Cattle bone derived biochar combined with phosphate solubilizing microorganisms as phosphorous fertilizer

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I, **Mahnoor khan** declare that this research work title "Cattle bone derived biochar combined with phosphate-solubilizing microorganisms as phosphorous fertilizer" is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-ur-Raman School of Applied Biosciences, NUST under the supervision of Dr. Saadia Andleeb. The material that has been used from other sources has been properly acknowledged/ referred.

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Dedication

I, with great pleasure and reverence, dedicate this work to the Holy Prophet (P.B.U.H) who inspired me through His saying "Learn from cradle to grave"

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List of Acronyms

Abbreviation	Meaning
FT-IR	Fourier Transform Infrared Spectroscopy
EDX	Energy Dispersive X-ray
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscopy
CB-BC	Cattle Bone biochar
$CB\text{-}BC_{850}$	Biochar produced at 850°C
CFU	Colony forming units
EC	Electrical conductivity
PSM	Phosphate solubilizing microorganisms
pH	Power of hydrogen ions
Rpm	Revolution per minute
HNO_3	Nitric Acid
ABC	Animal bone char
DAP	Diammonium Phosphate
RP	Rock Phosphate
Hap	Hydroxyapatite
CEC	Cation exchange capacity
P	Phosphorous
K	Potassium

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Abstract

Agriculture is the Foundation of Pakistan's economy_ and that sector is struggling. Phosphate is the most essential and limiting nutrient required for Plant growth. Fertilizer Sector invest huge amount on Chemical phosphate fertilizer production (DAP, TCP, SSP etc.) utilizing local phosphate rock deposits_ Phosphate rock is a source of heavy metal pollution of air, soil, water and food chain etc. While Biotechnology offers a cost effective and sustainable solution to mitigate these problems by utilizing low value Animal bone grist. Bone is a natural source which contain high phosphate calcium content (>30%) P_2O_5 and other essential nutrients, 100% pure and organic. The purpose of this study is to investigate the potential of biochar produced from cattle bones together with phosphate solubilizing bacteria as Bio-phosphate fertilizer. The Cattle bone biochar was produced at 850 ◦C for 20min under Ar inert atmosphere in a tube furnace. In the first phase of study Physiochemical- characterization of biochar was done ash content, moisture content, volatile matter, proximate analysis and the chemical characterization was achieved through SEM, EDX, XRD and FTIR analysis techniques. In the second phase of the study bacterial screening was performed for phosphate-solubilizing bacteria (PSB) on four bacterial strains, two strains SPARC10 and HF43 showed clear phosphate solubilization zones around colonies and further these two strains were used to prepare 1-Liter of PSB liquid bioinoculant formulation and stored in refrigerator. In the third phase the efficacy of processed biochar along with microbial dose was evaluated on growth of maize plant in greenhouse trial. control group (T0), Biochar (T1), PSB Inoculation (T2), Biochar and PSB Inoculation (T3), Rock phosphate (T4), Di-ammonium phosphate (T5). Inoculation of PSB together with CB-BC increased the Plant growth in terms of plant height, number of leaves, leaf length, shoot and root dry biomass, and total P uptake in maize plant compared to the other treatments. The combined use of PSB inoculant and CB-BC was more economical due to minimal cost and maximum returns. These results suggested that PSB inoculation along with CB-BC would be an appropriate substitute for chemical phosphate fertilizer application in sustainable agriculture systems.

Chapter 1

Introduction

Many modern agricultural systems have suffered from degradation and a lack of sustainability in recent years. It is acknowledged that intensive use of chemical products, such as fertilizers and pesticides, might result in a variety of environmental issues, is the most likely source of this problem. Phosphorus (P) is an essential macronutrient after nitrogen for growth of plants, their yield, and seed germination. Even though most agricultural soils contain significant quantities of inorganic and organic Phosphorous, but these are immobilized and thus mostly becomes unavailable. As a result, the plant has access to a very low concentration of P.(Soumare et al., 2019). The average soil contains about 0.05% (w/w) P, but only small portion 0.1% of the total P is available to plants due to its poor solubility and its fixation in soil (Seema B. Sharma, Riyaz Z. Sayyed, Mrugesh H. Trivedi, & Thivakaran A. Gobi, 2013). In order to satisfy crop nutritional needs, P is typically added to soil as chemical P fertilizer; however, the synthesis of chemical P fertilizer is highly expensive and energy intensive process. It has long-term environmental consequences in terms of eutrophication, soil fertility depletion, and carbon footprint, harmful impacts on the soil structure, composition, microflora and other properties of soil (Kaur & Reddy, 2015) **.** Furthermore, plants can only use a small portion of this P because about 80% of added P forms complexes with the metal-cations and rapidly fixed in soils. Such environmental concerns have prompted to find some sustainable method of supplying P to crops. Despite the fact that Pakistani soils are rich in P, they are P deficient in 80–90% of cases due to availability issues(Aimen et al., 2022) .

Worldwide P is being used at very high rates especially from 1961 to 2007(Lu & Tian, 2017). In rock phosphate mining and P fertilizer industries one of the major sources of concern in the is that, according to some estimates, these low-cost rock phosphate reserves could be depleted in as little as 60-80 years, as rock phosphate apatite material has become a limited and non-renewable resource(Cordell & White, 2014). Numerous crises resulting from P shortages encouraged researchers to increase P efficiency by reducing its losses in agro-ecosystems(Fernandez-Mena, Nesme, & Pellerin, 2016). Due to the shortage of fertilizer and the rising demand for food, it is important to boost farm production(Vos & Bellù, 2019).

In this regard the utilization of phosphate-solubilizing microorganisms (PSM) combined with residues of combusted animal bones combined can prove to be the best eco-friendly means for

P nutrition of crop. Results on using animal bone char in conjunction with P-solubilizing microbes have surfaced in the last few years(Singh et al., 2020). In one of the most recent research in this area, 97 bacterial isolates from various soils were chosen and tested for their capacity to solubilize ABC and enhance the development and health of plants(Postma, Nijhuis, & Someus, 2010). With rising demand for poultry and meat in developed nations like Pakistan, the production of animal waste, including bones, is on the rise. Every year, millions of tons of chicken, mutton, and beef bones are produced. Animal bones contain a high concentration of nutrients, minerals, and proteins, such as phosphorus (P), calcium (Ca), nitrogen(N), zinc (Zn) and manganese (Mn) and they are frequently discarded as municipal solid waste(Jeng, Haraldsen, Grønlund, & Pedersen, 2006). Biochar is currently gaining worldwide attention due to its CO_2 sequestration and long-term use in agriculture land practices (Mei et al., 2022). Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) making up a significant portion of both human and animal body tissues, because of its biocompatibility, has been widely employed as a bio ceramic (DileepKumar et al., 2022). Most HAPs studies focus on their use in biomedical (Sobczak-Kupiec et al., 2021) and their potential in agricultural applications, except for a few (Madusanka et al., 2017). According to Dong et al. (Pan et al., 2021) biochar functions as an slow-release fertilizer, as it gradually increases the soil's accessibility to nutrients and reduces nutrient loss through leaching (El-Naggar et al., 2019). Biochar thereby enhances crop production in addition to acting as a soil conditioner(Azeem et al., 2022). Hossain et al. (Hossain et al., 2020) also suggested that biochar can be used as organic fertilizer which not only improves soil physical properties but also enhances nutrient accessibility. It also has the water retention ability due to its unique surface area and porous nature. Hence the type of biomass feedstock , heating temperature, and other conditions provided during pyrolysis have the greatest influence on the morphological and physicochemical properties (Tomczyk, Sokołowska, & Boguta, 2020). Earlier, bone char from animal sources has been used as an biological fertilizer due to having an excessive amount of Ca/P(M. Wang, Liu, Yao, Han, & Liu, 2020)

A waste management method is used in the production of biochar, in which biomass is pyrolyzed (wood, food, and yard waste) and added to soil for soil amendment to sustain ecosystem services. Biochar additions have been demonstrated to meet scale-dependent parameters and criteria in soils, offering natural solutions like carbon uptake and moisture retention, cation exchange capability, soil organic matter (SOM), enhancing soil fertility and raising pH to support soil management and ecosystem services (Liang et al. 2006; Van Zweiten et al. 2010; Qian et al. 2014; Keesstra et al. 2018b). Though part of the nutrients from the used biomass are retained by biochar, it works best to hold onto and slowly release the nutrients to be utilized by the plants (Alling et al. 2014; Brantley et al. 2015; Hagemann et al. 2017), thus systematically regulating nutrient release. Depending on the manufacturing process, biochar production and application can also improve soil preservation, carbon sequestration, and biomass development (Jien and Wang 2013; Malghani et al. 2013; Thomas and Gale 2015). These elements also satisfy ecological services related to the natural remedies proposed by Keesstra et al (2018b). In contrast to other organic amendments, biochar is highly stable and resistant to biochemical degradation nonetheless, its porous nature can offer an ideal carrier material or shelter for the survival of microbes (Thies and Rillig 2009; Chen et al. 2012).

Interdisciplinary areas of engineering and science have come up with the growing attention in the advantages of biochar application (Ahmad et al., 2014). Biochar that is the product of thermal degradation and basically it is carbon-rich content and reported as "black carbon" (Lehmann and Joseph, 2009) and it is different from charcoal. the ability of biochar to use as "soil amendments". Biochar provides different services in the ecosystem in different aspects. (Lehmann *et al*.,2011). It is the organic matter that is burnt and generated intentionally to make it applicable to the soil for carbon C sequestration for the upgrading of soil properties (Lehmann and Joseph, 2009). Biochar applications can improve soil fertility, C sequestration, recycling of agricultural waste/ product and remediation of pollutants (Ahmad *et al*., 2014).

The properties of biochar like residence time, biomass type, pyrolytic temperature, and heat transfer rate are the important parameters that direct the changes in the properties of biochar. Organic matter and pyrolytic temperature chiefly affect the properties of biochar. Since both pyrolysis conditions and biomass govern physical and chemical properties, so the aim for biochar amendment can be evaluated by biochar manufacturers and designer. biochar can be generated by modifying the pyrolysis and feedstock protocol for remediation of a soil issue (Bagreev *et al.*, 2001).

The most riveting aspect of biochar is attributed to the reason that biochar is representative for easy-produced, low-priced and sustainable process costing not much, and effective way to improve soil fertility comparison, to products from chemical processes. In recent years biochar gained great consideration due to great number of applications with remarkable effects in vivid areas. These applications include purification of water, gas and energy storage and catalysis (Qian *et al.*, 2015). Biochar also play an essential role as absorbent due to high ability to adsorb contaminants from soil (Uchimiya *et al.,* 2010). It plays a significant role in environmental management. One of the essential aspects of using biochar is that carbon content present in biochar has half-life longer then thousands of years, that seeks great attention for carbon sequestration.

The authentic evidence from many researchers shows that in comparison with other soil amendments biochar is more stable and upsurge the availability of nutrients, these properties of biochar make it more influential as compared to other biomass present in the soil (Lehmann and Joseph 2009).

The topic of biochar is of increasing interest due to its vast applications like the alteration in the soil biota is due to applying biochar to the soil. This series of research is quite significant due to the diversity in microbial population and health in the soil is steps to evaluate the ecosystem services and function of soil, carbon storage capacity, proper aeration, and disease resistance. (Brussaard,1997). Biodiversity in the soil is greatly managed by these sorts of organic amendments. It is widely known that the trophic level food web in the soil is influenced by the excellence, amount and dispersal pattern of organic amendments (Moore *et al.,* 2004). It has been known that biochar has great applicability in improving storage of nutrients, upsurge the carbon content, maintain the soil pH, improves the water holding capacity, lessens the toxicity level of Al, tensile strength, greenhouse gas emission (CH_4 and N_2O), change the microbiology of soil, makes the soil habitat better for earthworm population and improves the effectiveness of fertilizer usage (Downie *et al.,* 2009). The perspective and multi-benefits of biochar in combination with its low cost and environmentally friendly feature for reclamation of soil offers motivation for further research.

Phosphate solubilizing microorganisms (PSMs) are a class of useful bacteria that can hydrolyze insoluble organic and inorganic phosphorus compounds. A number of bacterial (*pseudomonads* and *bacilli*) and fungal (*Aspergilli* and *Penicillium*) strains have been recognized as phosphate solubilizing microbes. It has been used as a biofertilizers for agricultural enhancement for years but have yet to be adequately commercialized (Ruzzi & Aroca, 2015). After wheat, rice, and cotton, maize ranked Pakistan's fourth-most significant crop. Additionally, it is equally important as a staple crop in other nations besides Pakistan. It is a member of the group with rapid growth, high biomass production, and high P need (Mengel and Kirkby, 2001).

Biochar plays a significant role in the production of crops, assuaging climate change, bioremediation in the environment that is adulterated with poisonous contaminants and reprocessing of agriculture waste, so we can say that biochar has multiple benefits and have both direct and indirect effect on the environment. For instances, the increase in the crop yield make plants to absorb more carbon dioxide to sequester the more carbon, and the remediation effect of biochar helps in removing the contaminants and helps in maintaining soil and make the soil disinfectant, clean and healthier that will guarantee the standard progress in development of various variety of crops. The numerous significances of biochar are interlinked through each other and in this way, and consequently, a virtuous circle is formed once one characteristic is promoted. Thus, in modern society, biochar can be potentially a striking alternative to resolve the environmental related problems in an economical way which is facing by our society due to the rapid increase in population. Win-win consequences can be obtained by using biochar in the right way which means that the appropriate amount of biochar at the contamination site (Novak et al., 2013).

Objectives

The objectives of the present study are:

- Synthesis and Characterization of Cattle bone derived biochar (CB-BC)
- Formulation of phosphate solubilizing bacterial liquid bioinoculant
- Evaluation of Cattle bone biochar and PSB liquid bioinoculant with the commercial fertilizers on Maize crops.

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Chapter 2

Literature review

2.1 Environmental impacts of phosphate fertilizer

There are various types of studies in the literature that are related to the environmental impacts of PF industries. Previously, quite enough research on radioactivity in phosphate rocks, fertilizers, and PF industrial waste had been published.

2.1.1 Phosphate Rock

Phosphate deposits can be found all over the world, almost on every continent. Approximately 93% of the phosphate rock produced is used to make mineral fertilizers, primarily di-ammonium phosphate (DAP), mono-ammonium phosphate (MAP), triple superphosphate (TSP), and single superphosphate (SSP). In order to produce PFs in Pakistan, Tufail et al. (Tufail, Akhtar, & Waqas, 2006) gathered two different types of PR samples from Jordan and Hazara deposits in Pakistan. Along with measuring the chemical composition of the PR samples, researchers also looked at their radioactive composition and that of the samples of collected fertiliser. The scope of the health harm posed by phosphate rocks was thoroughly disclosed by this investigation.

2.1.2 Phosphate Fertilizers Source of heavy metals

Heavy metals have both lithogenic and anthropogenic sources, weathering of rocks contributes to the high amount of heavy metals in the environment which is a natural way of their entry in the environment. Beside these anthropogenic sources include mining, industrial and agriculture waste.

In Pakistan, both imported and domestically produced phosphatic fertilisers are utilized. (Khan & Abbasi, 1998) measured the natural radioactivity in these fertilisers. The study's findings showed a greater level of radioactivity that could expose operating personnel to dangerous levels of radiation because of the risk of radon buildup in poorly ventilated regions. The concentration of several harmful heavy metals (Cr, Cd, Cu, Ni, Pb, and Zn) in the various fertiliser types (DAP, TSP, and NPK) was measured by (Giuffréde López Carnelo, de Miguez, & Marbán, 1997). The findings demonstrated examined materials had Cd and Pb concentrations that were much greater than those found in soils naturally. According to the study, constant fertilization of soils may cause heavy metal levels to exceed natural soil abundances and enter the human food chain.

2.1.3 Effects of heavy metals

Massive concentrations of heavy metals are extremely harmful to the environment's health, the production of crops and livestock, the quality of food and water, and ecotoxicology. (Bååth, 1989). Due to fast industrialization, urbanization, and unsuitable environmental policies, heavy metal concentrations have dramatically increased in developing world like Pakistan, raising concerns for both the environment and human health (Xu et al., 2014). Furthermore, because heavy metals are not biodegradable, they bioaccumulate via the food chain, and occasionally organisms may enhance these harmful metals, converting them into organic complexes that produce more toxic forms(Bai & Abraham, 2003) They also have detrimental effects on water bodies and the quality of the atmosphere, which can directly or indirectly endanger human health. Excessive use of non-essential heavy metals like cadmium and lead is dangerous because they are carcinogenic and can lead to various types of life-threatening cancer, renal dysfunction, and changes in the hematology (Martin and Griswold, 2009). These metals have the ability to impede biodegradation and interact with proteins (enzymes), which hinders cellular metabolism.

2.1.4 Effects of heavy metal on biotic and abiotic factors of environment

In the ecosystem, they pose long-lasting toxic effects and shows a bad influence on physical and biological processes and activities. Due to their excessive use in the emerging fields of industry and pyrotechnics, and high bioaccumulation and toxicity, heavy metals act as the major source of abiotic stress agent for living organisms, and due to their high reactivity, they can directly impact the growth, energy synthesis processes and senescence (Maksymiec W, 2007). Other than their influence on abiotic factors these metals also have adverse effects on plants, animals, fungi and other microorganisms. Even if essential trace elements exceed a certain concentration and exposure times, it becomes toxic to the soil, animals thus affecting abundance, diversity and animals' distribution in the environment (Lee et al.,2002)

The main anthropogenic sources of heavy metals include industrial waste disposal and combustion activities, as well as hazardous solid transportation. Additionally, long-term pesticide use can add potential poisons including cadmium, nickel, zinc, and copper. Other activities like mining can also generate heavy metal pollution in an ecosystem's soil, water, and air (Nicholson, Smith, Alloway, Carlton-Smith, & Chambers, 2003). Through a variety of human and geochemical activities, these heavy metals are exported from their initial waste source to the surroundings through intricate processes. The major factors that have a negative impact on the solubility of these metals include pH, the presence of complexing reagents including organic acids, carbonates, chlorides, and sulphates, as well as the characteristics of the redox potential and solid waste phase. Even if they only slightly exceed the threshold value, these heavy metals are hazardous to organisms at all levels and have been shown to be carcinogenic, and mutagenic as well. Numerous studies have shown that heavy metals can change the physiochemical and biological characteristics of the environment and of organisms, respectively. the biggest issue facing the environment.

2.2 Biochar

In the absence of oxygen, organic waste (such as woodchips, peanut shells, spent tea leaves, agricultural waste, chicken dung, animal bone, and poultry manure) is thermally decomposed to produce biochar. (Lehmann and Joseph, 2009). It has a smaller molecular weight, a porous structure, a high carbon content, and a low to medium temperature range (450 to 650°). (Lehmann et al., 2011). The production of biochar from these materials transforms carbon (C) into a recalcitrant form that may persist for hundreds to thousands of years (J. Wang, Xiong, & Kuzyakov, 2015), indicating that biochar may help in carbon sequestration as a negative greenhouse gas emission technologies with concomitant benefits for sustainable development (Smith et al. 2019).

2.2.1 Main components of Biochar

The major components of biochar are hydrogen(H), carbon(C), phosphorus(P) nitrogen (N as well as oxygen(O) that contains that function groups (Park et al., 2013). These surface charges, functional groups and large surface area of biochar are major determinants for immobilization of heavy metals (Zhang et al., 2013).

2.2.2 Biochar production

The thermal degradation of biomass produces syngas, bio-oil, and char, and a higher heating rate influences the final conversion of biomass (Fushimi et al., 2003) Biochar is best described as a "soil conditioner" since biochar has a high degree of porosity and this porous structure provides the active site for adsorption and hydrostatic pressure further increase the water retention. Furthermore, it improves soil fertility.

2.2.3 Biochar properties

Biochar have been demonstrated to enhance environmental quality over shorter time scales by sorbing heavy metals and organic contaminants, have a positive impact on soil water holding capacity for a longer time (e.g., Lentz et al. 2019; Kammann et al. 2011), carbon sequestration (e.g., Fuertes Mendizábal et al. 2019; Borchard et al. 2018; Jefery et al (e.g., Laird et al. 2017; Novak et al. 2016; Liu et al. 2013). The final biochar product can be significantly influenced by feedstock choice, pyrolysis temperatures, and pyrolysis types (Cao et al. 2017; Cha et al. 2016). Therefore, deeper insight of the relationship between feedstock, heating temperature, and production method would facilitate stakeholders on biochar in making better decisions about its use.

2.2.4 Application of biochar

- Biochar act as bio fertilizer so it increases the overall yield and growth of plants.
- It is purely carbonaceous in nature, so it enhances the total carbon pool of soil.
- Biochar is extremely porous in nature therefore it maximizes the water holding capacity of soil.
- Biochar is alkaline in nature if it is introduced into acidic soil it elevates the soil Ph.
- Biochar helps in sequestration of carbon.
- Whenever biochar is amended into the soil it enhances the micro biota of that particular soil.
- Biochar is recalcitrant to microbial degradation, so it can remain in the soil up to 800 or 1000 years.
- Biochar remediates the soil and water which are contaminated with heavy metals.
- Biochar plays essential role in conservation of food, because it has capability to absorb the humidity.
- Biochar can be used for the sanitation purposes because it is an excellent absorber.
- The application of biochar in reducing the greenhouse gases is remarkable.

2.3 Factors affecting biochar characteristics

2.3.1 Type of feedstock

The various physicochemical aspects of biochar, such as how its characteristics are greatly influenced by the type of feedstock used, as well as its interaction with feedstock at various pyrolytic temperatures, are the main determinants of the uses and functions of biochar. The physical and chemical qualities of biochar affect its various characteristics, including surface area, porosity, and adsorption capacity. Corn, wheat straw, and rice straw/husk were the main crop wastes used to make biochar. The manures/biosolids dataset also contains biochar created from papermill sludge, bovine and dairy manure, and other manure from different animal sources, poultry manure and litter, animal bone, other waste solids etc. The most frequent feedstocks used to make biochar were biosolids, cattle and dairy manure, pig manure, poultry manure, and pig manure.

Figure 1. Types of Feedstock

The most common source of the feedstocks used to manufacture biochar is solid waste, such as municipal garbage and agricultural waste. They are cheap and abundantly available (Schmidt et al., 2012). Additionally, non-native plant species are converted to biochar in this way, which is significant for managing invasive plants and environmental management. As a result, converting biomass to biochar, which acts as a great adsorbent, is a crucial step in improving environmental management (Cao et al., 2009). A variety of thermal processes, such as gasification, fast pyrolysis, flash carbonization, slow pyrolysis, and hydrothermal carbonization (HTC), are used to produce biochar. These processes use a variety of feedstocks, such as municipal waste, tea waste, wood biomass, crop residues, and animal manures (Meyer et al., 2011).

2.3.2 Temperature

The synthesis of biochar occurs through thermal conversion, and temperature is an important factor that influences the properties of biochar, such as the presence of functional groups on the surface of biochar, and thus they control the capacity of heavy metal sequestration in soils

(Uchimiya et al., 2010). Others have demonstrated that a specific surface area increased with rising pyrolysis temperatures (Ji et al., 2022) as a result of the solid matrix compression, which relatively reduces large pores and raises total surface area (Weber & Quicker, 2018); de Mendonça et al. 2017). Nutrient retention and pollutant sorption have historically been linked to surface area, but water availability and soil aeration are thought to be affected by pore volume, which typically rises with pyrolysis temperature (Ajayi, 2016); Qambrani et al. 2017).

As the carbonate and hydroxide phases in the resulting biochar inside the ash increase, it alternatively increases the pH values, it is evident that as heating temperature increase it also increases the ash content and pH of prepared. Temperature affects the surface area of biochar, micro porosity, and hydrophobicity so biochar produces at high temperature have a high rate of sorption due to all above mention factors, however biochar produced at low temperature is more suitable for the removal of contaminants by oxygen-containing functional group by electrostatic forces of attraction (Ahmad et al., 2014).

2.3.3 Effect of biochar on soil properties

The surfaces of biochar can absorb the nutrients and cations present in the soil (Ennis, Evans, Islam, Ralebitso-Senior, & Senior, 2012)due to which there occurs a rise in the level of nutrients available for microbial metabolism. The uptake of potassium due to biochar amendments is directly related to the increase in the pH of the soil (Yamato *et al.*, 2006)

Biochar composed of different type of nutrients, for example, N, K, Mg, P and Na and because of the ability of sorption there occurs increases in nutrients in soil because of its greater surface area and higher pore volume and its negatively charged surface (Ahmed & Schoenau, 2015)

Another important physiological factor that influences the microbial diversity and activity in the presence of biochar is pH. In the presence of biochar, pH remains stable in the favorable range from 5-6.4 (Leah Herbert *et al.*, 2012). Generally, biochar neutral to alkaline pH but some acidic biochar has been reported. There are several parameters that affect the pH of biochar like thermochemical process and type of the feedstock effects the intensity of pH. If the soil is acidic then alkaline biochar must limit the effect that ultimately increases the productivity of plants. Moreover, pyrolysis temperature also affects the acid neutralizing capacity of biochar.

It has been reported that biochar is generally alkaline when they are produced but by increasing the pyrolysis temperature from 300 to above 800 ° C results in increasing pH from 7.48 to

11.62. This occurred due to an increase in the ash content in the biochar produced at high temperature.

2.4 Biochar of animal origin

Animal bone derived biochar is a very attractive approach. Each year, millions of tonnes of bones from chicken and mutton are produced. Animal bones are rich source of many essential nutrients, vital minerals and proteins and are frequently included in municipal solid trash. Incineration and pyrolysis are the primary methods of disposal; both generate enormous volumes of ash and solids, the valorization of which is of great significance. Additionally, macrospores in bone-derived biochar contain carbon (10–24%), inorganics (Ca and Mg 75–90%), and P2O5 fertiliser (10–30%),(Azeem et al., 2021). Some of these traits make biochar made from bone stand out as a possible soil supplement and remediating agent with qualities similar to natural organic fertiliser(Koron, Lavrič, & Someus, 2018).

Hydroxyapatite is a major component of bone, which has been widely used as a bioceramic(Manalu, Soegijono, & Indrani, 2015). The majority of HAPs research focuses on their biomedical applications (Katti et al., 2008; Mateus et al., 2008; Ferraz et al., 2008). Except for a few (Montalvo, McLaughlin, & Degryse, 2015) their potential in agricultural applications has not been thoroughly investigated. There are naturally occurring phosphate rocks that are a type of hydroxyapatite and are commonly used as phosphorus fertiliser, but they have little solubility. As a result, phosphorus solubility in the form of bone-derived HAP is very likely to increase. Industrial animal bone residues are distinguished by their high calcium and P content (up to 47%). Consequently, due to bone residues energy content, the fertiliser sector can be used to valorize these wastes. As of now, the rehabilitation of heavy metal-contaminated sites has proven to be quite successful when soil enrichment is done using thermally processed bone meal byproducts. The low metal content of heat-treated bones is one of their most significant advantages. Although rock phosphates contain cadmium, lead, copper, arsenic, and other heavy metals, which bone char and bone derived biochar do not.

2.4.1 Microbial Solubilization of Animal Bone char

Results on the use of animal bone char (ABC) in conjunction with P-solubilizing microorganisms have emerged in recent years. One of the most recent studies in this area selected and tested 97 bacterial isolates from various soils for their ability to solubilize ABC and improve plant growth and health(Postma et al., 2010). ABC is a good biocontrol agent carrier because it provides them with a sheltered niche, delivers phosphate to plants, and simultaneously recycles P from food chain wastes. The highest concentrations of P were dissolved by Pseudomonas sp and Bacillus pumilus, followed by and three Streptomyces isolates. Different phosphate sources, including pyrolyzed animal bone char, which has not formerly been assessed for its role as a P fertiliser, were compared in another recent article by the same group. In that investigation, 12 distinct soils were incubated with animal bone char, Gafsa phosphate rock (GPR), and triple superphosphate fertiliser (TSP). The pH and P sorption of the soil were the two key factors in defining P solubilization from ABC. pH 6.1 (ABC) and pH 5 of the soil did not significantly affect phosphorus solubility (GPR). Additionally, it was shown that the amount of dissolved P increased with decreasing pH (Warren, Robinson, & Someus, 2009).

2.4.2 Reasons to select Cattle bone for biochar production

Numerous uses for bone char are possible in the agricultural, food production, and environmental sectors due to its high adsorption capacity for big organic molecules and distinctive macro-porous mineral structure, including:

- Organic P/Ca Fertilizer Recovered
- Biochar has low-cost production and they require minimal requirement for pretreatment. During production, no harmful substances are produced.
- Availability of biomass on large scale. Origin in the environment (from organic substances such as crop residues or animal waste)
- Soil microbiological carrier for integrated organic fertilization, biocontrol, and plant growth enhancement.
- The decolorization and refinement of sugar, the treatment of drinking water, the highly effective treatment of sewage water, contaminated subsurface water, and industrial process water.

2.5 Availability of phosphorous in soil

Phosphorus is an essential macronutrient and does not exist as elemental form in the soil. Both inorganic and organic phosphorus are present in the soil solution but mostly they are insoluble (Bueis, Bravo, Pando, Kissi, & Turrión, 2019). Because there is no exchange with the atmosphere and no other source can be made biologically available, its cycle in the biosphere might be regarded as "sedimentary". Therefore, phosphorus deficiency substantially limits crop development and production (Zhu, Li, & Whelan, 2018). About 0.05% of the soil is phosphorus. Although soil test results are typically significantly higher, the majority of them, 95 to 99%, are insoluble phosphates(S. B. Sharma, R. Z. Sayyed, M. H. Trivedi, & T. A. Gobi, 2013). The amount of soluble Phosphorous in soil solution is often quite low, ranging from parts per billion in extremely deficient soils to 1 mg/L in soils that have received heavy fertilization. Plant cells may absorb phosphorus in a variety of forms, however the majority is taken mostly as phosphate anions or depending on the pH of the soil. The application of phosphorus fertilisers is the principal source of inorganic P in agricultural soil (Bhattacharya, 2019). Nearly 70 to 90 percent of the phosphorus fertilisers added to soils are transformed from inorganic to organic phosphorus by cations. These are unavailable because they are insoluble forms. If these accumulating phosphates in agricultural soils could be transformed into soluble P forms by phosphate solubilizing microbes, they would be enough to maintain good crop production globally for many years (Kalayu, 2019). Therefore, it has become more important to find an alternative system with affordable technology that can provide enough P to plants.

2.6 Phosphorus solubilizing microorganisms (PSM)

Numerous microbiological species, including bacteria, fungi, actinomycetes, and algae, have the capacity to solubilize and mineralize P, mechanism of phosphate solubilization is shown in the figure below. The solubilization and mineralization of weakly accessible phosphorus by soil microorganisms has been documented various strains of Azotobacter (Alori, Glick, & Babalola, 2017), Bacillus (Alori et al., 2017). PSMs capable of converting insoluble phosphorus to soluble forms can be used as biofertilizers to improve the utilization of phosphorus accumulated in soils. This increases the amount of soluble phosphorus (Zhu et al., 2012).

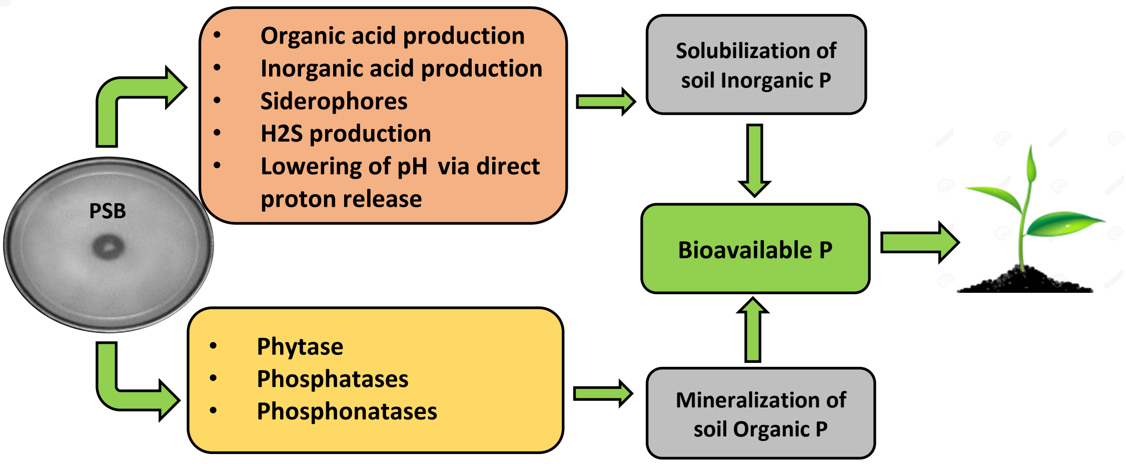

Figure 2. Mechanism of Phosphate solubilization

The use of phosphorus biofertilizers is a promising approach to improving food production by increasing agricultural yield because it is preferable to use an environmentally friendly approach to solving infertile soil problems. The use of phosphate-soluble microorganisms in saline-alkaline soil improves its fertility and suitability for agricultural use without posing the same environmental or health risks as long-term usage of synthetic fertilisers. Some of the phosphate solubilizing bacteria along with effect on host plants have given in the table below (Kalayu, 2019).

Table 1: Effect of PSM on growth and yield performance of different crops

PSMs	**Host plant**	**Reference**
Azotobacter	**Wheat**	**[9]**
Azospirillum spp.	**Maize, sorghum, and wheat**	**[9]**
Bacillus	**Peanut, potato, and wheat**	**[9]**
Bacillus megaterium and Azotobacter chroococcum	**Wheat**	**[9]**
Pseudomonas	**Zea mays L.**	**[6, 38]**
Pseudomonas chlororaphis and P. putida	**Soybean**	**[36]**
Pseudomonas fluorescent	**Peanut**	**[39]**
Pseudomonas putida and P. fluorescens	**Canola, lettuce, rice, wheat and tomato**	**[9]**
Lactobacillus plantarum	**Tomato**	**[116]**
L. plantarum	**Radish, tomato**	**[35, 109]**
L. Acidophilus	**Wheat**	**[120]**

Chapter 3

Materials and methods

3.1 Biomass collection

Cattle bones were collected from a local slaughter house. Bones were first washed with distilled water, sun-dried for 4-5 days then crushed into small piece via hammer and stored in a polyethene bag. The prepared feedstock was then used for pyrolysis.

Figure 3: Feedstock for CBBC

3.1.1 Biochar production

The cattle bones were first sterilized at 140°C for 30min at 1 bars of pressure in a vacuum oven. The pre-weighed sterilized bones were placed in boat crucibles and pyrolyzed at 850 ◦C for 20 minutes under Ar inert atmosphere in a tube furnace with rise rate of 10 ◦C min^{-1}. The pyrolyzed bone were taken out after the furnace is cooled down. After pyrolysis, a fine powder was made by grinding the biochar in mortar and pestle. Several trials were run to get the desired amount of biochar before to its application. The prepared cattle bone derived biochar was stored in polythene bags and kept closed prior to use.

Figure 4: Synthesis of Cattle bone biochar

3.2 Biochar characterization

To determine the physiochemical analysis of biochar various techniques were performed to analyze the physical and chemical aspects relate to biochar which is as follows

3.2.1 pH and EC determination

The Biochar pH and EC (electrical conductivity) was determined by the following protocol: Initially pH and EC meter were calibrated accurately. Biochar was soaked with deionized water at a 1:1 ratio for about 20 minutes in a shaking incubator at 28°C. After this the sample was passed through the Whatman filter paper no. 42 and then pH and EC were measured using pH meter ion lab pH 7110 and EC meter SM 301 Milwaukee, respectively (Richard 1954).

3.2.2 Proximate analysis

The value of moisture, ash, and volatile content in percentage values were calculated by following the ASTM standard procedure.

Biochar yield %

Dried bones were weighed and placed in boat crucibles and pyrolyzed at 850 ◦C for a residence time of 20min under Ar inert atmosphere with gradient rate of 10 ◦C min^{-1}. The biochar was weighed and yield was calculated as:

$$\text{Biochar yield \%} = \text{weight of biochar in grams/ weight of dry biomass} \times 100$$

Moisture percentage

The resulting biochar was placed in dry oven at 150°C for 2-3 hours. The crucible was left open throughout this procedure. The moisture was calculated to be:

$$\text{Moisture \%} = [(A-B)/A] \times 100$$

Where A= weight of biochar in grams, B= weight of biochar after drying at 150°C.

Volatile matter percentage

The sample was then placed in muffle furnace at 950°C for 7 minutes with the capped crucible and the volatile matter was determined as follows:

$$\text{Volatile matter \%} = [(B-C)/B] \times 100$$

Where C= weight of biochar after drying at 950 °C.

Ash content %

The uncapped crucible containing sample was then placed in muffle furnace at 750°C for 6 hours to determine ash content. The formula mentioned below was applied to determine ash:

$$Ash \% = D/B \times 100$$

Where D= the residue left.

Fixed carbon %

The fixed carbon was calculated as:

$$\text{Fixed carbon \%} = 100 - (\text{Ash\%} + \text{Volatile matter\%} + \text{Moisture content})$$

3.2.3 Fourier transform infrared spectroscopy FTIR analysis

To evaluate the effectiveness of biochar's surface organic surface functional groups for $CBBC_{850}$ were examined using Fourier transform infrared (FTIR) spectroscopy utilizing a Perkin Elmer FTIR instrument in the 4000-400cm-1 wave number range. One of the key aspects of FTIR is that the substrate exhibits characteristic emission or absorption in the IR spectral region, and depending on this property, both qualitative and quantitative results can be analyzed. This technique is used to obtain the infrared spectrum of emission or absorption of liquid, gas, or solid (**Bacsik et al., 2004)** About 1g of finely ground biochar was analyzed using FTIR to determine the functional groups of the biochar, such as the various functional groups present on the surface of the biochar

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3.2.4 X-Ray Diffraction XRD analysis

X-Ray Diffraction (XRD) is a laboratory-based technique commonly used for identification of crystalline materials and analysis of unit cell dimensions. Identification of crystallographic structure in the biochar produced was done, Data was collected over 2 theta from 20 to 60 degree. Identification of peaks was done by contrasting the obtained XRD patterns with standards referring from literature.

3.2.5 Scanning electron microscopy SEM analysis

The biochar was scanned with an electron beam using the scanning electron microscopy technique to view images of the particles made from various feedstocks at various temperatures. Scanning electron microscopy was used to study the surface morphology of biochar. The morphological changes that occurs in the surface of the biochar were evaluated by using scanning electron microscope that is armed with energy dispersion X-ray spectroscopy (EDX)

3.2.6 Energy dispersion X-ray spectroscopy EDX analysis

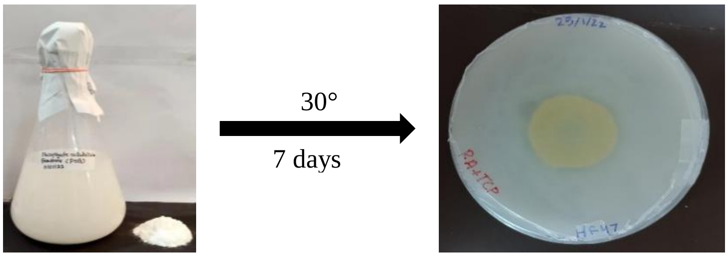
Energy-dispersive X-ray analysis is used in conjunction with (SEM) to determine the types and amounts of elements. To determine the quantitative values of certain elements, energy dispersive X-ray spectroscopy (EDX) was used to determine the elemental composition of prepared biochar.

3.3 Selection of PSB and liquid inoculum formulation

3.3.1 Screening for TCP solubilizing ability

Phosphate solubilization **s**creening test was performed on four bacterial strains. The potential of the microbial strains to solubilize phosphate was tested in a screening test. On sterilized petri plates containing Pikovskaya medium and tricalcium phosphate (TCP), the isolates were point-inoculated. The plates were incubated for 4–7 days at 37°C. After incubation, the size of the halo zones surrounding the colonies was measured on the plates. The following formula was used to compute the phosphate solubilization index. **(Premono et al., 1996).**

Solubilization Index (SI)= colony diameter + clearing zone diameter/colony diameter



Pikovskaya agar medium **Point inoculation**

Figure 5: Phosphate solubilization screening on Pikovskaya medium

While Phosphate solubilization efficiency of the strains were calculated according to formula below **(Nguyen et al., 1992)**

Phosphate solubilization efficiency = Solubilization diameter/ Colony diameter × 100

3.3.2 Liquid bioinoculant formulation

- For the preparation of liquid bioinoculant, a loopful of 24 h culture of SPARC 10 and HF43 in mrs agar media was inoculated into 1litre MRS broth each separately and incubating on a mechanical shaker at 150 rpm, 30 °C, for 24 h.
- The following day growth in the broth was observed, cultures were harvested by centrifugation for 25 min at 8500 rpm, 4 °C, in PRIMO R centrifuge.
- The pellet will be collected at the bottom, while the supernatant will be discarded. Finally, the pellet was gently cleaned with distilled water.
- The cell pellet was resuspended with sterile distilled water to make a final volume of 500ml for each strain. To form the consortia 500ml of each SPARC 10 and HF43 were mixed at the end to obtain 1liter liquid formulation.
- The number of cells in formulation was adjusted to 1×10^7 cfu/ml by measuring the optical density at 540 nm wavelength $OD_{(540nm)}$.

Figure 6: Liquid bioinoculant formulation

3.4 Plant material

Maize crop seeds were attained from a local shop. Initially seeds were first washed with sterile distilled water for 3–4 times and then were stored in a polythene bag.

3.5 Experimental design

Greenhouse field experiment were directed in a completely randomized block design, consisting of 6 treatments: control soil, soil + biochar, soil + PSM consortium, soil + Biochar + PSM consortium, soil + RF, soil + DAP diammonium phosphate fertilizer. Each treatment plot was of 4 m × 4 m (16 m^2) size. The plot was divided into 6 sections and 3 rows were maintained in each section. Three replicates were set up for each treatment in this way. Biochar was added in soil at the rate of 15 g per 3 kg of soil and mixed properly. Rock phosphate was amended in soil at the rate of 15g per 3 kg of soil before sowing. Similarly, Diammonium phosphate (DAP) was added at a rate of 15 g per 3 kg of soil. The PSB inoculum was applied once every week at the rate of 1ml/seed for 65 days.

Rows were 30 cm apart, and the seeds were sowed in each row with a gap of 20 cm between the seeds. Five air-dried seeds of Maize crop were immediately sown at 2 cm depth in each row. The germination rate was calculated one week after sowing and seedlings were thinned down to three in each row. All the plots were irrigated once before sowing to ensure proper germination of seeds and then regularly during crop growth as per agronomic practices.

Figure 7: Greenhouse trial on Maize crop

3.6 Parameter studied

Following parameters were studied during the growth of Maize crop plant

3.6.1 Germination rate

After one week of sowing, germinated seeds were counted and the germination rate was calculated by the following formula:

% Germination = (Number of seeds germinated/ Number of seeds sown) × 100

3.6.2 Plant Growth parameters

After 65 days of Vegetative growth cycle of maize crop, shoot length, no of leaves, leaf length, Stem girth of all the plants in each treatment is measure using a measuring tape. Whereas fresh of roots and shoots (including leaves) along with root length were determined in replicates of three from each treatment on a weighing balance after that root and shoot samples were kept in drying oven at 60°C for 24 hours. The dry weight of shoot and roots were measure on weighing balance and the chlorophyll content was determined via Chlorophyll Soil Plant Analysis Development (SPAD) meter (SPAD 502, Minolta, Japan).

The plants were harvested taking all the precautions so that roots remained intact.

3.7 Post harvested soil analysis

After the experiment, the physico-chemical properties of the soil were examined. To achieve this, soil samples were collected, air dried, smashed in a pestle and mortar, and sieved through using a 2mm sieve. Soil cores were selected from each treatment group and examined as a composite.

3.7.1 Determination of Soil pH and EC

The pH and EC (electrical conductivity) of composite soil sample was determined by the following protocol. Initially pH and EC meter were calibrated accurately. 1g of soil sample was soaked in 10ml deionized water at a 1:1 ratio for about 20 minutes in a shaking incubator at 28°C. The soil sample was allowed to settle for about 30 minutes after then pH and EC were measured using pH meter ion lab pH 7110 and EC meter SM 301 Milwaukee, respectively (Richard 1954).

3.7.2 Available Phosphorous, Nitrate-nitrogen and potassium content in post-harvest soil

For estimation of available phosphorus, Ammonium Bicarbonate-DTPA method for multi-element soil analysis test was used (Soltanpour and Workman 1979). Potassium content of the soil samples along with Nitrate Nitrogen (NO_3-N) were also determined via this method.

Procedure

Extraction:

- Weight 10 g air dry soil into 125 ml conical flask.
- Add 20 ml extraction solution (Ammonium bicarbonate-DTPA)
- Shake on a reciprocal shaker for 15 minutes, now filter the suspension using Whatman No. 42 filter paper.

Nitrate-Nitrogen Analysis

Add 1 ml of soil extraction to a 25 ml test tube. Add 3 ml of the working copper sulphate solution. Add the working solution of hydrazine sulphate. Add 3 ml of the working NAOH solution. In a water bath at 38 °C for 20 minutes, thoroughly combine. Take out of the water bath. Now stir in 3 ml of color development reagent. After 20 minutes, check the absorbance of the blank, the standards, and the samples on the spectrophotometer at 540 nm. Create the standards calibration curve by graphing absorbance vs sample nitrate-nitrogen concentration. Now read NO_3-N concentration of the samples from the calibration curve.

Phosphorous Analysis

Add 1ml soil extract to 10 ml of distilled water. To avoid sample loss due to excessive foaming, carefully add 2.5 ml color developing reagent. Stir thoroughly. After 30 minutes, use the Spectrophotometer to measure the absorbance of the blank, standards, and samples at 880 nm. Prepare the calibration curve for standards by plotting absorbance versus sample P concentrations. Using the calibration curve, determine the P concentration of the samples.

Potassium Analysis

Available potash in soil extract can be directly determined using a spectrophotometer and a K hollow cathode lamp. Run a number of appropriate K standards first, then create a calibration curve. Obtain K concentrations from soil samples by analyzing emission levels. In accordance with the calibration curve, compute the K concentrations now. The soil texture was determined through Hydrometer method, explained by DAY 1965.

3.8 Plant analysis for phosphorous uptake

Plants were harvested in replicate from each treatment taking all the precautions so that roots remained intact. The oven-dried (at 60 ◦C for 48 h) shoot and root samples were processed for the estimation of total phosphorus content. Total phosphorus content of plant samples was estimated by Rashid (1986) method. This method consists of wet digestion of plant material using HNO_3-$HClO_4$ followed by UV- spectrometry.

Procedure

Weigh 1g dry and ground plant material and then transfer into a 100 ml conical flask. Add 10 ml (2:1) nitric acid perchloric acid mixture, sample was placed in oven for 1 hours at 150 ◦C. After the sample is cooled down, place funnels in the mouth of tubes to extract the digested sample. After 30 minutes, use the Spectrophotometer to measure the absorbance of the blank, standards, and samples at 880 nm. Prepare the calibration curve for standards by plotting absorbance versus sample P concentrations. Using the calibration curve, determine the P concentration of the samples.

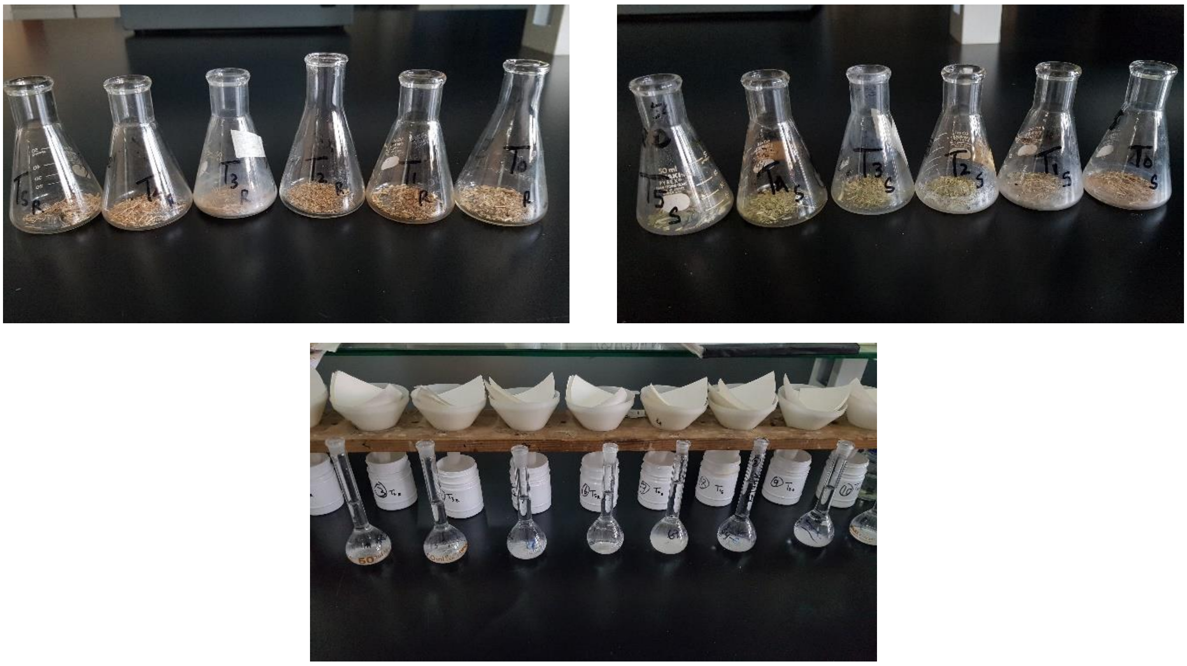


Figure 8: Acid-digestion for plant phosphorous analysis

3.9 Statistical analysis

Results are stated as the mean ± standard deviation (SD) of multiple independent replicates as mentioned for different experiments. The values are analyzed by Origin software at 5% level of significance $P \leq 0.05$. (*$P<0.05$ **$P<0.01$ ***$P<0.005$ ****$P<0.0001$)

Chapter 4

Results

4.1 Biochar characterization

To determine the physiochemical analysis of biochar various techniques were used to analyze all the physical and chemical aspects related to biochar which is as follows:

The pH of cattle derived biochar was 9.14 and that shows the alkalinity of CB-BC, with increase in pyrolytic temperature that results to the separation of alkali salts from organic materials that might be responsible for alkaline nature of biochar. Similarly, increase in value of **ECe** 920 μS depicts the alkaline nature of CB-BC. With the increase in temperature, the pH value of biochar increased with the possibility of the concentrated non-pyrolyzed inorganic elements, that were already a part of the original raw material (Novak et al., 2009). (Chan and Xu, 2009) reported that the high pH values of biochar's are because of concentration of basic cations. Electrical conductivity of samples evaluates the amount of TDS total dissolved solids or the concentration of total dissolved ion (Ding Y *et al*., 2010). At high temperature, loss of volatile matter caused an increase of EC value that promoted the relative amount of salts in the ash content. According to literature, due to application of biochar, soil pH and EC values increased (Khanna et al. 1994).

Table 2: pH and EC determination of $CB\text{-}BC_{850}$

Parameter	pH	EC (μS/cm)
BC_{850}	9.14	920 μS

4.1.1 Proximate analysis of $CB\text{-}BC_{850}$

The estimated biochar yield for cattle bone biochar was 65.4**%** (CB-BC), **(Table 1)** at high pyrolysis temperature of 850 ◦C. Results of proximate analysis are given in Table 1, illustrating that CB-BC has moisture content and volatile matter as 4% and 12.5% respectively. Moreover, ash content and fixed carbon were found to be 64% and 19.5% respectively. Proximate analysis results are listed in (Table 3

Table 3: Proximate analyses for CB-BC_{850}

Parameters	CB-BC_{850}
Yield%	65.4
Moisture%	4
Volatile matter%	12.5
Ash%	64
Fixed carbon%	19.5

4.1.2 FT-IR Properties

FT-IR spectra of biochar produced from cattle bone is shown in following figure (9). The FTIR spectra agreed well with the elemental composition of the biochar. The bending and stretching vibrations of the PO_4^{3-} groups resulted in significant bands of natural hydroxyapatite in the CBBC samples at 570 cm1 and 1038 cm1. The peaks at 1474 cm1 and 1636 cm1 correspond to the C-H and C=C groups in the organic phase of the bone matrix. The 2934-2203 and 2017 bands showed the presence of an alkyne group, C≡C. The band at 3436 cm–1 is assigned to OH stretching of the hydroxyl group and suggests that the OH of hydroxyapatite remained after pyrolysis process, a characteristic band indicating hydroxyapatite.

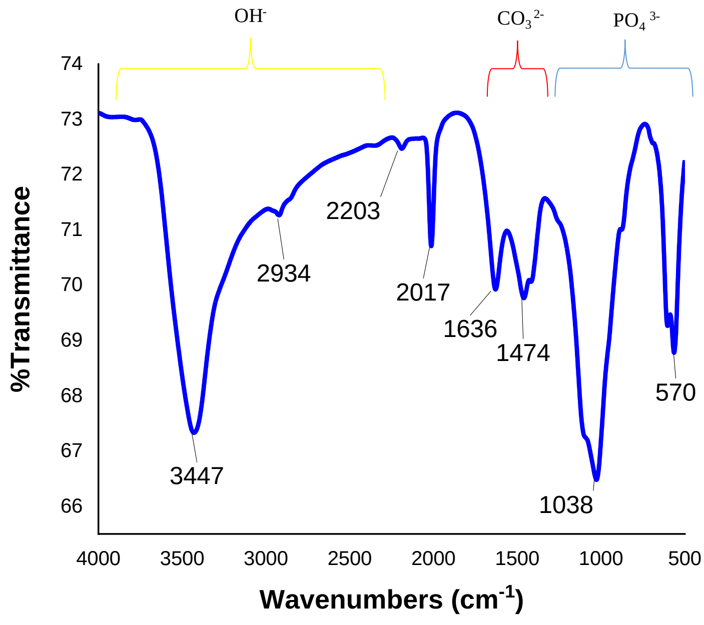


Figure 9: FT-IR spectra of $CBBC_{850}$

4.1.3 X-Ray Diffraction (XRD)

The structure and composition of cattle bone biochar were studied using x-ray diffraction analysis. The XRD pattern of bone char fits well with the hydroxyapatite phase, sharps peaks can be observed that shows the crystalline structure of the cattle bone biochar Figure (10). Since the chemical nature of HAP was remained unaffected and no other peak was seen in addition to HAP, it can be seen that the stability of HAP in the bone matrix was not disrupted when bone was heated up to 850 °C.

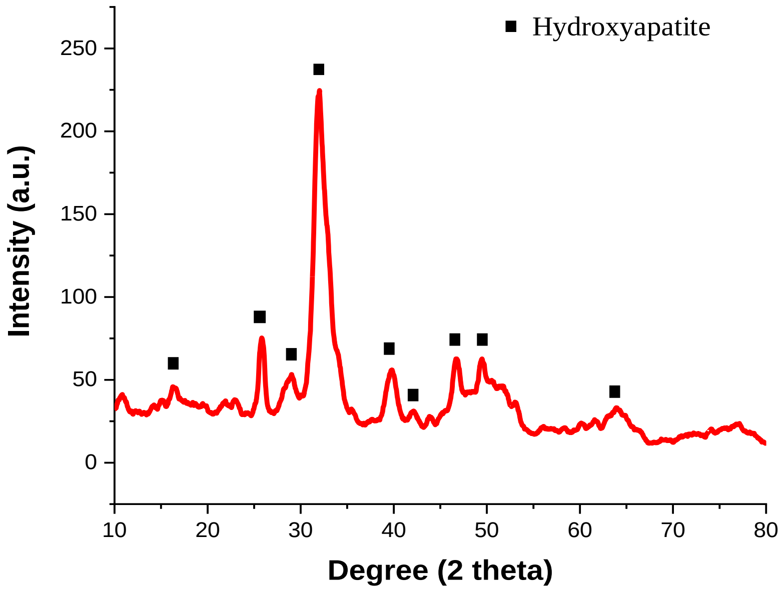


Figure 10: XRD spectra of $CBBC_{850}$

4.1.4 Morphological analysis by SEM

The surface morphology of cattle bone biochar was visualized by using scanning electron microscopy, as shown in the figure (11).

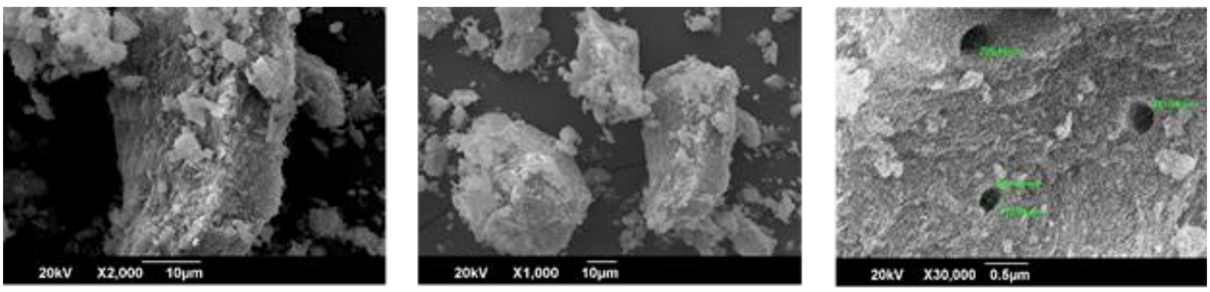


Figure 11: SEM micrograph of CB-BC_{850}

Cattle bone derived biochar presented an irregular rough surface morphology. At higher magnification, the structure of biochar was observed having a pore size diameter ranging from 236.46nm to 281.35 nm. This was in agreement with the results of prior investigations on bone char, which showed a surface with few or no holes and an undefined shape (Rojas-Mayorga et al., 2016**),** due to the clustering of dense constituents represented its bulky structure at high temperature**.** Cattle bones were subjected to a high thermal treatment of 850 degrees Celsius, which reduced the bulk porosity of the resulting bone char. Krzesinska and Majewska provide similar evidence that thermal degradation of animal bones at high temperatures results in stiffness of bone char surface morphology.

4.1.5 Energy Diffraction X-ray EDX

Energy Dispersive X-ray spectroscopy (EDX) of CBBC at 850°C was carried out to confirm the presence of major elements (**Figure 2)**. High amounts of Ca, P, and O, which are thought to be the main components of hydroxyapatite, were identified in bone char., as shown in **Table**. The Ca/P ratio was found to be 1.696 in bone char, which was substantially equivalent to previous value reported elsewhere.

Table 4: Energy Diffraction X-ray for CB-BC$_{850}$

Sample	**Ca**		**P**		**O**		**C**	
Cattle Bone char	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %	Weight %	Atomic %
	36.6	19.4	16.7	11.4	30.3	40.1	16.5	29.1

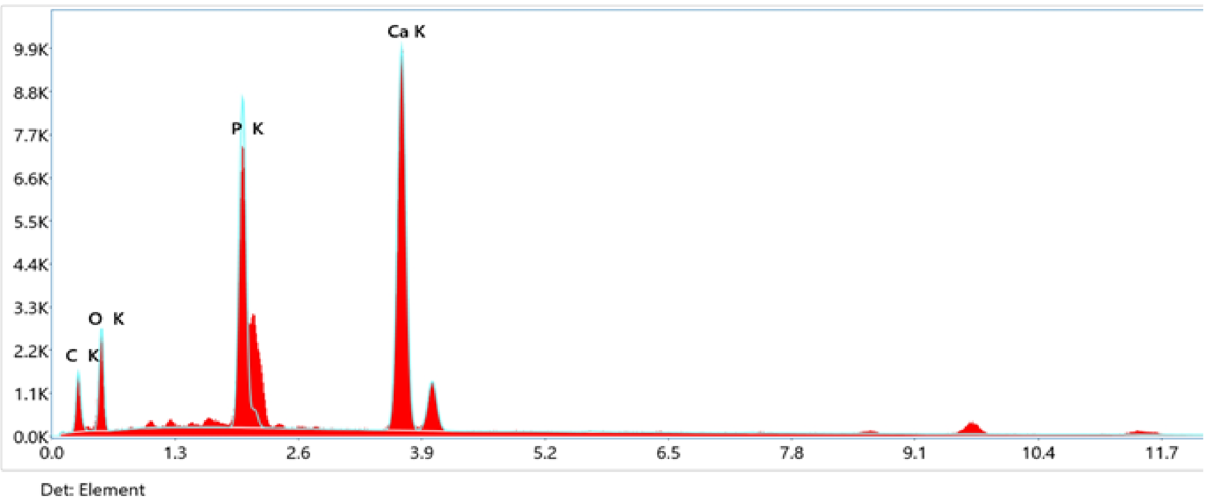


Figure 12 EDX micrograph of CB-BC$_{850}$

4.2 In-vitro screening

In the initial screening, point-inoculation of four bacterial strains on Pikovskaya agar plates, two bacterial strains SPARC 10, HF43 showed very distinct clearing zone around the colonies as shown in the Figure. Higher P solubilization efficiency (E) was demonstrated by SPARC 10 then followed by HF 43 (Table 5)

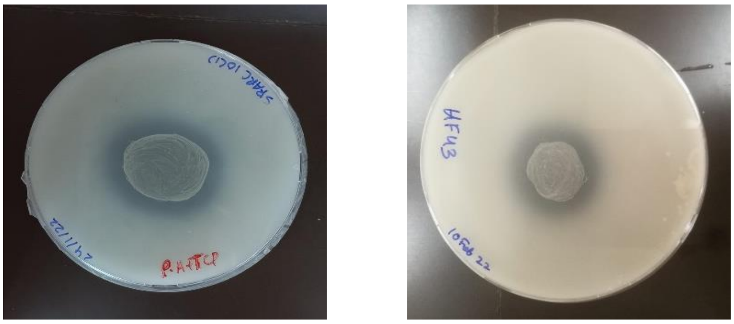


Figure 13: Phosphate solubilizer forming clear zones SPARC10 Left, HF43 (Right)

Table 5. Phosphate solubilization Efficiency (PSE) of Selected PSB strains

Strain	Colony diameter (cm)	Halo zone diameter (cm)	Phosphate solubilization index (PSI)	Phosphate solubilization efficiency %
SPARC 10	2.5±0	4±0	2.6±0	160±0
HF43	2±0	3±0	2.5±0	150±0

4.3 Glasshouse evaluation

4.3.1 Plant growth parameters

Generally, all treatments showed significantly greater growth than control plants grown in pure soil. The addition of biochar influenced plant growth positively, and inoculation with PSM inoculum improved growth even more. Thus, in most cases, the combination of biochar and PSM inoculum to soil proved to be the best, followed by soil + biochar. (Figure 15). The percent germination of Maize under Glasshouse conditions showed no significant difference among the treatments. Plant shoot height was the highest in soil + Biochar + PSM consortium (193.1cm), soil + biochar (184 cm), soil + PSM consortium (156 cm), and were significantly higher when compared to soil + RF (145.3 cm), soil + DAP (153 cm), and lowest plant height was observed in Control (135 cm). The no of Leaves and stem girth in all treatments showed slight differences as compared to control as shown in figure 15 (a, b, d). Length of leaves were highest in soil + Biochar + PSM consortium (99.7 cm), then soil + biochar (94 cm), soil + PSM consortium (88.5 cm), and were significantly higher when compared to soil + RF (86.75 cm), soil + DAP (87.75 cm), and lowest leaf length was observed in Control (83.75 cm) figure 15 (c).

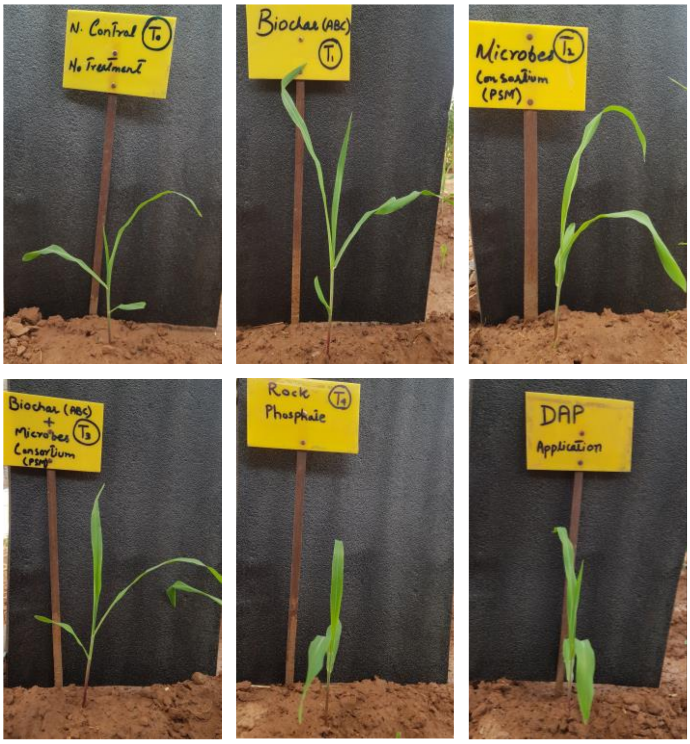
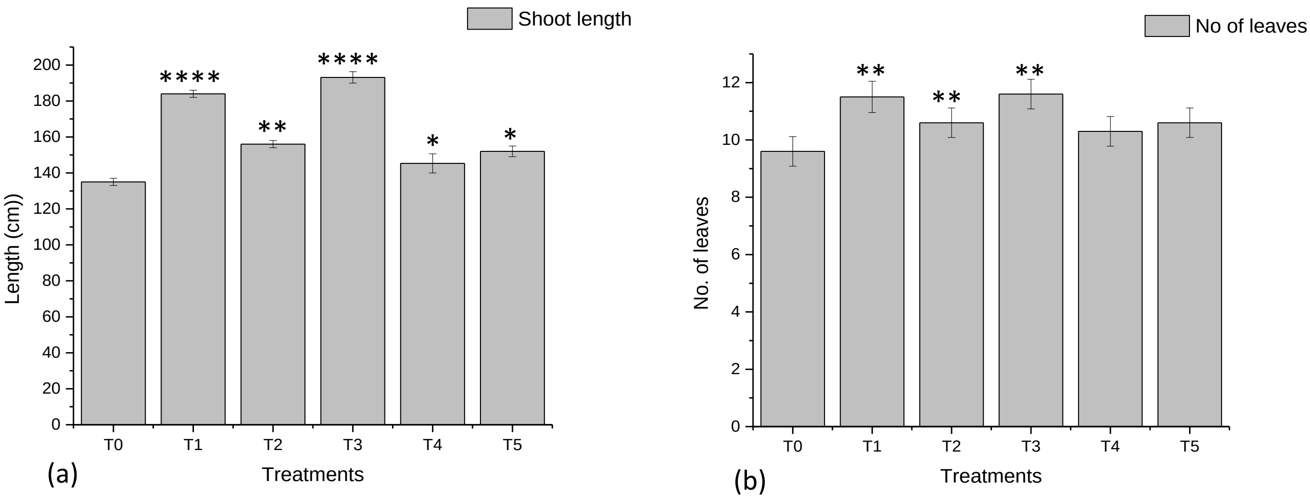
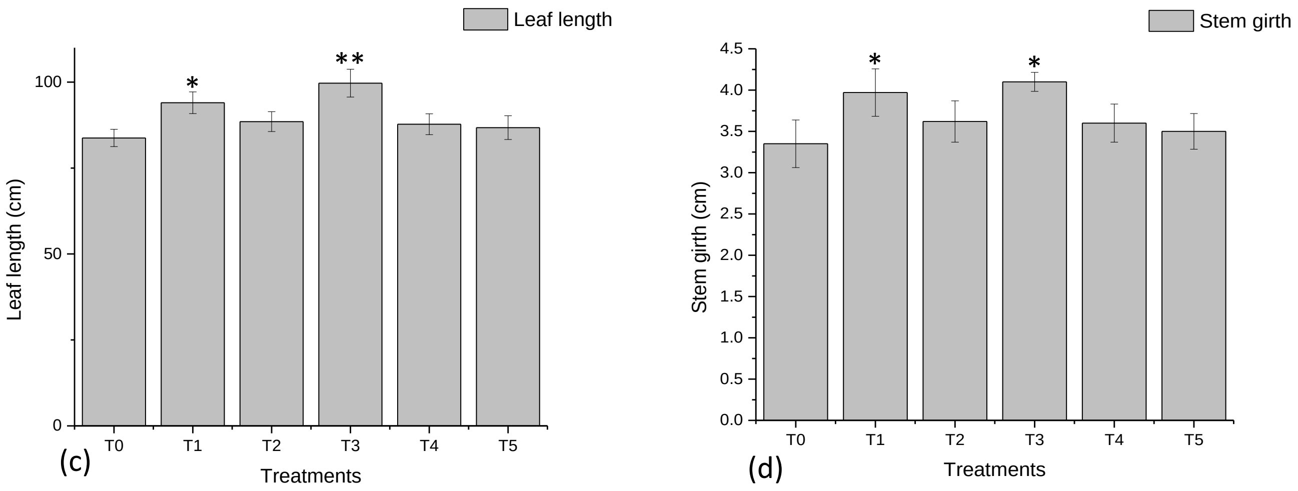


Figure 14: Maize crop growth in control and treated groups





Similarly, the root length was observed highest in the treatment consisting of soil + Biochar + PSM consortium (22.6cm), then soil + biochar (18.6cm), in comparison to all the other treatments. The chlorophyll content was found comparatively highest in soil + Biochar + PSM consortium group (45.1) Figure 15 (e, f)

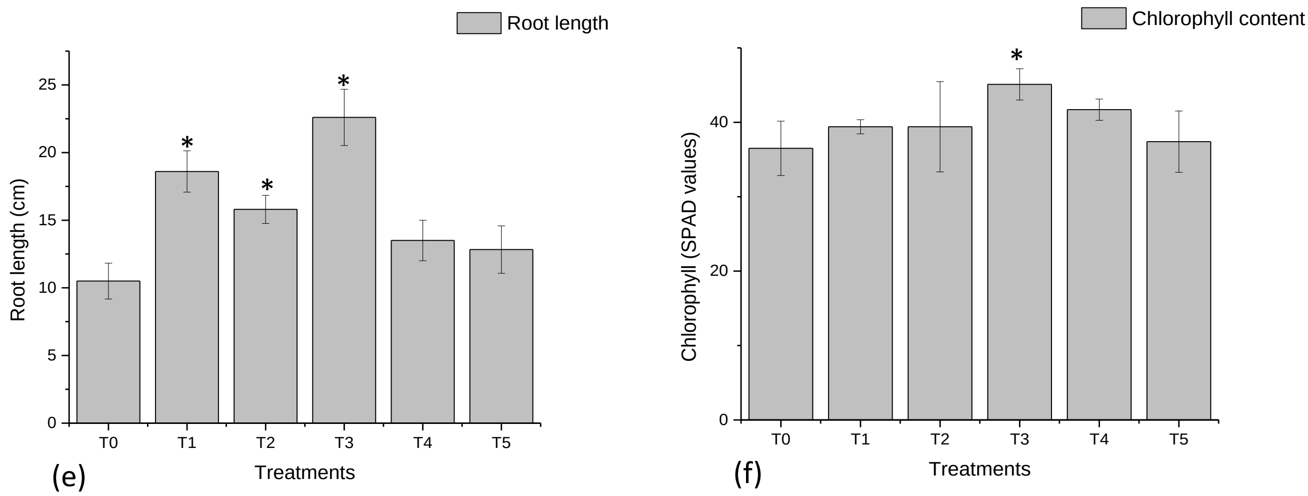
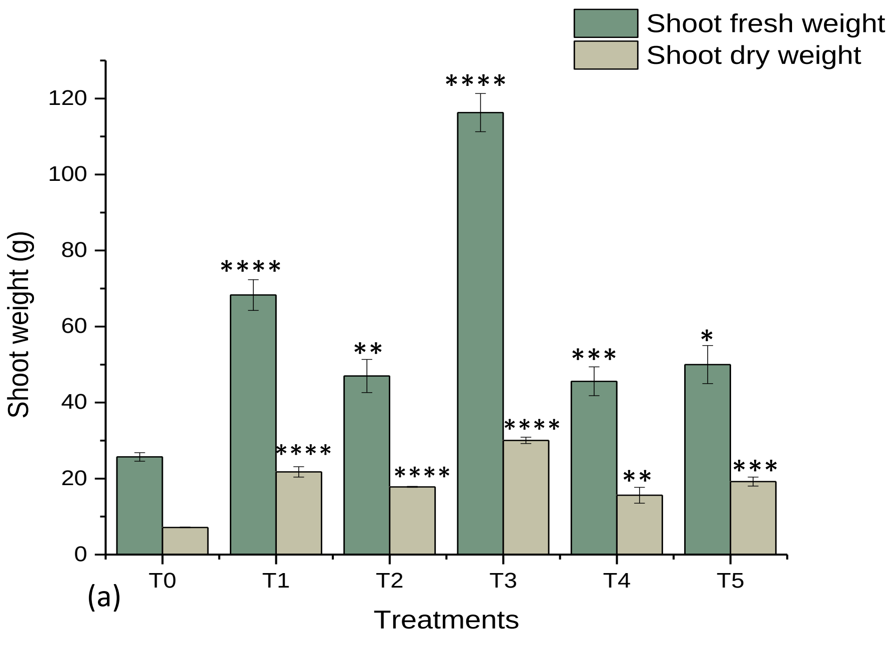


Figure 15: Shoot length (a), No. of leaves (b), Leaf length (c), Stem girth (d), Root length (e), Chlorophyll content (f) of control and treated groups. The values are analyzed by Origin software at 5% level of significance ($P \leq 0.05$). *P<0.05 **P<0.01 ***P<0.005 ****P<0.0001

Figure 16: Root proliferation in various treatments (T0) Control, (T1) Biochar, (T2) PSM bioinoculant, (T3) Biochar + PSM bioinoculant, (T4) Rock phosphate fertilizer and (T5) Diammonium phosphate fertilizer

4.3.2 Shoot and root biomass

In case of shoot fresh and dry biomass after 65 days of Vegetative growth cycle, they were significantly higher in the treatment consisting of soil + Biochar + PSM consortium (116.3g, 29.14g), soil + biochar (68.3g, 21.76g) and soil + DAP (50g, 19.22g) respectively. Similar trend for root fresh and dry biomass was observed (Figure 17)



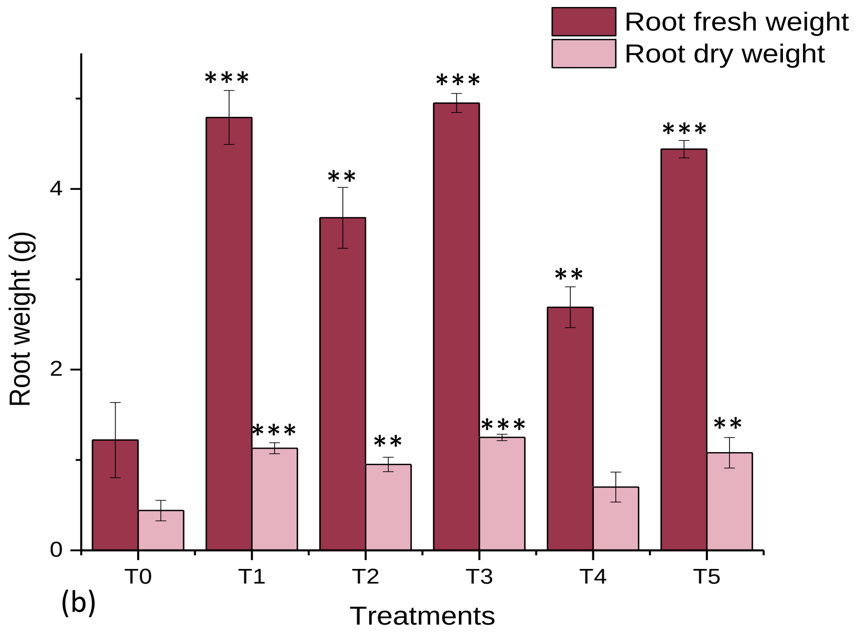


Figure 17: Shoot fresh and dry weight(a), root fresh and dry weight(b) in control and treated groups. The values are analyzed by Origin software at 5% level of significance ($P \leq 0.05$). *P<0.05 **P<0.01 ***P<0.005 ****P<0.0001

4.4 Post harvested soil

Soil was analyzed after 65 days of harvest, the values obtained for Available P from Biochar + PSM consortium and DAP treated soils were significantly improved as compared to control. The Available No_3.N was observed highest in DAP treatment, in case of Available K all treatments were significantly higher that control. Our findings further demonstrate that the addition of biochar made from cattle bones increased soil pH, causing soil alkalization, which increased soil ECe and Available P.

Table 6. Physico-chemical characteristics of post-harvested soil

Treatment	PH	EC (µS cm−1)	Soil Class	Available P	Available No_3.N	Available K
	1:1 Ratio			mg/kg		
T0	7.62	436	Sandy Loam	4.95	1.00	150
T1	7.80	715		8.71	0.65	508
T2	7.95	206		8.57	0.91	268
T3	8.23	585		9.64	0.67	370
T4	8.21	370		8.04	0.31	262
T5	8.22	337		9.80	1.78	268

4.5 Shoot and root phosphorous content

Shoot and root P contents in all amended treatments were significantly better than the control. Interestingly, P content was maximum in biochar= PSM bioinoculant and soil + DAP treatment while all other treatments showed significant enhancement Figure (18). Thus, cattle bone derive biochar is a potent P fertiliser, particularly when combined with phosphate solubilizing bacteria.

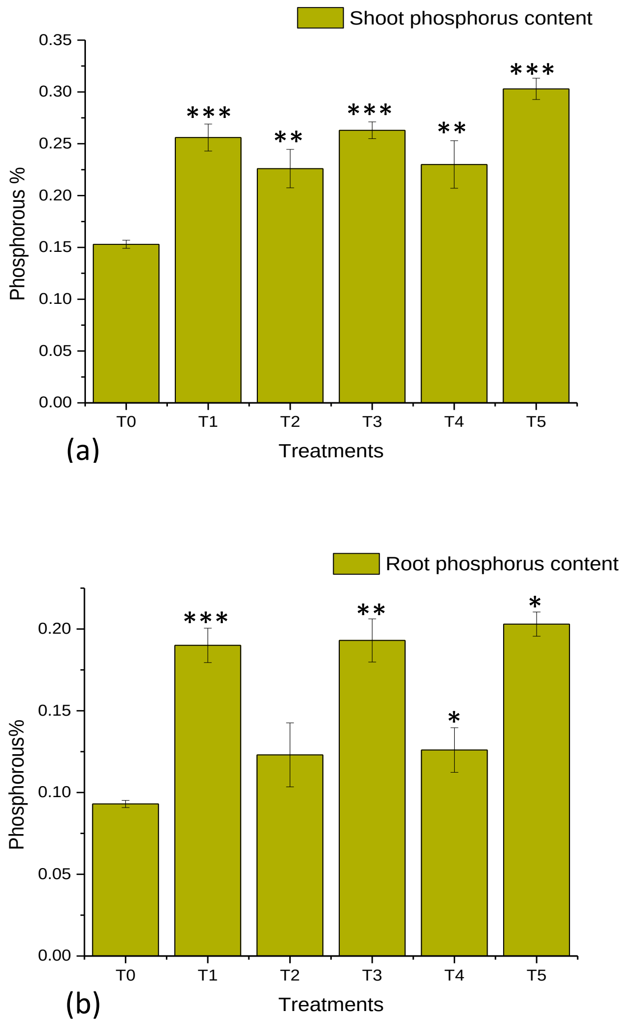


Figure 18: Shoot phosphorous content (a), root phosphorous content (b). The values are analyzed by Origin software at 5% level of significance ($P \leq 0.05$). *P<0.05 **P<0.01 ***P<0.005 ****P<0.0001

Figure 19: Maize growth in biochar treatment and control.

Chapter 5

Discussion

This study's objective was to produce biochar from cattle bones and examine how well it worked with PSM consortium as a phosphorus fertiliser to boost maize crop development. The Cattle bone derived biochar has shown to possess high pH and EC_e as it was synthesized at high temperature. Ash levels more than or about similar to 15 in biochar are regarded as desirable materials for pyrolysis. (Ippolito et al., 2020). The results indicated that high pyrolysis temperature lowered the volatile matter and moisture content, whereas ash content and fixed carbon levels showed the opposite trend (Alkurdi, Al-Juboori, Bundschuh, Bowtell, & McKnight, 2020). The volatile content of prepared biochar decreased significantly after thermal decomposition of raw biomasses. Li et al. confirmed that when biomass is heated to high temperatures, the yield of biochar is reduced and volatile contents are lost (Li et al., 2018). For CB-BC, the proportions of fixed carbon and ash contents were raised to 64% and 19.5%, respectively. This rise in the amount of ash and fixed carbon may be the result of recalcitrant carbon forming as a result of the deposition of carbon and minerals at high pyrolysis temperatures. According to Cao and Harris, ash content also rises as pyrolysis temperature rises because there is a large increase in the amount of organic residues and minerals present, even while volatiles are lost.(Cao & Harris, 2010).

The surface morphology revealed the biochar has porosity but at higher temperature the bulk porosity decreases which was in arrangement with the results of other studies on bone char(M. Krzesińska & Majewska, 2015) due to the clustering of dense constituents represented its bulky structure at high temperature(Rojas-Mayorga, Mendoza-Castillo, Bonilla-Petriciolet, & Silvestre-Albero, 2016). Cattle bones subjected to an 850 ◦C high thermal treatment which reduced the biochar's overall porosity. Similar research from Krzesinska and Majewska demonstrates that the rigidity of bone char surface shape is a result of thermal breakdown of animal bones at high temperatures. (Marta Krzesińska, Majewska, & Pyrolysis, 2015). The results showed that the Ca/P ratio was found to be 2.19 in $CBBC_{850°}$, which are higher than that seen for stoichiometric Hap (Rahavi, Ghaderi, Monshi, & Fathi, 2017). EDX results also showed that besides calcium and phosphorus, traces elements such as Na, N, Mg, K, Mn, Cu, and Zn were present in the biochar. The presence of these micronutrients is essential for the growth of plants (Abdoli, 2020).

Most apatite from biological source is non-stoichiometric due to the presence of the trace elements that replace the Ca in the apatite lattice, which might be the reason for higher Ca/P ratio(Akram, Ahmed, Shakir, Ibrahim, & Hussain, 2014). The XRD pattern of cattle bone derived biochar fits well with the hydroxyapatite phase (Manalu et al., 2015). Since the chemical composition of hydroxyapatite was unaffected and no other peak was discovered in addition to hydroxyapatite, it can be seen that the stability of hydroxyapatite in the bone matrix was not disrupted when bone was heated up to 850 °C. (Ayatollahi, Yahya, Asgharzadeh Shirazi, & Hassan, 2015). The FTIR spectra agreed well with the elemental composition of the biochar. Natural hydroxyapatite bands were found in significant amounts in the CBBC samples. (Han, Li, Wang, Jia, & He, 2007). Numerous bands in the spectra (including 570, 631, 873, 1038, 1474,1663,2017, 2203, and a broad band found between 3300 and 3600 cm-1) corresponded to bands in the HAP reference spectrum and showed strong agreement. (Lü, Fan, Gu, & Cui, 2007). Carbonate groups are present, according to FT-IR analysis, at 1410–1450 cm-1 and 873 cm-1, while hydroxide groups are present at 3200–3500 cm-1. The presence of these bands for the phosphate group at 1030–1090 cm-1, 1950–2200 cm-1, and 570 cm–1 may have resulted from the elimination of all organic matter from the raw cattle bone and the production of HAP crystals. The substance made from biological sources, such as cattle bone, is in fact hydroxyapatite. Because of this, the natural HAP produced by pyrolysis at 850 °C exhibits the necessary characteristics. In the initial screening, bacterial strains SPARC 10, HF43 showed very distinct clearing zone around the colonies, which indicated their ability to solubilize phosphate (Shrestha, Kim, & Park, 2014). A study on Lactobacillus strains revealed that Lactic acid bacteria has been shown to solubilize phosphate (Giassi, Kiritani, & Kupper, 2016) likely through the production of organic acids. To increase the agronomic value and assimilate organic matter such as lignin and cellulose materials, lactic acid bacteria breakdown and bio-stabilize animal and plant waste (Hidalgo, Corona, & Martín-Marroquín, 2022). The absorption of P by plants and crop production are both increased by using microorganisms that solubilize phosphate as inoculants. *Pseudomonas, Bacillus,* and *Rhizobium* strains are some of the most effective phosphate solubilizers (Rodríguez & Fraga, 1999). In a study, rice plants' height, biomass, root growth, and P uptake were all dramatically boosted by the use of phosphate-solubilizing bacteria as inoculants, phosphorus solubilizing bacteria (PSB), which may convert insoluble forms of phosphorus (P) to accessible forms and has an ameliorative effect on reclaimed soil recovery (Q. Chen & Liu, 2019). Most treatments considerably outgrew control plants cultivated in untreated soil in terms of growth. All the plant development metrics were shown to be affected by the addition of biochar and PSM liquid bioinoculant formulation. In most instances, the best results were obtained when biochar was added to soil together with PSM inoculum. (Biswas et al., 2022).

Recently, established microorganisms in agriculture and the environment were used to validate lactic acid bacteria and other bacillus-based biofertilizers. Crop yield is increased by microbial-based biofertilizers, which also hasten the mineral supplementation of plant roots. Additionally, they improve the catabolism of organic substances. For commercial applications, the spray and soil injection techniques come highly recommended. It is predicted that spraying a lactic acid bacterium -based liquid fertiliser on the soil and plant will improve plant health. As biofertilizers and biocontrol agents, fermented cocktails of phototrophic bacteria, yeast, and lactic acid bacteria are employed. At the same time, biofertilizers based on bacilli and lactic acid bacteria demonstrated high crop yields and accelerated the breakdown of organic materials.

Similarly, the root length was observed highest in the treatment consisting of soil + Biochar + PSM consortium followed by soil + biochar as compare to all the other treatments and control group. While he chlorophyll content was found comparatively highest in soil + Biochar + PSM consortium group as compare to all the other treatments and control group. In case of shoot fresh and dry biomass were significantly higher in the treatment consisting of soil + Biochar + PSM consortium followed by soil + biochar and soil + DAP respectively. Similar trend for root fresh and dry biomass was observed (Figure.). According to a study, using low grade rock phosphate that has been PSB inoculated on crops could help crops save about 50% of their commercial P fertiliser without reducing crop yields (Biswas et al., 2022). Additionally, phosphate solubilizing bacteria and Azotobacter chroococcum dual inoculation had a stronger favorable impact on potato plants' height, which could be attributed to the availability of P and N (Faccini, Garzón, Martinez, & Varela, 2007). In recent study, by adding either charcoal or Bacillus sp. to the soil, separately or together, French bean roots, shoots, and biomass all dramatically increased (Saxena, Rana, & Pandey, 2013). Because they are environmentally friendly, microorganism-based biofertilizers and biochar can make a significant contribution to sustainable agriculture (Ali et al., 2021).

In sustainable agriculture systems, PSB inoculation together with RP fertilisation would be a suitable replacement for the application of chemical phosphate fertiliser. Because of the small investment and high returns, it is more economical (Kaur & Reddy, 2015).

Soil was analyzed after 65 days of harvest, the values obtained for Available P from Biochar + PSM consortium and DAP treated soils were significantly improved as compared to control. The Available No_3.N was observed highest in DAP treatment, in case of Available K all treatments were significantly higher that control. The composite of Biochar + PSM consortium acted as a slow-release organic fertiliser, significantly improving soil properties, according to Koron et al., who reported that biochar derived from plant sources has less impact on soil fertility as compared to that of bone char, which comprises about 90% mineral composition and 10% carbon content. (Koron et al., 2018). There is a lot of promise for increasing P availability in calcareous soils by using PSB as a bio-fertilizer (Adnan et al., 2022). Cattle Bone biochar can be used as an organic fertiliser because it has the property of hydroxyapatite and an appropriate amount of Ca and P contents, according to Chen et al. (H. Chen et al., 2020) who also observed that the addition of bone char promoted carbon mineralization in soil. Butnan et al (Butnan, Deenik, Toomsan, Antal, & Vityakon, 2015) demonstrated through an experimental investigation that adding biochar to soil enhances soil characteristics such as Available P, ECe, and PH as well as nutrient availability. Our findings further demonstrate that the addition of biochar made from cattle bones increased soil pH, causing soil alkalization, which increased soil ECe and Available P. Identical results were achieved for the application of chemical fertiliser. The application of CB-BC and PSM consortia is thought to have stimulated soil microbial activity, most likely through the process of soil microbial solubilization, which made the macro and micronutrients accessible to the plants (Rawat, Sanwal, & Saxena, 2018). As a result, both the Biochar + PSM consortium and the Biochar treatment alone strengthen their bonds with the plant by making more nutrients available in comparison to the control treatment.

All modified treatments had significantly higher shoot and root P contents than the control. Interestingly, the P content was highest in the Biochar + PSM consortium and soil + DAP treatments, while all other treatments showed significant improvement over the control. Thus, cattle bone derive biochar is a potent P fertiliser, particularly when combined with phosphate solubilizing bacteria. Plant access to soil P is influenced by biochar, which calls for careful treatment to increase P availability in soil as well as in plant (Zwetsloot et al., 2016).

A recent study examined soil quality, maize growth, and heavy metal remediation using sheep bone derived biochar at. The results showed improved Zn and Cd immobilization in smelter-affected soils, increased bacterial abundance and microbial function (urease, phosphates), and improved plant growth (Azeem et al., 2021).

In the experiment, biochar was added to the soil at a rate of 5 g/kg. The kind of soil and the crops affect how much biochar should be applied. Its application rate has not been the subject of numerous investigations. Based on the data and information provided in the Li and Huang (2014) paper regarding the effect of hydroxyapatite, the primary ingredient in our synthetic biochar, on Pakchoi (Brassica chinensis L.), it can be deduced that at low concentrations (5g kg^{1}) it improves the growth of Pakchoi but has reduced its growth at higher concentrations (Liu & Lal, 2014). According to our study the bioapatite synthesized from cattle bone has shown to improves the growth of maize plants. Extensive research has produced interesting findings, such as the reduced rate of crop yield that was observed when more biochar was applied to the soil in a pot experiment (Rondon, Lehmann, Ramírez, & Hurtado, 2007). Another trial carried out in the USA revealed that peanut hull and pine chip biochar applied at 11 and 22 t ha-1 decreased maize yields below those attained in the control plots under conventional fertiliser management (Gaskin et al., 2010).

We therefore introduced a small amount of biochar and got good results, avoiding the bad effects of biochar. While, using alone biochar proved to be second-best in many cases, the use of biochar in conjunction with PSM consortium enhanced overall growth and made this combination the best in many cases. As a result, it contributes to greater vegetative growth by encouraging the growth of soil microorganisms, accelerating mineral absorption, and enhancing root system strength. It also offers the benefit of shielding plants from disease and pests. The isolate chosen for the study displayed numerous traits that encouraged plant growth, such as IAA and phosphate solubilization (Shrestha et al., 2014). These traits demonstrate the potential of using liquid bioinoculant formulation as a biofertilizer (Sahu & Brahmaprakash, 2016). In a recent study, it was discovered that adding biochar to Lettuce increased the plant's intake of nitrogen, phosphorus, and potassium (Nigussie, Kissi, Misganaw, & Ambaw, 2012). According to Uzoma et al (Uzoma et al., 2011) the addition of biochar to the tropical environment increased nutrient uptake. Compared to all other treatments, the PSM liquid bioinoculant formulation and biochar made from cow bones had the greatest effect on plant growth. The increased soil qualities and plant nutrient uptake in soils treated with biochar have been attributed primarily to the presence of plant nutrients and ash in the biochar, as well as to the material's high surface area, porous nature, and ability to serve as a medium for microorganisms.

Conclusion and future prospects

This study's objective was to produce biochar from cattle bones and examine how well it worked with PSM consortium as a phosphorus fertiliser to boost maize crop development. It is clear that both the biochar made from cattle bones and the liquid bioinoculant PSM formulation have the potential to improve the overall growth of the maize crop, making them suitable for use in sustainable agriculture. Massive amounts of recyclable bio-waste, in particular waste bones can be put to good use by converting them into biochar. The addition of biochar to soil along with PSM bioinoculant resulted in greater vegetative growth of maize than commercial. fertiliser, reducing the use of chemical fertilisers.

The use of biochar has also resulted in significant improvements in biochar amended soils' physicochemical and biological properties. As a result, phosphorus-solubilizing microorganisms with multifunctional properties, combined with the high adsorption capacity of cattle bone biochar as a carrier material for the solubilization of immobilized phosphorous in soil, could be an ideal candidate. The current study's encouraging maize crop development results may be attributable to the abundance of vital elements (Ca, P, N, Zn, and K) in the CB-BC and their high bioavailability in the soil. The morphological and biochemical characteristics of the maize plants have most likely been significantly affected by the biochar application as comparatively, it had demonstrated more growth in soils treated with biochar than in controls or with chemical fertiliser (DAP). The study offers optimism for a gradual transition away from chemical fertilisers and toward biological and organic fertilisers. However, additional fieldwork needs to be conducted in future experiments

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