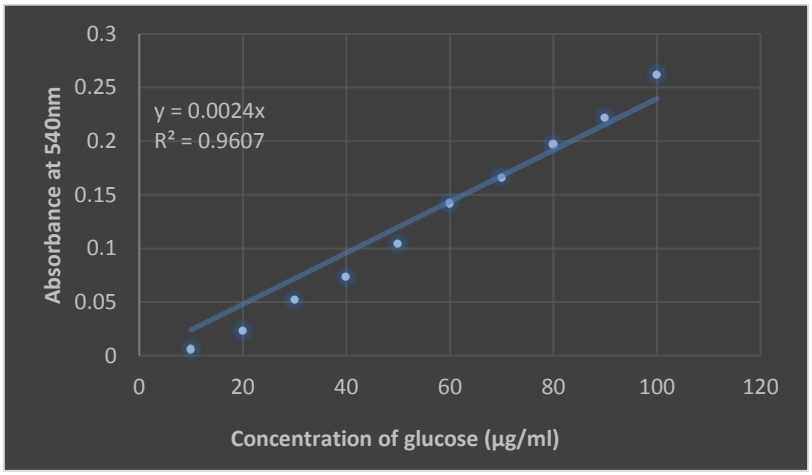


Figure 36: Standard calibration curve for reducing sugar quantification.

Using different concentration of glucose, the standard calibration curve was developed to calculate the amount of reducing sugar present in the acid treated lignocellulosic biomass broth. The calibration curve (Figure 36) showed linear line in correspondence to absorbance at 540 nm. The R^2 value was found to be 0.960 indicating the graph was reasonably accurate as standard calibration curve. Thus, the value of constant 'a' for equation Y=ax was found to be 0.002 and taken for subsequent calculation of amount of reducing sugar in culture media of different isolates of *Bacillus megaterium.*

4.5.1.1 Reducing sugar utilization test for *Bacillus megaterium:*

The test that is employed to determine the reducing sugar is called Dinitro salicylic acid (DNS) test. The 3,5-dinitrosalicylic acid is a monohydroxy benzoic acid consisting of 2-hydroxybenzoic acid having nitro substituent at the 3- and 5-positions. It is used in colorimetric testing for the presence of free carbonyl groups (C=O) in reducing sugar. Rochelle salt used in this test prevents the reagent from dissolving oxygen and essential to color stability (Miller, 1959). Since the reducing sugars contain free carbonyl group, have the property to reduce many of the reagents. All monosaccharide and some disaccharide are reducing sugars. When alkaline solution of 3,5-dinitrosalicylic acid reacts

with reducing sugars (like glucose, lactose) it is converted into 3-amino-5-nitrosalicylic acid with orange color. This color formation strongly absorbs light at 540 nm.

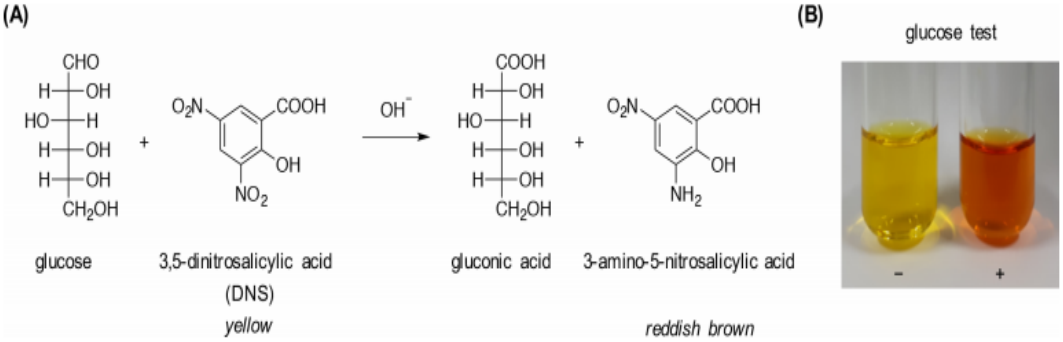


Figure 37: Reaction of reducing sugar with DNS reagent.

So, DNS test was performed to check the amount of reducing sugar present in the lignocellulosic biomass (Figure 38). This indicated that there is the presence of reducing sugar, albeit the nature of reducing sugar cannot be defined from this work.

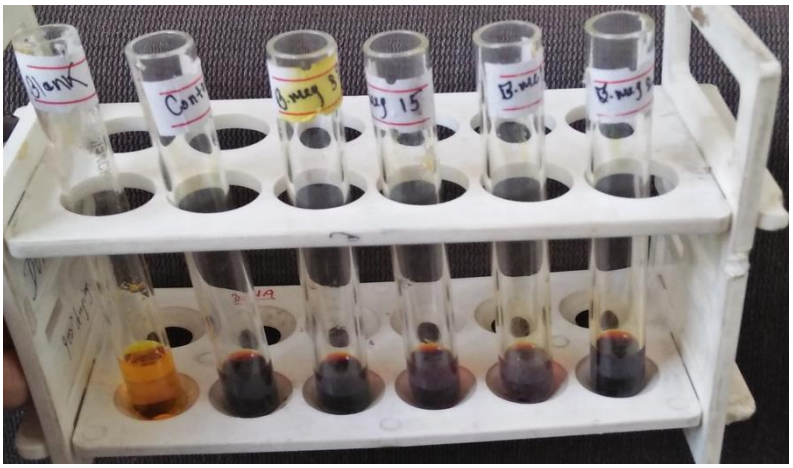


Figure 38: DNS test for *Bacillus megaterium.*

The formation of the dark red color after the addition of reagent (DNS reagent) and boiling for 5 mins. indicated the presence of reducing sugar in the broth. The broth in test tubes were all dark red in color except for the tube labeled as blank. This indicated the presence of reducing sugar in all test tubes except for the blank which contained water.

Amount of reducing sugar was quantified in the acid pretreated sludge where the bacteria were incubated for the successive period of 16 days with the regular measure of cell growth and reducing sugar test (DNS test) after the interval of 2 days. It was found that the successive bacterial cell growth resulted in decrease in the amount of reducing sugar with the increase in incubation time (Figure 39) indicating that the cells used these reducing sugars for their growth.

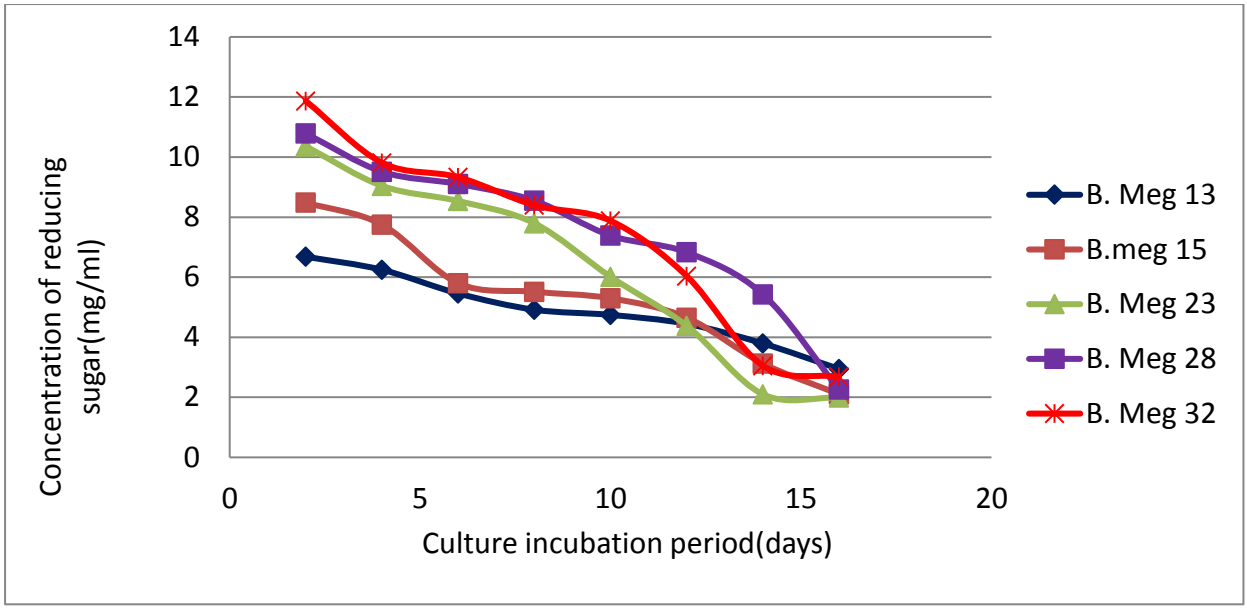


Figure 39: Quantification of reducing sugar in acid pretreated sludge by *Bacillus megaterium.*

This indicated that the bacteria were able to survive by utilizing the reducing sugar which were released after the acidic pretreatment of *Saccharum spontaneum* (a potent lignocellulosic biomass). Comparison was made between the concentration of 2nd and the 16th day of reducing sugar of incubation of *Bacillus megaterium*. Concentration was determined after multiplication by the dilution factor. Among the isolates *Bacillus megaterium* 32 had the best consumption ratio (Table 7).

Table 7: Concentration of reducing sugar during 2nd and 16th day of incubation of isolates.

S. N	Concentration of reducing sugar(mg/ml) at 2nd day of incubation	Concentration of reducing sugar(mg/ml) at 16th day of incubation
1.*Bacillus megaterium* 13	6.681 mg/ml	2.943 mg/ml
2. *Bacillus megaterium* 15	8.484 mg/ml	2.103 mg/ml
3.*Bacillus megaterium* 23	10.356 mg/ml	1.989 mg/ml
4.*Bacillus megaterium* 28	10.783 mg/ml	2.256 mg/ml
5.*Bacillus megaterium* 32	11.865 mg/ml	2.684 mg/ml

4.5.2 Quantification of furfural in the bacterial inoculated broth:

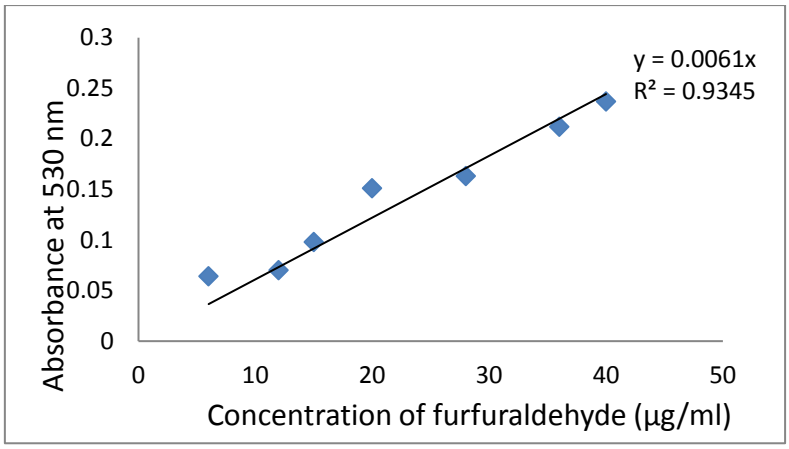


Figure 40: Standard calibration curve for furfural quantification.

Using different concentration of furfuraldehyde, the standard calibration curve was developed to calculate the amount of furfural present in the acid treated lignocellulosic biomass broth. The calibration curve (Figure 40) showed somewhat linear line in correspondence to absorbance at 530 nm. The R^2 value was found to be 0.9345 indicating the graph was reasonably accurate as standard calibration curve. Thus, the value of constant for equation Y=ax found as 0.006 for 'a' was taken for subsequent calculation of amount of furfural in culture media of different isolates of *Bacillus megaterium.* During the detection of furfural in the sample, the aniline and furfural react in the presence of hydrochloric acid (HCl) to form a deep purple compound, the corresponding acid salt of 1-phenyamino-5-phenylamino-2-hydroxypenta-2,4- diene and this color formation strongly absorb light at 530 nm.

4.5.2.1 Furfural utilization test for *Bacillus megaterium*:

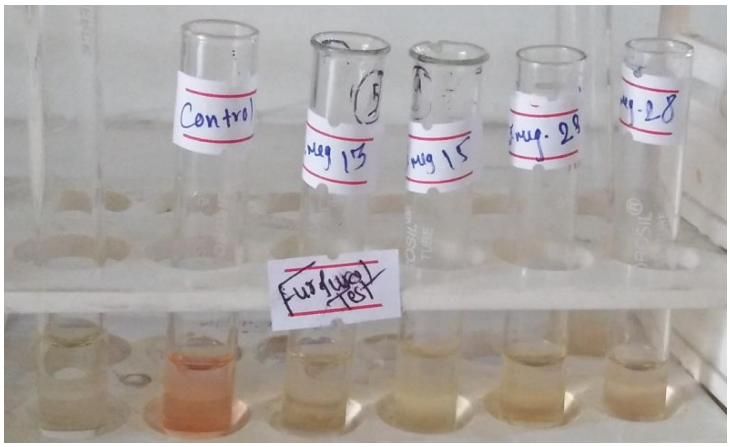


Figure 41: Furfural test for *Bacillus megaterium.*

Formation of pink color indicated the presence of furfural in the broth. Comparatively the broth in all test tubes were colorless except for the tube labelled as control. It indicated

that since no bacteria was presented in the tube labelled as control and appeared pink due to the presence of furfural. But the other tubes except control they appeared colorless due to the utilization of furfural by the incubated bacteria. Furfural the hydrolyzing product of pentoses is one of the inhibitors of fermentation process. Furfural is exclusively produced from lignocellulosic biomass by dehydrating pentose (Eseyin and Steele, 2015). It can contaminate the product streams of lignocellulose breakdown in the production of sugar from biomass. This contaminant inhibits the fermentation of glucose, and thus prevents efficient formation of the product. It is reported that the furfural reduce the specific growth rate, the cell-mass yield on ATP, volumetric and specific ethanol productivities (Palmqvist and Hahn-Hägerdal, 2000). These bacteria are supposed to utilize the furfural (Taylor *et al*., 2012). So, the amount of furfural was quantified in pretreated sludge incubated with the bacteria at the regular interval of 2 day and continued for the successive period of 16 days (Figure 42). The amount was found to be reduced with the successive increase in the incubation time.

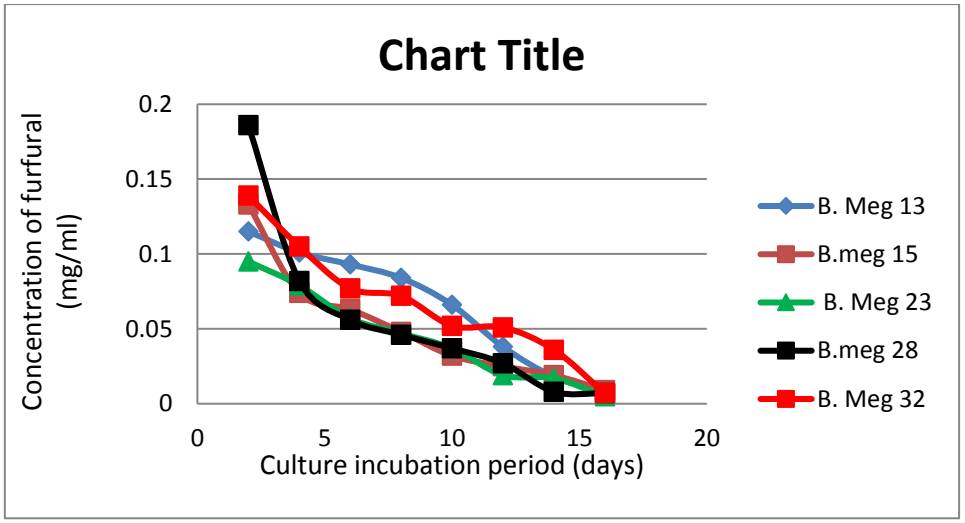


Figure 42: Quantification of furfural in acid pretreated sludge by *Bacillus megaterium.*

When isolated putative *Bacillus megaterium* was incubated for 16 days the amount of furfural significantly decreased. Among the different isolates, isolate 28 was most efficient in decreasing furfural (Figure 42). Thus, it can be stated that the isolates were able to utilize furfural. Since the reducing sugar test gave presence of reducing sugar in the broth utilization of furfural was intriguing. *Bacillus* species are known to be regulated by Carbon Catabolite Repression (CCR) (K. D. Singh *et al.,* 2008) except for some that can simultaneously use glucose and mannose (Vinuselvi *et al.,* 2012) the presence of reducing sugar must have prevented use of furfural as sole reduced carbon sources. Because of furfurals toxic effect the bacteria must have degraded this to three carbon intermediates of Entner-Doudoroff Pathway (ED) (which takes place only in prokaryotes) during breakdown of sugar that does not show CCR or could have been converted to pentose

that could be used as building block for making DNA, RNA and other cofactors. This pathway is not governed by central carbon metabolism reaction (Sudarsan *et al.*, 2014).

Table 8) Concentration of furfural during 2nd and 16th day of incubation of isolates.

S. N	Concentration of furfural (mg/ml) at 2nd day of incubation	Concentration of furfural (mg/ml) at 16th day of incubation
1.*Bacillus megaterium* 13	0.115 mg/ml	0.008 mg/ml
2.*Bacillus megaterium* 15	0.133 mg/ml	0.009 mg/ml
3.*Bacillus megaterium* 23	0.095 mg/ml	0.005 mg/ml
4.*Bacillus megaterium* 28	0.186 mg/ml	0.007 mg/ml
5.*Bacillus megaterium* 3	0.139 mg/ml	1.07 mg/ml

4.5.3 Quantification of phenol in bacteria inoculated broth:

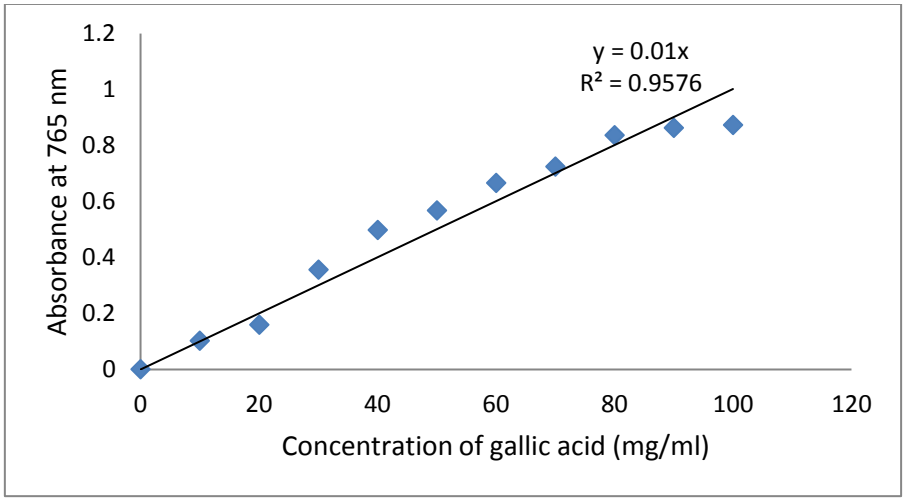


Figure 43: Standard calibration curve for phenol quantification.

Using different concentration of gallic acid the standard calibration curve was developed to calculate the amount of phenol present in the acid treated lignocellulosic biomass broth.

In order to determine the amount of phenol in the acid treated lignocellulosic biomass, folin and Ciocalteu's phenol reagent was used. This reagent is most commonly used in the Lowry method for determining protein concentration. The Folin-Ciocalteu method is described in several pharmacopoeias. And has also been used for the quantification of total phenolics (Waterborg and Matthews, 1994). This reagent does not contain phenol, rather, it reacts with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. Sodium carbonate used in this test form a

blue colored complex, which was measured at 765 nm (Molybdenum–tungsten blue method) (Qarah *et al.,* 2017). The blue chromophore constituted by a phosphotungstic phosphomolybdenum complex is during this reaction where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds (Blainski *et al.*, 2013).

The calibration curve (Figure 43) showed linear line in correspondence to absorbance at 765 nm, The R^2 value was found to be 0.957 indicating the graph was reasonably accurate as standard calibration curve. Thus, the value of constant for equation Y=ax found as 0.01 for the value of 'a' was taken for subsequent calculation of amount of phenol in culture media of different isolates of *Bacillus megaterium.*

4.5.3.1 Phenol utilization test for *Bacillus megaterium:*

Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. During hydrolysis of the lignocellulosic biomass the lignin is hydrolyzed to the phenolic compounds. Phenolic compounds are toxic even at ppb level (Fang and Chen, 1997).These phenolic compounds cause partition and loss of integrity of the cell membrane of the fermenting organisms thus reducing cell growth and glucose assimilation (Palmqvist and Hahn-Hägerdal, 2000b). Phenol is highly toxic to all life form in all concentration (5-2000 mg/L) (Hasan and Jabeen, 2015)

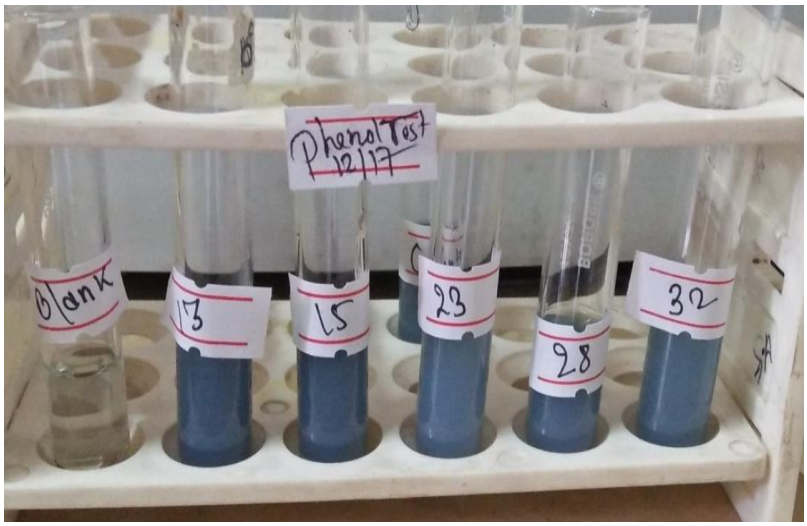


Figure 44: Phenol test for *Bacillus megaterium.*

Formation of blue color after the addition of reagent (Fc reagent) and incubation for 15 mins indicated the presence of phenol in the broth. So, phenol test was performed to quantify the amount of phenol initially present in the broth and finally at 16th day of incubation. And to check if the isolated organisms were able to survive in spite of presence

of toxic phenolic compounds. The screening of the bacteria was done in phenol as sole reduced carbon source and it survived and the bacteria were able to grow in the phenol media, thus it was presumed that the isolate would utilize phenol. However, the screening protocol was performed in the media containing phenol along with agar in plate assay it was not clear whether the bacteria survived through utilizing phenol or agar as reduced carbon source. So, as to confirm the growth was actually by not consuming the agar as a carbon source but by utilizing the Phenol. The bacteria were incubated in the acid pretreated sludge where the degradation product of lignin would release phenol (Figure 44). Since the bacteria grew in the medium it was not clear whether the bacteria really consumed the phenol present in the medium. The results of incubation showed reduced amount of phenol in successive days indicating that the phenol has been utilized in one or another form.

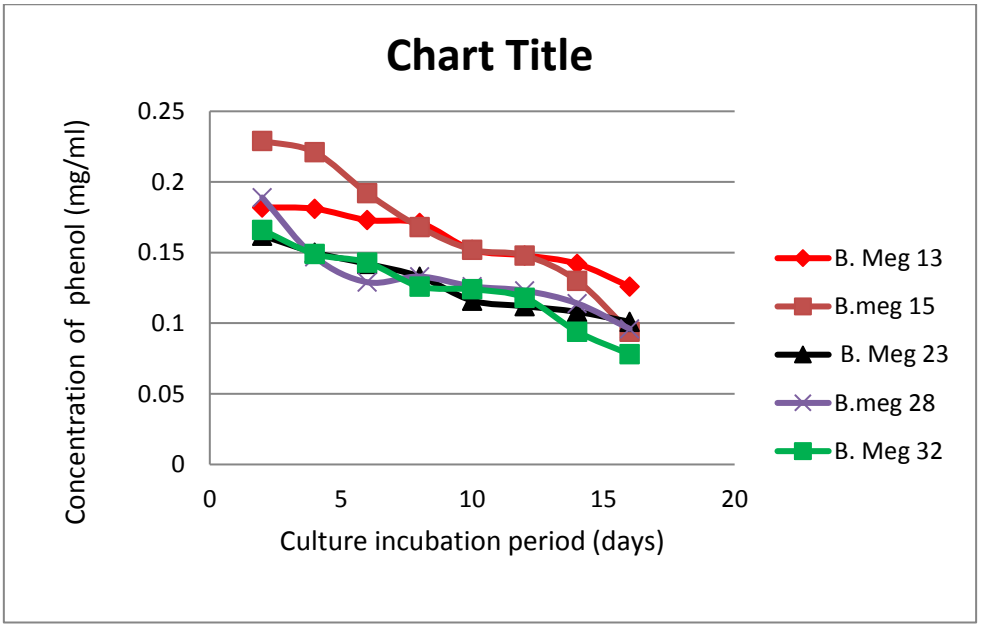


Figure 45: Quantification of phenol in acid pretreated sludge by *Bacillus megaterium.*

For phenol utilization isolate 15 was more prominent than other isolates (Table 9). This result indicated that if these inhibitors have to be neutralized then most probably different isolates or consortium has to be developed. Moreover, utilization of phenol in the mixture of different reduced carbon sources indicates that these strains could be used for neutralizing inhibitors produced in bioethanol industries. The probable mechanism (Figure 52) should have allowed the bacteria to utilize phenol in running TCA cycle for energy generation and use of other reduced carbon source for making amino acids, DNA, RNA and cofactors.

Table 9: concentration of phenol during2nd and 16th day of incubation of isolates

S. N	Concentration of phenol (mg/ml) at 2nd day of incubation	Concentration of phenol (mg/ml) at 16th day of incubation
1.*Bacillus megaterium* 13	0.182 mg/ml	0.126 mg/ml
2.*Bacillus megaterium* 15	0.299 mg/ml	0.094 mg/ml
3.*Bacillus megaterium* 23	0.162 mg/ml	0.101 mg/ml
4.*Bacillus megaterium* 28	0.189 mg/ml	0.096 mg/ml
5.*Bacillus megaterium* 32	0.166 mg/ml	0.078mg/ml

4.6 Reducing sugar utilization test for *Bacillus* subtilis:

Figure 46: DNS test for *Bacillus* subtilis.

The formation of the dark red color after the addition of reagent (DNS reagent) and boiling for 5 mins indicated the presence of reducing sugar in the broth. The reducing sugar utilization test was performed for the putative *Bacillus* subtilis in a manner similar to *Bacillus megaterium* (Figure 46) but *Bacillus megaterium* had higher reduction rate. The concentration was determined after multiplication by the dilution factor. This indicated that the bacteria were able to grow using the available reducing sugar in the mixture of pretreatment sludge.

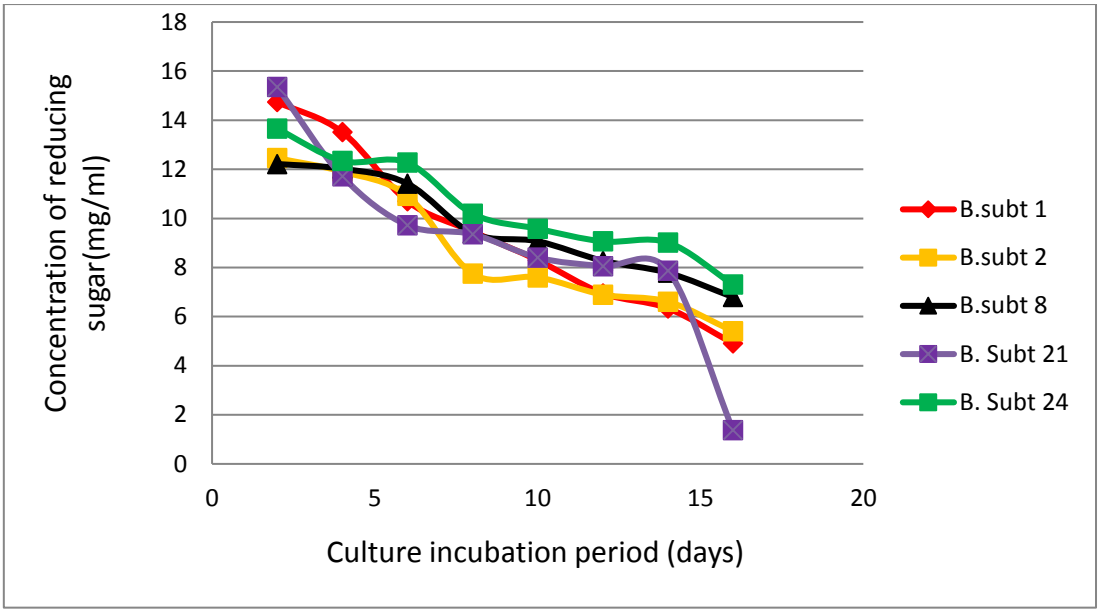


Figure 47: Quantification of reducing sugar in acid pretreated sludge by *Bacillus* subtilis.

It was found that there was decrease in the amount of reducing sugar with the increase in the incubation period (Table 10). The consumption of reducing sugar was relatively low compared to *Bacillus megaterium* except for one isolate. Low consumption of the reducing sugar could be because *Bacillus megaterium* has big size than *Bacillus subtilis* thus (Eppinger *et al.,* 2011) must have consumed much sugar during its cell growth. Isolate 21 had the highest consumption rate indicating that his strain had higher growth.

Table 10) Concentration of reducing sugar during 2nd and 16th day of incubation of isolates.

S.N.	Concentration of reducing sugar(mg/ml) at 2nd day of incubation	Concentration of reducing sugar(mg/ml) at 16th day of incubation
1.*Bacillus subtilis* 1	14.742 mg/ml	4.907 mg/ml
2.*Bacillus subtilis* 2	12.455 mg/ml	5.407 mg/ml
3.Bacillus subtilis 8	12.216 mg/ml	6.744 mg/ml
4.*Bacillus subtilis* 21	15.353 mg/ml	1.368 mg/ml
5.*Bacillus subtilis* 24	13.656 mg/ml	7.308 mg/ml

4.7 Furfural utilization test for *Bacillus* subtilis:

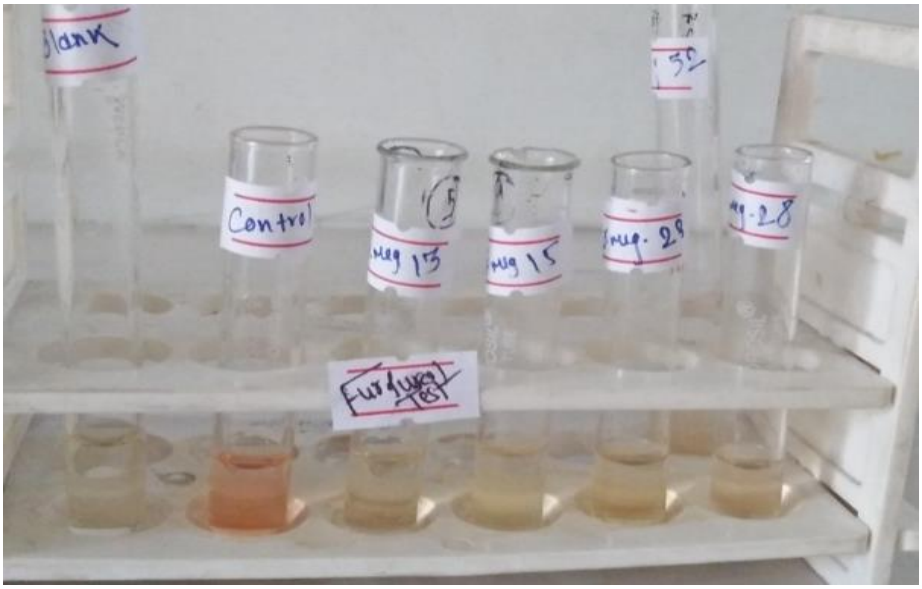


Figure 48: Furfural test for *Bacillus subtilis*.

Formation of pink color indicated the presence of furfural in the broth. Furfural utilization test was also performed in *Bacillus subtilis* in a manner similar to that for *Bacillus megaterium*. Most of the strains completely utilized furfural. This could be as described for *Bacillus megaterium*. This suggests that *Bacillus* species could have some transporters for transporting furfural even in the presence of reducing sugar. Furfural is known to be overexpress cytosine transporter (van der Pol *et al.*, 2016) indicating that this transporter could have some role in furfural transport. The group also found that the exposure of *Bacillus coagulans* to furfural prior to incubating with lignocellulosic pretreated biomass gave higher amount of lactic acid indicating that the exposure to furfural increases the adaptability of the organism.

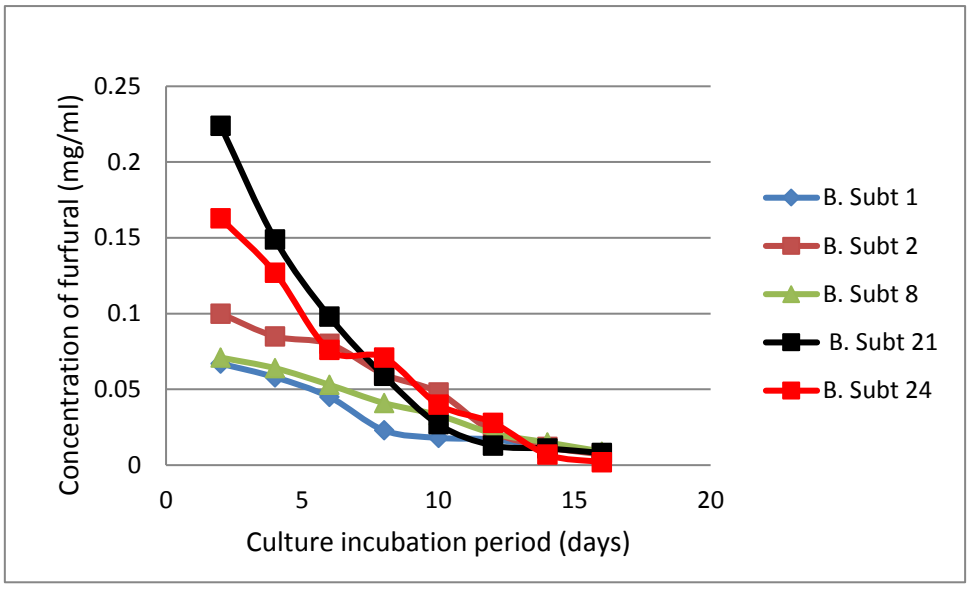


Figure 49: Quantification of furfural in acid pretreated sludge by an isolated *Bacillus subtilis*.

Table 11) Concentration of furfural during 2nd and 16th day of incubation of isolates

S. N	Concentration of furfural (mg/ml) at 2nd day of incubation	Concentration of furfural (mg/ml) at 16th day of incubation
1.*Bacillus subtilis* 1	0.067 mg/ml	0.008 mg/ml
2.*Bacillus subtilis* 2	0.100 mg/ml	0.007 mg/ml
3.*Bacillus subtilis* 8	0.071mg/ml	0.009 mg/ml
4.*Bacillus subtilis* 21	0.224 mg/ml	0.008 mg/ml
5.*Bacillus subtilis* 24	0.163 mg/ml	0.002 mg/ml

4.8 Phenol utilization test for *Bacillus* subtilis:

Figure 50: Phenol test for *Bacillus* subtilis.

The formation of blue color after the addition of the reagent (FC reagent) and incubation for 15 mins. at room temperature indicated the presence of phenol in the broth**.** For *Bacillus subtilis* also phenol utilization test was performed in a manner similar to that of *Bacillus megaterium***.** And it was found reduction in the amount of phenol in the broth after the successive period of 16 days. Isolate 24 had the highest rate of consumption of phenol. Thus, it is suggested that different isolates with different characteristics could be integrated for co-culturing.

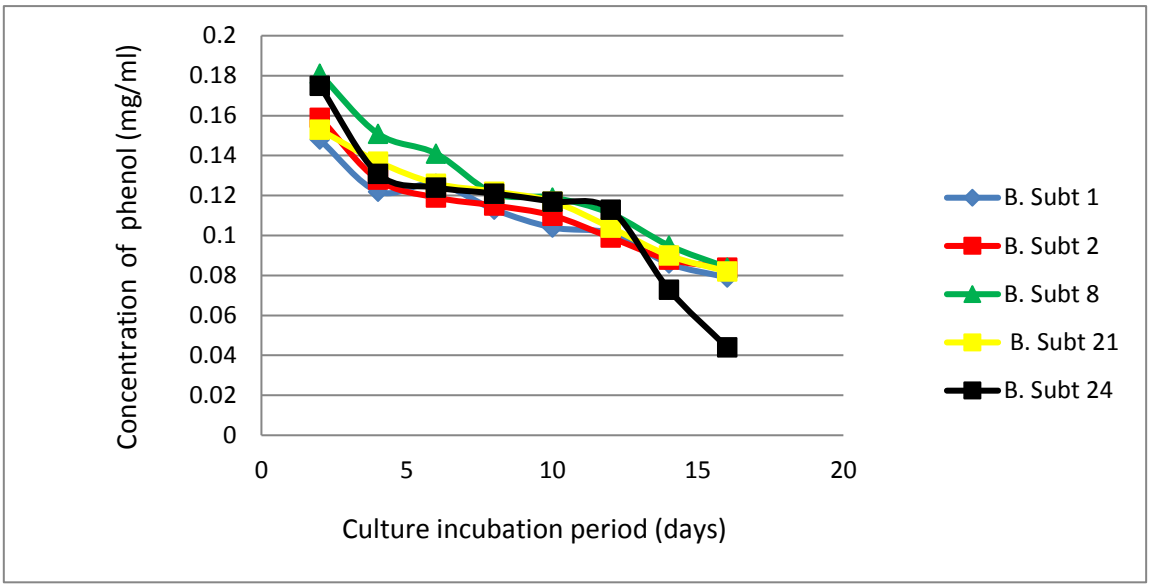


Figure 51: Quantification of phenol in acid pretreated sludge by isolated *Bacillus subtilis*.

Table 12) Concentration of phenol during 2nd and 16th day of incubation of isolates

S. N	Concentration of phenol (mg/ml) at 2nd day of incubation	Concentration of phenol (mg/ml) at 16th day of incubation
1.*Bacillus subtilis* 1	0.148 mg/ml	0.079 mg/ml
2.*Bacillus subtilis* 2	0.159 mg/ml	0.084 mg/ml
3.*Bacillus subtilis* 8	0.181 mg/ml	0.084 mg/ml
4.*Bacillus subtilis* 21	0.153 mg/ml	0.082 mg/ml
5.*Bacillus subtilis* 24	0.175 mg/ml	0.044 mg/ml

As world is moving towards removing fossil fuel alternative fuel would gain upward increase in consumption. The bio-fuel industry that would require reducing sugar for making any forms of bio-fuel would require this in high amount that could have impact to the food security. Thus, lignocellulosic biomass has been of interest. But the inherent flaw in using this is release of fermentation inhibitors during the pretreatment process to release cellulose that could be later converted to monomeric sugar, glucose, for fermentation and/or bio-fuel production. The most potent inhibitors are furfural, HMF and phenol.

Phenol is produced naturally by the breakdown of plant materials during microbial degradation (Jeong, 2003). Among the different pollutants of aquatic ecosystems; phenols and its derivatives are considered as priority pollutants since they are harmful to organisms even at ppb levels (Fang and Chen, 1997). Residues of phenols have been found worldwide in soil, water and air samples, in food products, in human and animal tissues and body fluids (Bahdod *et al.,* 2009). Low concentration of these compounds can inhibit the growth of microorganisms present in biological wastewater treatment systems (Barrios-Martinez *et al*.,2006).

It was reported that *Bacillus* stearothermophilus BR219, degraded phenol at levels to 15 mM at a rate of 0.85, uM/h (4 x 106 cells). The solubilized phenol hydroxylase was NADH dependent, exhibited a 55°C temperature optimum for activity, and was not inhibited by 0.5 mM phenol (Gurujeyalakshmi and Oriel, 1988). Hydroxylation of phenol is the first step during phenol degradation. In mesophilic microorganisms like *Pseudomonas species* inhibition of phenol hydroxylase activity in their whole cells was reported at 0.25 mM phenol (Janke *et al*., 1981). Neujahr and Gaal, 1973) demonstrated phenol hydroxylase inhibition above 0.25 mM phenol with highly purified enzyme from *Trichosporoncutaneum.*

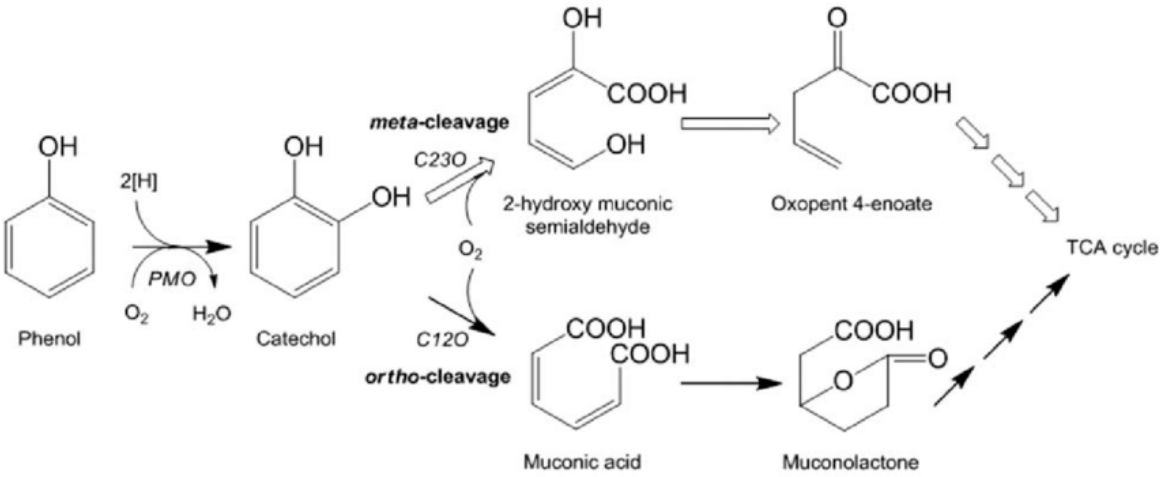


Figure 52: Probable mechanism of phenol degradation pathway (Hasan and Jabeen, 2015).

The genera of bacteria like A*cinetobacter*, Alcaligenes, *Thauera*, *Azoarcus*, *Comamonas*, *Pseudomonas*, *Bacillus* use a typical aerobic degradation pathway of phenolic compounds, which has two critical steps: the ring hydroxylation of adjacent carbon atoms and ii) the ring cleavage of the resulting catecholic intermediates. In the phenol degradation, the first step is the conversion of phenol to catechol (an initial intermediate of the central pathways of aromatic compound catabolism. Phenol hydroxylase (PH) enzyme is involved for such degradation (Nešvera*et al.,* 2015). The mechanism involved here is that the PH (a monooxygenase) attached a hydroxyl group at ortho position with the benzene ring and catechol is formed. Catechol is degraded either by Catechol 2, 3-dioxygenase (via meta-cleavage) leading to the formation of pyruvate and acetaldehyde (Merimaa *et al.,* 2006) or by ortho-cleavage by Catechol 1,2- dioxygenase leading to the formation of succinyl-CoA and acetyl-CoA. These various citric acid cycle intermediates are formed and enter into the TCA cycle for energy. Thus, bacteria degrade the phenolic compounds. So, such phenol degrading microorganisms can be used in bioremediation technologies, and also have proved to be a promising option for the removal of many phenolic pollutants, with obvious economic and environmental benefits. Furthermore, the remaining of the roots after the crops are harvested the phenolic compounds would be generated after their decomposition and impact the soil microbiome. These phenolic compounds could hinder the growth of other beneficial microbes of the soil and if the phenolic compound degrading bacteria is also present in the soil, they will degrade the phenolic compounds and help in creating favorable environment in the niche environment.

4.9 Hydroxyapatite nanoparticle synthesis (HA nanoparticle):

Excessive P inputs and other inappropriate P management practices are the main causes of severe eutrophication of lakes, rivers, and other water bodies. P is accumulated in large amount in manure from animal feeding that has increased the potential for P release following agricultural land. Attention on manure management and potential for P in runoff can prevent the accelerated eutrophication problems. Use of insoluble form of phosphate fertilizer can be one of the solutions for such problems. Hydroxyapatite [Ca10(PO4)6(OH)2] is the inorganic and natural source of phosphate found in the calcareous soil (Li *et al.,* 2015).

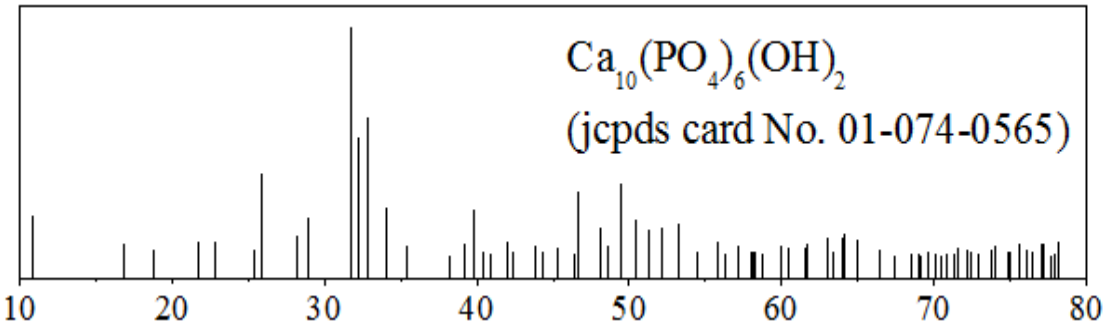


Figure 53: Joint Committee on Powder Diffraction Standards (JCPDS) peak range of HAP.

The nHA has been synthesized from raw buffalo bone as described in the materials and methods. The produced nHA was characterized by XRD. Its XRD pattern has matched somewhat with the standard JCPDS data of HA (Figure 53). It was found that the HA has been formed in crystalline structure. Based on the XRD data of different protocols used for making nHA, the calcination process performed at 900°C has favored the removal of organic compounds present in the raw bone (Figure 54).

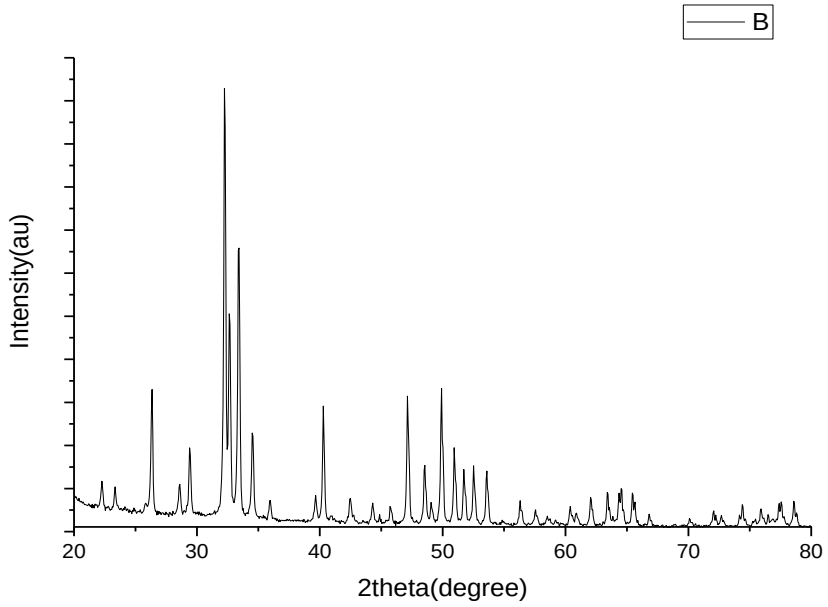


Figure 54: XRD spectra of HAp extracted from Buffalo bone calcined at 900^{o}C.

Thus, the synthesized nHA was thought to be used as P-fertilizer since use of bone directly might affect the sentiments of Hindu and if swine bones are to be used then it might hurt the sentiments of Muslims. Hence, solubilization of synthesized nHA by the isolated *Bacillus* species was investigated as these are known P solubilizers.

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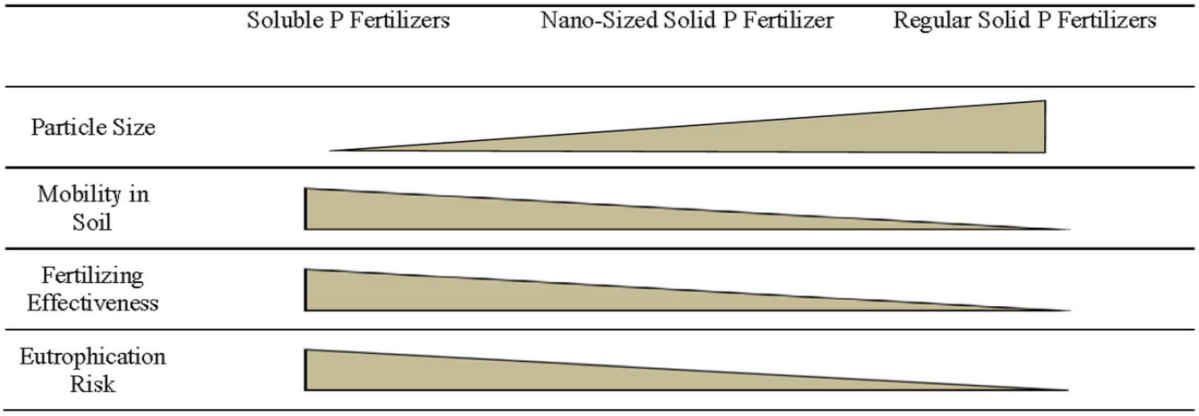

Figure 55: A schematic comparison of soluble P, nano-sized solid P, and solid P on their environmental properties.

(note: some of nanoparticle properties are hypothetical).

Animal bone is huge source of both calcium and phosphorous that if effectively be utilized for fertilization could be one of the best ways of recycling phosphorous source. Thus, HA nano-particle was synthesized. To improve P efficiency, hydroxyapatite nanoparticles (n-HAP) were evaluated as a potential fertilizer based on the hypothesis that nano sized

particles can potentially move in the soil and reach the plant roots. The use of sparingly soluble calcium phosphates such as hydroxyapatite has been studied as an alternative to conventional water-soluble P fertilizers for acidic and strongly P sorbing soils (Bertran *et al*., 2015).

4.10 Potential of nanohydroxyapatite (nHA) solubilization by *Bacillus megaterium:*

As *Bacillus megaterium* is known as phosphate solubilizer (Y. P. Chen *et al.,* 2006), it was assumed that the isolates screened as putative *Bacillus megaterium* could have potential to solubilize HA and consume it as phosphate source for its growth. The Na-acetate media used to for initial screening was slightly modified where HA was used at 0.33 gm/l that is equivalent to 0.00197 mole of phosphate taking HA [$Ca_{10}(PO_4)_6(OH)_2$] as sole source of phosphate. The phosphate in this media supplied by K_2HPO_4 and KH_2PO_4 was removed in the media composition. It was assumed that those bacteria that can survive in this media have ability to solubilize HA and use phosphate released from it.

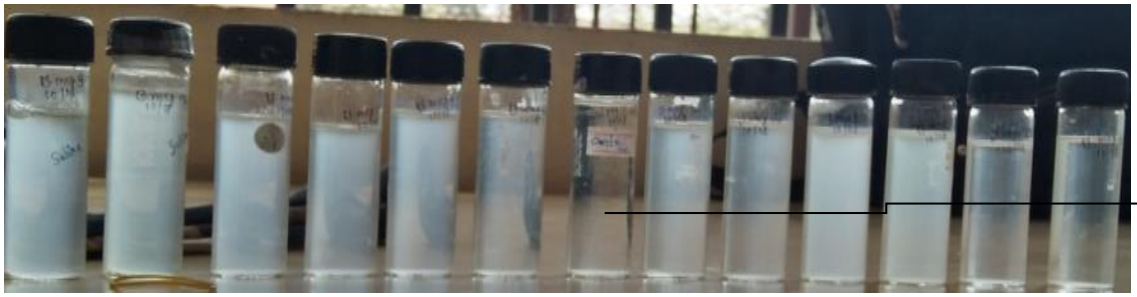

Figure 56: Growth of *Bacillus megaterium* in HA media after 10th day incubation.

The screened different *Bacillus megaterium* isolates overnight culture were incubated into the media containing nano-hydroxyapatite (nHA), an insoluble form of phosphate and growth was observed. All the 33 isolated colonies of putative *B. megaterium* were tested and only 12 isolates showed growth (Figure 56). These bacteria that were incubated in nHA media at 28°C for the successive time period of 20 days were periodically measured for cell growth at OD 600 nm and also presence of soluble phosphate in the media was detected by titration and measuring aborbance at 827nm after the regular interval of 5 days. The highest growth and the highest amount of phosphate solubilization by bacteria were found to be when incubated for the time period of 10 days (Figure 57 and 58). This could be probably the bacteria entered the stationary phase. The presence of soluble phosphate in the media indicated that these isolates can solubilize HA and release some soluble phosphate in the surrounding indicating that it could be utilized by other organisms that live around these isolates niche environment.

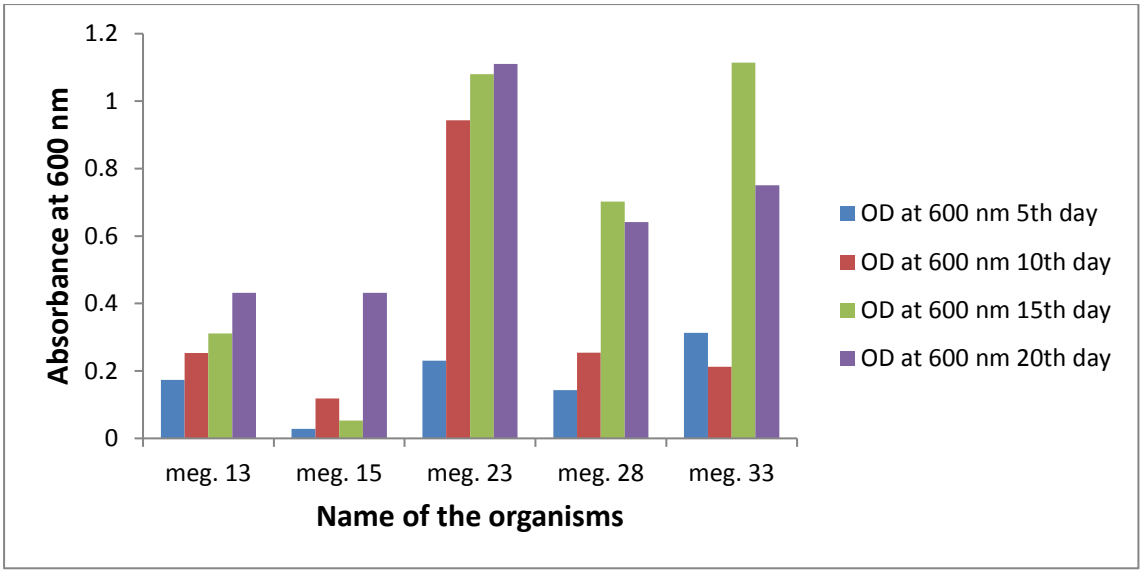

Figure 57: Absorbance at 600 nm at 5th, 10th, 15th and 20th day of incubation of *Bacillus megaterium.*

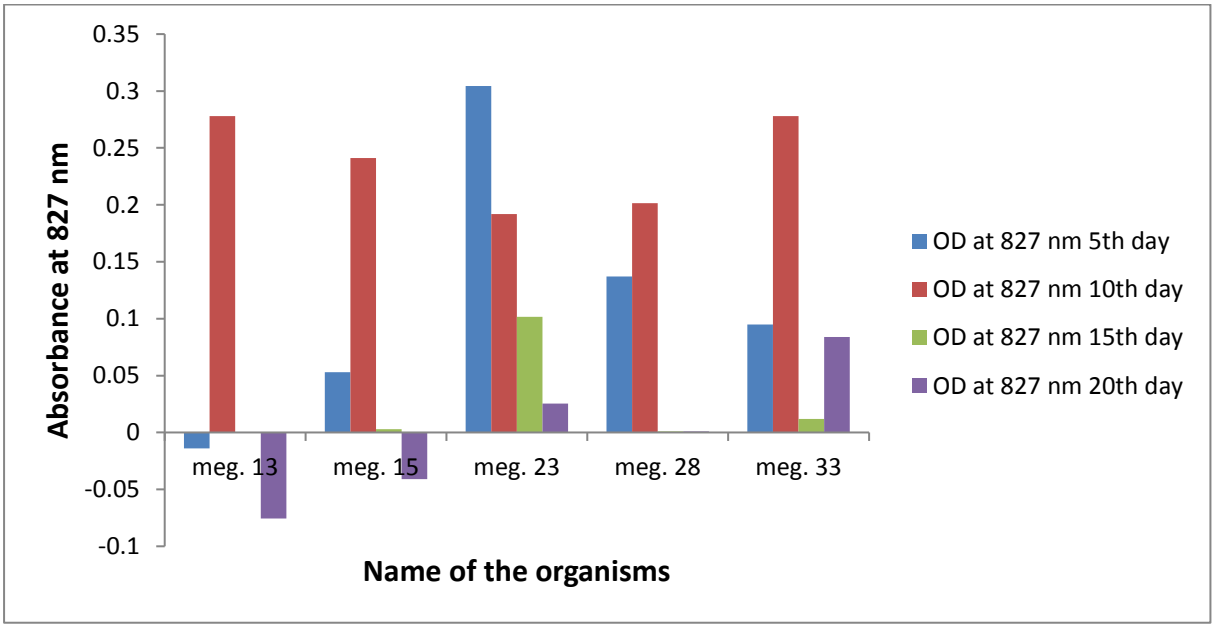

Figure 58: Absorbance at 827 at 5th, 10th, 15th and 20th day of incubation of *Bacillus megaterium.*

Since *Bacillus megaterium* is known as rhizospheric (A. P. Chakraborty *et al.,* 2015) and endophytic (McInroy and Kloepper, 1995) bacteria it can presume that the amount of soluble phosphate in the surround of the these isolates extracellular environment could be potentially be used by plants for their phosphate requirement. This clearly indicated that HA could be supplemented as phosphorous source. As it is in insoluble it cannot be washed and the isolates screened have potential to solubilize HA to phosphate and calcium leaving some in extracellular environment supplementing plants with these valuable ions. Thus, it is suggested that HA along with the isolates could be developed as sustained phosphate fertilizer.

4.11 Potential of nanohydroxyapatite (nHA) solubilization by *Bacillus subtilis:*

As *Bacillus subtilis* is known as phosphate solubilizer (Elkoca *et al.,* 2007), it was assumed that the isolates screened as putative *Bacillus subtilis* could have potential to solubilize HA and consume it as phosphate source for its growth. The Na-acetate media was slightly modified where HA was used. The phosphate in this media supplied by K_2HPO_4 and KH_2PO_4 was removed in the media composition. It was assumed that those bacteria that can survive in this media have ability to solubilize HA and use phosphate released from it.

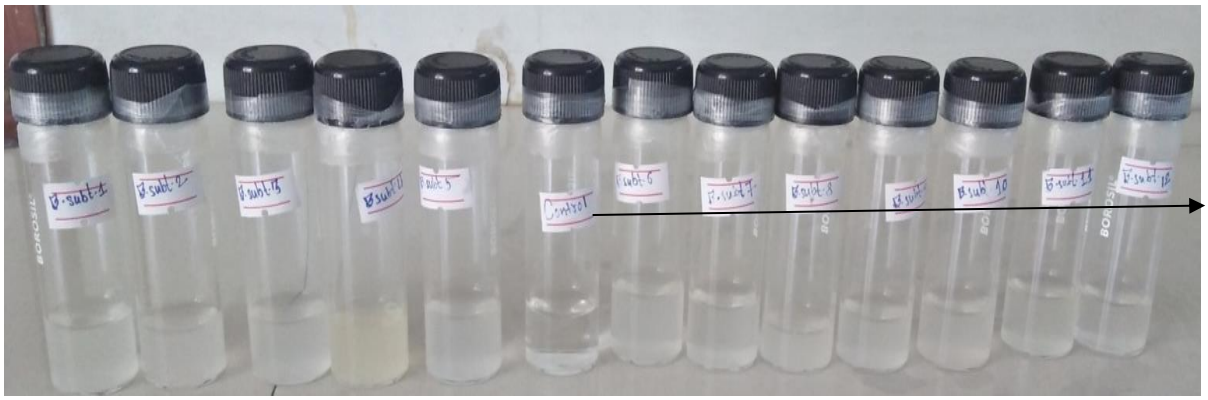

Figure 59: Growth of *Bacillus* subtilis in HA media after 10th day incubation.

Similar to that of *Bacillus megaterium*, the screened *Bacillus subtilis* were incubated into the media containing nano-hydroxyapatite (nHA), an insoluble form of phosphate and growth was observed. All the 30 isolated colonies of putative *Bacillus subtilis* were tested and only growths of 12 isolates were shown (Figure 59). These bacteria that were incubated in HA media at 28°C for the successive time period of 20 days were periodically measured for cell growth at OD 600 nm and also presence of soluble phosphate in the media was detected by titration and measuring absorbance at 827 nm after the regular interval of 5 days. The highest growth and the highest amount of phosphate solubilization by bacteria were found to be when incubated for the time period of 15 days and 10 days respectively (Figure 60 and 61). This could be probably the bacteria entered the stationary phase. The presence of soluble phosphate in the media indicated that these isolates can solubilize HA and release some soluble phosphate in the surrounding indicating that it could be utilized by other organisms that live around these isolates niche environment.

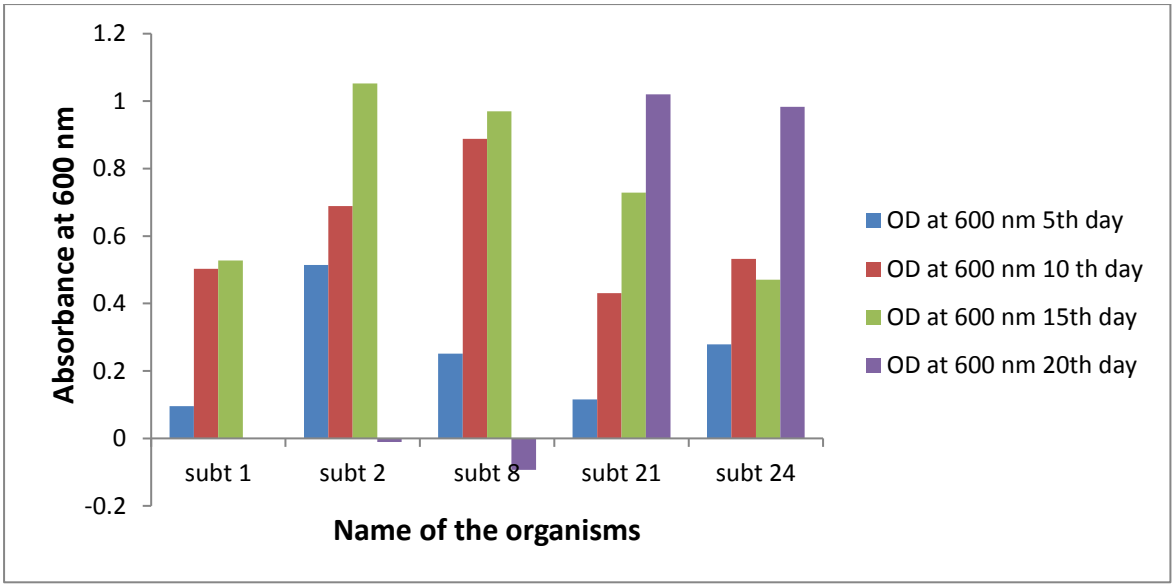

Figure 60: Absorbance at 600 nm at 5th, 10th, 15th and 20th day of incubation of *Bacillus* subtilis.

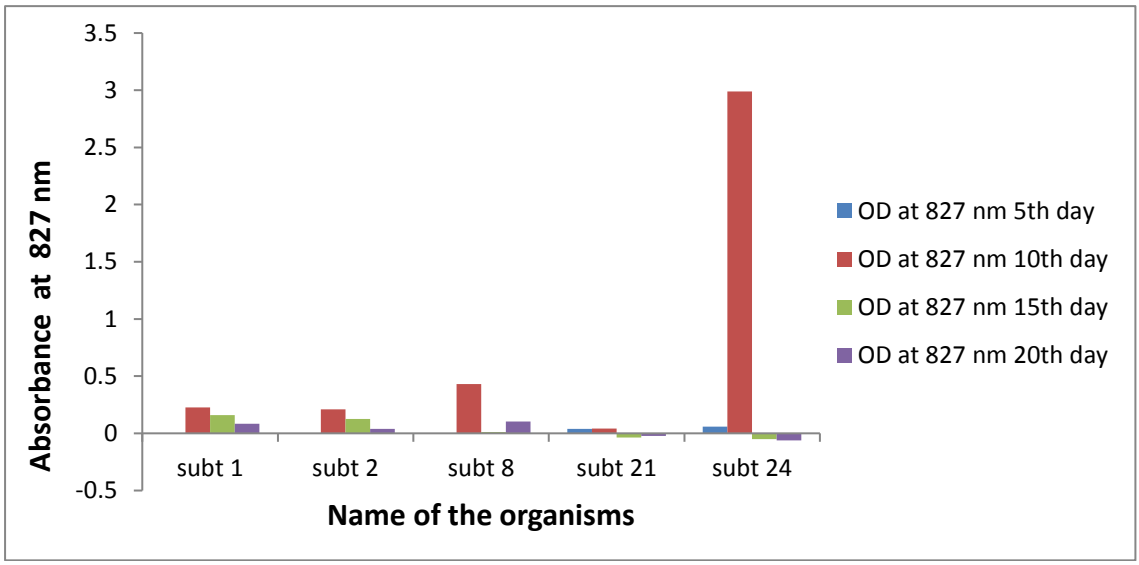

Figure 61: Absorbance at 827 nm at 5th, 10th, 15th and 20th day of incubation of *Bacillus subtilis.*

4.12 Quantification of phosphate present in HA by the isolated bacteria:

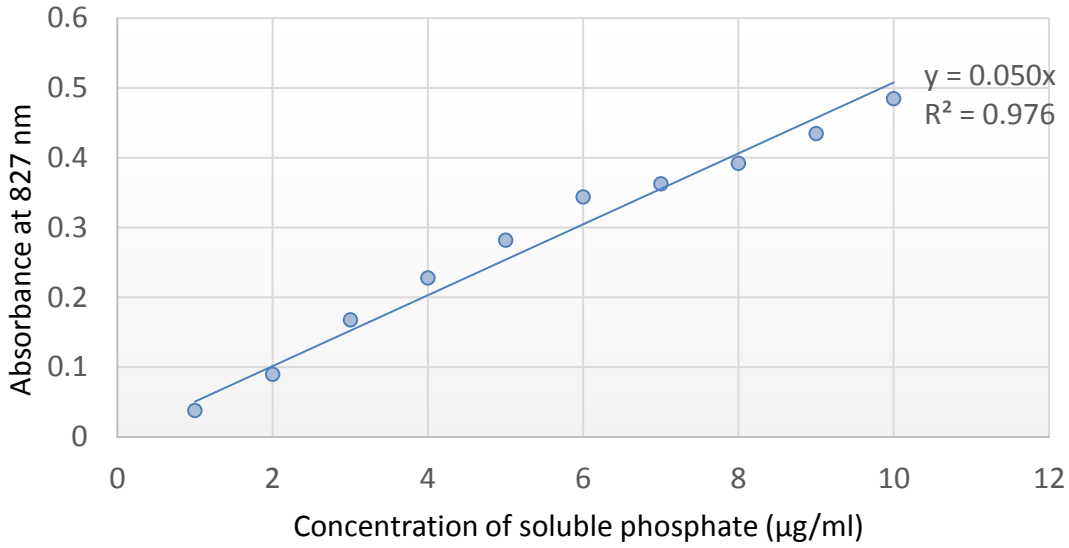

Figure 62: Standard calibration curve for soluble phosphate quantification.

Using different concentration of KH_2PO_4 the standard calibration curve was developed to calculate constants to quantify the amount of soluble phosphate in the culture media that has been released from HA present in the culture media. The amount of soluble phosphate was quantified using "A Single solution reagent method" (Murphy and Riley, 1958). The sulfuric acid and ammonium molybdate in the reagent exert a very marked effect in the formation of phospho-molybdic acid. The ascorbic acid reduces this heteropoly acid of molybdate to intense blue color that has absorbance peak maxima of 827 nm.

The calibration curve (Figure 62) showed linear line in correspondence to absorbance at 827 nm of phospho-molybdic acid formed upon reaction of molybdate with soluble phosphate with linear increment with concentration of soluble phosphate. The R^2 value was found to be 0.976 indicating the graph was reasonably accurate as standard calibration curve. Thus, the value of constant for equation Y=ax found as 0.050 for 'a' was taken for subsequent calculation of amount of dissolved phosphate in culture media of different isolates of putative *Bacillus megaterium.*

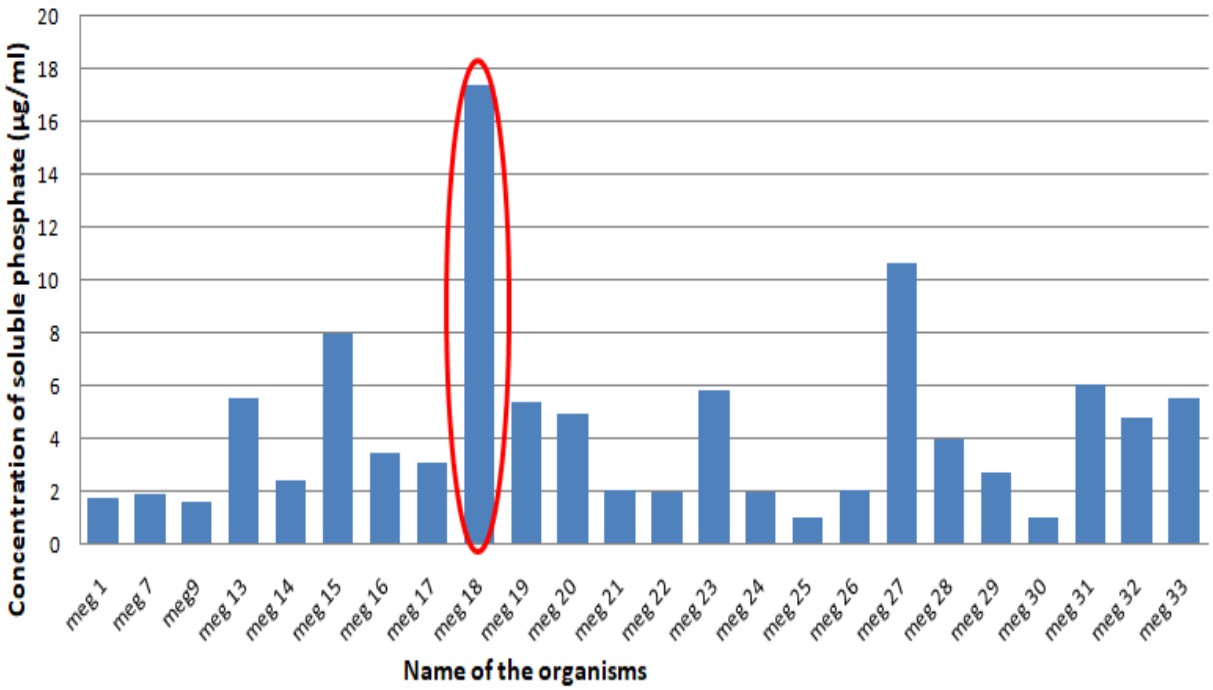

Figure 63: Quantification of soluble phosphate (µg/ml) by isolated *Bacillus megaterium.*

As described above the growing of isolates in the media, the isolates were incubated in the media with nHA as sole phosphate source for 10 days at 28°c and the amount of soluble phosphate in the media was calculate after titration using the constants derived from calibration curve. The amount of dissolved phosphate in the media of different isolates varied but isolate *Bacillus megaterium* 18 had around 17.38 µg soluble phosphate/ml media. This was the highest amount followed by 10.25 µg soluble phosphate/ml media with *Bacillus megaterium* 27. Thus, this clearly indicates that blending of screened putative *Bacillus megaterium* along with nHA could be developed as potential phosphorous fertilizer with judicious use of this non-renewal limited resource that is critical for every life including that of human.

Moreover, the growth of the bacteria seems to be linear further indicating that it has consumed the soluble phosphate for the cellular growth pertaining the optimal availability of other nutrients such as N-source, sulfur and potassium and other minerals and metals. This indicates that the P-solubilizing bacteria sense abundance of other nutrients and solubilizes HA to the extent where it can match stoichiometric amount of P-required with respect to other nutrients for bacterial population growth before entering the stationary phase.

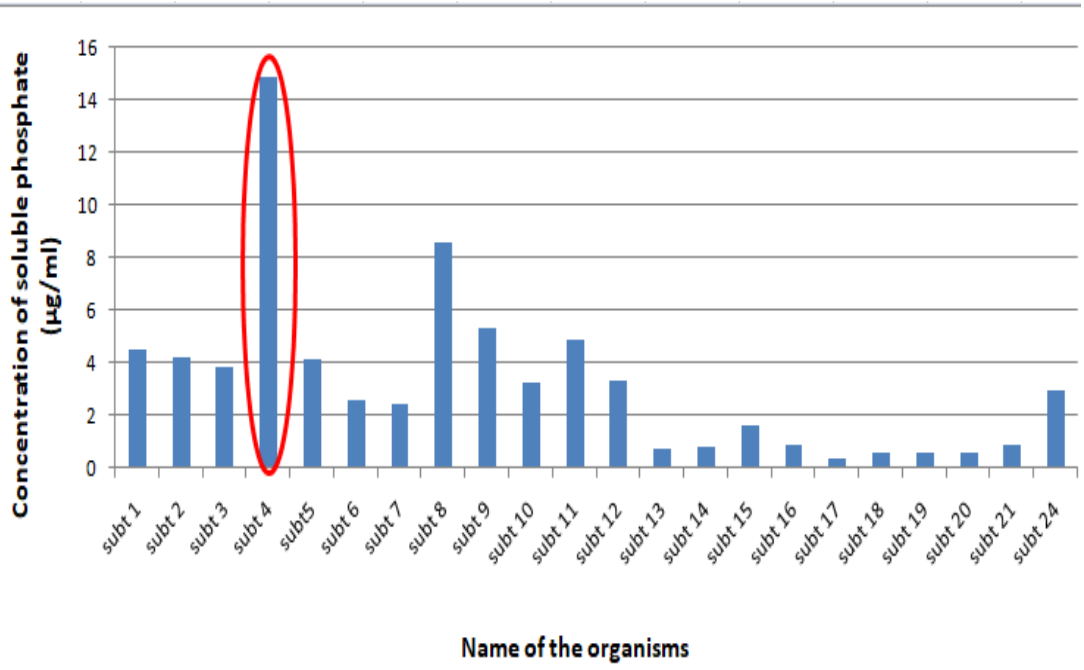

Figure 64: Quantification of soluble phosphate (µg/ml) by isolated *Bacillus subtilis*.

The isolates were incubated in the media with nHA as sole phosphate source for 10 days at 28°c and the amount of soluble phosphate in the media was calculate after titration using the constants derived from calibration curve. The amount of dissolved phosphate in the media of different isolates varied but isolate *Bacillus subtilis* 4 had around 14.88 µg soluble phosphate/ml media. This was the highest amount followed by 8.6 µg soluble phosphate/ml media with *Bacillus subtilis* 8. Thus, this clearly indicates that blending of screened putative *Bacillus subtilis* along with nHA could be developed as potential phosphorous fertilizer with judicious use of this non-renewal limited resource that is critical for every life including that of human.

Chapter 5
SUMMARY

5 SUMMARY

Global population growth and increased anthropogenic activities are perceived to have created threat to the environment. Use of fossil fuel has been one of the concerns of global climate change and there is mounting pressure to find alternative to the fossil fuel. In addition, with the increased income change in food habit has been observed thus the rising competition for food, feed and fuel has changed the society from past to now as same cereals are used creating "Triple F" effect. Thus, the sustainable development of bio-fuel production from more ecofriendly option is sought to some extent. Substituting cereals with lignocellulosic biomass for bio-ethanol production has been done. However, during the production of bio-ethanol from the lignocellulosic biomass the lignin has to be removed and pretreatment is done by strong acid/alkali that results in the formation of fermentation inhibitors that has to be separated from the fermenting broth. This is another issue that need to be addressed. So, to overcome these problems use of microorganisms that can degrade these inhibitors could be better options but same microorganisms should not be pathogenic and have market value. *Bacillus megaterium* and *Bacillus subtilis* that are in GRAS category which are also said to be probiotics and are PGPB that has ability to solubilize embedded insoluble phosphate in agriculture practice could be an option. Thus, these strains were isolated, screened and molecularly characterized. The protocol for isolation of these strains have been developed and validated. These bacteria were incubated into the acid treated lignocellulosic biomass i.e. *Sachharum sponatneum*. The growth of bacteria indicated that they utilized the reduced carbon present in the lignocellulosic biomass pretreated solution as the carbon source for their growth as these organisms are heterotrophs. Moreover, they were able to degrade the inhibitor products like furfural and phenol as was shown by their successive growth and decrease in the furfural and phenol content. This indicated that the pretreatment sludge of lignocellulosic biomass based bio-ethanol producing facility could be used to propagate the beneficial bacteria that would not only reduce the cost of managing these wastes but would also add additional income to the industry from sales of these bacteria that could potentially make the bio-ethanol industry to compete in the market with fluctuating fossil fuel price.

Moreover, as these bacteria are the strong phosphate solubilizer both organic and inorganic which are also known to solubilize the sparingly soluble or insoluble (like HA) form of phosphate source they could be good PGPBs for phosphate fertilizer

management. Thus, to have “Cyclic Economy” of non-renewal and fast depleting phosphate sources bovine bone was used to make nHA and it was synthesized from raw buffalo bone to be used as a phosphate source. Since nHA is insoluble and if it is applied as P fertilizer with these bacteria it could potentially minimize the problems created by the water-soluble phosphate fertilizer that creates eutrophication in downstream water bodies. The phosphate solubilizing ability of these isolates were shown by their growth in the media containing nHA and the amount of solubilized phosphorus was quantified using “A single solution reagent method”. This clearly indicated that these bacteria when applied to the field they will not only release the phosphate present in the soil as insoluble compound for their growth but for other organisms to make soil healthy but also to the plant roots to support agricultural productivity. The applied nHA could support in supplying sustained source of phosphate to be used in agriculture without it being washed since extending over last 80 years have shown that only 10-20% of applied phosphorus is utilized by the plants. Thus, it is suggested that an industry that encompasses production of bio-fertilizer and bio-ethanol could be developed to supply fuel and fertilizer from lignocellulosic biomass and reduce the country’s import cost by reducing import of the fossil fuel (present day diesel engine can easily be blended with 15% ethanol) and chemical fertilizer.

Chapter 6

CONCLUSION

6 CONCLUSION

Bacillus megaterium and *Bacillus subtilis* that have potential to survive in phenol, catechol or furfural along with the phosphate solubilizing potential bacteria were screened using different reduced carbon sources to differentiate them with other bacteria. These bacteria were screened based on their ability to grow in catechol and phenol thus were used to utilize the fermentation inhibitors like furfural, HMF and phenol produced during pretreatment of lignocellulosic biomass to release cellulose for saccharification and subsequent fermentation. Since management of pretreatment sludge is costly its utilization to develop additional value-added product would not only reduce the cost of bio-ethanol production but would generate additional revenue from sales of these bacteria thus making the bioethanol production more feasible. In addition, their P solubilizing ability was determined by incubating them into the media containing nHA. Thus, HA solubilizing bacteria could be one of the options of supplementing phosphate in the soil because HA nanoparticles could be made from the bones, the phosphate provided from the cereals to build-up bones could be reused to provide phosphate to plants to produce cereals and making “Cyclic Economy” of phosphate. Use of HA nanoparticles made from bones, if possibly be used as fertilizer can provide both phosphate and calcium which plays pivotal role in plants host pathogen interaction to module plant immunity. It would be better to refer as HA in a county like Nepal because it would not affect the Hindu sentiment and can use wasted bone as fertilizer. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases which play a major role in the mineralization of insoluble organic phosphorous in soil, hence, these bacteria could be used as fertilizer supplement to release phosphate from nHA. Thus, an integrated approach of industry that is involved in both bio-fertilizer and bio-ethanol production can benefit the globe. This could economically support bioethanol production facility that could be profitable even by using agricultural wastes as sugar sources.

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8 APPENDICES:

Appendix 1: List of media used in the study

Sodium acetate agar media (1000 ml, pH 7.0)

Sodium acetate	10 gm
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm
$MgSO_4$.7H2O	0.5 gm
$CaSO_4$.7H2O	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml
Agar	12 gm

Nutrient agar media (100 ml)

Pepton	0.5 gm
Beef extract	0.3 gm

Agar	1.5 gm
Sodium chloride	0.5 gm

Tryptophan media (1000 ml, pH 7.0)

L-tryptophan	0.010 gm
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm
$MgSO_4$.7H2O	0.5 gm
$CaSO_4$.7H2O	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml
Agar	12 gm

Catechol media (1000 ml, pH 7.0)

Catechol	0.0055 gm
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm
$MgSO_4$.7H2O	0.5 gm
$CaSO_4$.7H2O	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml
Agar	12 gm

Phenol media (1000 ml, pH 7.0)

Phenol	0.0047 gm
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm

$MgSO_4.7H2O$	0.5 gm
$CaSO_4.7H2O$	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml
Agar	12 gm

Maleic acid media (1000 ml, pH 7.0)

Maleic acid	0.0058 gm
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm
$MgSO_4.7H2O$	0.5 gm
$CaSO_4.7H2O$	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml
Agar	12 gm

Mineral mixture (1000 ml)

Magnesium chloride	3 gm
Manganese sulphate	0.5 gm
Sodium chloride	1 gm
Ferrous sulphate	0.1 gm
Calcium chloride	0.1 gm
Cobalt chloride	0.1 gm
Zinc chloride	0.13 gm
Aluminium chloride sulphate	0.01 gm
Boric acid	0.01 gm
Sodium molybdate	0.025 gm

Vitamin mixture (1000 ml)

Biotin	2 mg
Folic acid	2 mg
Pyridoxine	10 mg
Riboflavin	5 mg
Thiamine	5 mg
Nicotonic acid	5 mg
Pantothenic acid	5 mg
Vitamin B 12	0.1 mg
P-aminobenzoic acid	5 mg

Appendix 2: Preparation of working solutions and buffers used in the study

Tris-cl (1 M, pH 7.5-8)

Tris-cl buffer was prepared by adding 12.11 gm of Tris base in 80 ml of Distilled water and pH was adjusted to 7.5-8 by adding the concentrated HCl. And the final volume was adjusted to 100 ml.The solution was autoclaved and stored at 4°c.

EDTA (0.5 M, pH 8.0)

Disodium EDTA.2H2O of 18.61 gm was dissolved in 80 ml of Distilled water, stirred vigorously on magnetic stir and pH was adjusted to 8.0 by adding NaOH. Finally, the volume was maintained to 100 ml. The solution was autoclaved ad stored at 4°c.

TE1 solution

50 mM Tris-cl (pH 7.5)

50 mM EDTA (pH 8.0)

TE2 solution

50 mM Tris-cl (pH 8)

50 mM EDTA (pH 8.0)

STEP solution

SDS 0.5%

Tris-cl 50mM (pH 7.5)

EDTA 0.2mM (pH 8)

Proteinase K 1 mg/ml

Lysozyme (1 mg/ml)

Lysozyme (1 mg) was dissolved in 1 ml of 10 mM Tris-cl (pH 8). The solution was prepared immediately before use.

50x TAE buffer (500ml)

Trizma base (Tris buffer) 121 gm

Glacial acetic acid 28.55 gm

EDTA 9.3 gm

Ethidium bromide (10 mg/ml)

Ethidium bromide of 100 mg was weighed and dissolved in 10 ml MilliQ water. The solution was protected from the sunlight by wrapping with aluminum foil and stored at room temperature**.**

Stock solution of phosphate

0.1757 gm of anhydrousKH_2PO_4 was dissolved in distilled water and was diluted to 1 liter. This solution was stored glass bottle for the further use.

5N H_2SO_4 solution

Concentrated H_2SO_4 of 70 liters was diluted to 500 ml.

4% ammonium molybdate solution

20 gm of ammonium molybdate was dissolved in distilled water and diluted to 500 ml.

0.1M Ascorbic acid solution

1.76gm of ascorbic acid was dissolved in 100 ml distilled water.

Mixed reagent

125 ml of 5N H_2SO_4 and 37.5 ml of 4% ammonium molybdate solution was mixed. To this solution, 75 ml of 0.1 M ascorbic acid solution was added and diluted to 250 ml. The mix reagent should be prepared an hour before use.

Appendix 3

Primer details and PCR conditions for 16s rRNA gene amplification

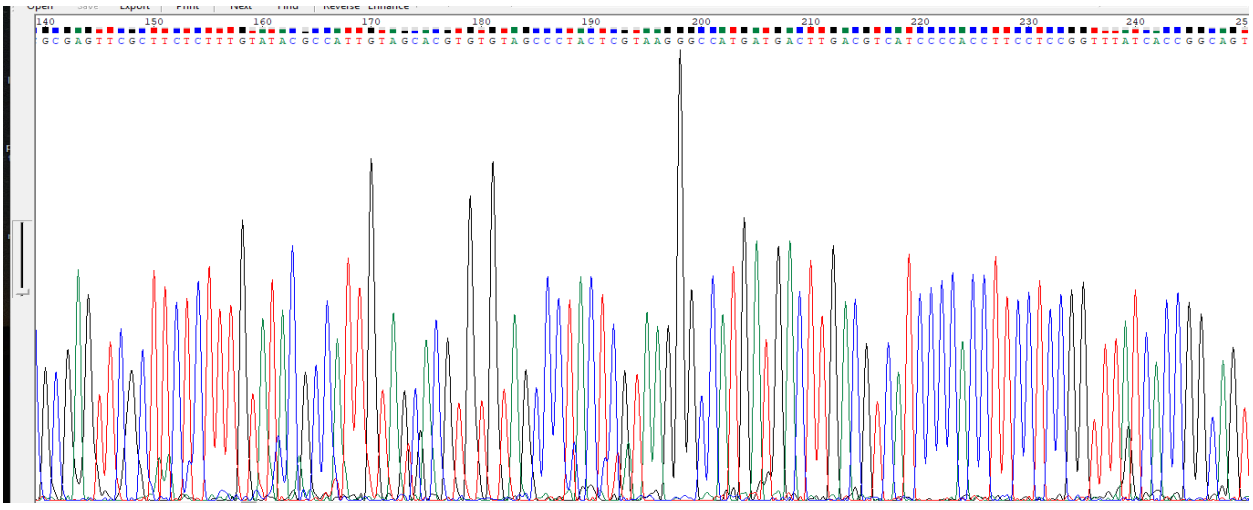
Name of primer	Tm	Sequences	Amplicon length (bp)
27F	56.4	AGAGTTTGATCCTGGCTAG	1500
1492R	56.4	CGGTTACCTTGTTACGACTT	1500

Volume of reagents used in PCR

Reaction mixture	Volume
DNA template	1 µl
Master mix	5 µl
Forward primer	1 µl
Reverse primer	1 µl
Nuclease free water	2 µl
	10 µl

S. N	Steps	Temperature (°C)	Time
1.	Enzyme activation	95	2 mins.
2.	Denaturation	95	30 secs.
3.	Annealing	52	30 secs.
4.	Extension	72	2.5 mins.
5.	Final extension	72	5 mins.
6.	Final hold	4	∞

Total 35 cycles



The 16S rRNA genomic DNA sequence chromatogram of isolated putative *Bacillus subtilis* S24 for forward primer.