ADDIS ABABA UNIVERSITY
SHOOL OF GRADUATE STUDIES
Institute of Biotechnology

Isolation and *in-vitro* characterization of extracellular phytase producing bacterial isolates for potential application in poultry feed

By
Lubaba Amede

JULY, 2020
Addis Ababa, Ethiopia

ADDIS ABABA UNIVERSITY
SHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

This is to certify that the thesis prepared by Lubaba Amede entitled "Isolation and *in-vitro* characterization of extracellular phytase producing bacterial isolates for potential application in poultry feed" submitted in partial fulfillment of the requirements for the Degree of Masters of Science in Biotechnology complies with the regulations of the university and meets the standard with respect to originality and quality.

Signed by the Examining Committee

______________________	_________________	_______________
Internal Examiner	Signature	Date
____________________	_________________	_______________
External Examiner	Signature	Date
Diriba Muleta (PhD)	________________	_______________
Major Advisor	Signature	Date
Mesfine Tafesse (PhD)	_________________	_______________
Co-Advisor	Signature	Date

Table of contents

List of Tables

List of figures

LIST OF TABLES IN THE APPENDIX

List of figures in the appendix

ACKNOWLEDGEMENTS

First I would like to thanks my God. I wish to express my sincere gratitude and heartfelt thanks and appreciation to my major advisor Dr. Diriba Muleta for his valuable comments, guidance and encouragement from proposal development up to the Thesis write-up. I am also grateful to my co advisor Dr. Mesfin Tafesse for his close supervision and invaluable suggestions throughout the research period. Without close guidance and professional expertise of both advisors, the completion of this work would have been impossible.

I would like to thank Addis Ababa University, for giving me the opportunity to pursue my post graduate study through female scholarship program.

My special and grateful thanks go to the Biotechnology Laboratory at Addis Ababa Sciences and Technology University and its staff members for assisting and providing me with all the necessary laboratory materials for my work. I wish to express my special thanks to Mr. Umer Ahmed a researcher at Biotechnology Laboratory for his help in routine microbiological biochemical tests, screening methods, close assistance in availing the necessary equipment, the possibility of finding a place to conduct the research and in giving valuable comments during the Thesis write- up.

I would like also to thank Mr. Kefie Mhretie, Endashaw Abate and Mr. Gessese Kebedea researcher in Biotechnology Laboratory, for their close support in availing the necessary equipment and providing training on basic microbial biotechnology laboratory techniques.

I would like to thank Mr. Eshetu Kebede, for his great assistance in selection of the bacterial isolates for sequencing and for their type cooperation and support while allocation the same laboratory during my thesis research work.

Thank you my family, friends and others who had given me support during my study!

List of abbreviations/ Acronyms

NCBI:	The national center for biotechnology information
AAM:	Acetone-acid-molybdate
LB:	Luria broth
OD	Optical density
OVAT	One variable at a time
rpm	Revolutions per minute
spp	Species
RS	Rhizospheresoil

Abstract

Phytase is an enzyme that can be breaking down the complex organic forms of phosphorous such as phytate into simpler forms by sequential hydrolysis of phosphate ester bonds and liberate the usable form of inorganic phosphates. Supplementation of feeds with microbial phytase enhances the bioavailability of phosphorus and micronutrients. Hence, the aim of this study was to isolate and characterize phytase producing bacteria from Rhizospheresoil, fresh poultry excreta, Cattle shed in order to evaluate their potential in improving poultry feeds. Phytase producing bacterial were isolated using Wheat Bran Extract Medium. A total of 169 bacterial isolates were purified and screened for phytase activity. Out of which36 were found positive for the desired purpose. The bacterial isolates were identified by cultural, morphological, and biochemical features. Finally, the isolates were identified to species level by 16S RNA genes sequencing using universal primers. The isolate (RS1, RS8, RS10 and RS15) correlated with shared the highest similarity indices, Enterobacter asburiae, Klebsiella pneumonia, Pseudomonas aeruginosae and Escherichia coli respectively. all isolates alone possible to increase the phytase production in cultures medium (wheat bran extract medium) by supplementing with 1% galactose and ammonium sulphate, temperature (50^{o}C), pH (6), incubation time (72 hr) and size of inoculum (at 800µl). *Proximate composition analysis after supplementation of phytase released the bound phytate phosphorus and improved bioavailability of phosphorus, calcium, potassium and sodium in poultry feed. Overall, this study is to improve the nutritional value of poultry feed, reducing the cost of diets and supplementation inorganic phosphate. Lastly, it will be maximizing poultry products by supplementation of phytase with poultry feed.*

Keywords: Phytase, rhizosphere soil, wheat bran, industries and poultry

1. Introduction

Poultry isan agricultural practice in which domesticated and commercialized types of birds kept and managed forproduction of eggs andmeat for humans. It isthe second most widely eaten foods globally.Poultry provides nutritionally beneficial foods (meat and eggs) that contain high-quality protein accompanied by a low proportion of fat (Melkamu, 2013, FAO, 2010and Reta, 2009).In Ethiopia poultry production is an important part of the mixed crop-livestock farming system practiced by most households where it makes a vital function through the provision of meat and eggs for home consumption and for the generation of income (Birhanu, *et al.*, 2015) The sector in the country can be characterized into three major production systems namely village, small scale commercial and large scale commercial poultry production on the basis of the parameters such as breed, flock size, housing, feed, health, technology, and bio-security (Tadelle, *et al.*, 2003).

In Ethiopia there is huge demand of poultry meat and eggs which has lead to an rising establishment and expansion of modern and organized poultry farms in the entire country particularly in per urban and urban areas (Dana,*et al.*, 2010).These emerging farms contribute a lot to improve the livelihood, food security and poverty reduction as well as providing a handsome return in per-urban and urban areas in the tropics(pica and otte, 2010). The demand of protein food is progressively growing with the improvements of society's income and population growth (FAO,2010).Therefore, Poultry meat and egg are important sources of edible animal protein(FAO, 2010). Moreover, poultry production is considered as a fulfilling the nutritional requirement of the poorest sections of the society(Reta,2009).Thus, if food self-sufficiency is to be achieved and to combat malnutrition in developing countries particularly in Ethiopia, there is a need to give due attention to poultry production (Melkamu, 2013).

Factors affecting chicken under intensive production systems are numerous, however feed, marketing constraints, diseases and bio-security are the most important one (Matawork, 2016).Poultry feed is one of the most critical constraints to poultry production in the rural smallholder and large-scale systems and account 60-70% of production costs under intensive production systems(Yenesew, *et al.*, 2015).The problem is mainly associated with lack of processing facilities, inconsistent availability and distribution and sub-standard quality of processed feeds when available(Haftu, 2016).Therefore, poultry production is seriously constrained due to poor quality poultry nutrition among others in the case of a large quantity of phosphorus in grain based poultry diet exists in phytate-bound form. As a result, poultry cannot use bound form of phosphorus due to absence of phytase enzyme in their gastro-intestinal tract (hailu *et al*., 2017).

Phytate is reduces bioavailability of some important cations and is known to reduce digestibility of protein. In order to overcome this problem, food, and feed can be supplemented with phytase for improving phosphorous bioavailability and reducing phosphorous excretion in the areas of intensive livestock populations (Sasirekha, *et al.*, 2012).

Phytase is widespread in nature and can be derived from plants, animals and microorganisms.(Konietzny *et al*.,2002).Generally, isolating phytase producing microbes can improve poultry feed, making the poultry farm economically useful. Some of the phytase producing microorganisms contain bacteria such as *Bacillus subtilis, Escherichia coli,* fungi such as *Aspergillus niger,A.oryzae, A. flavus* and Penicillium sp., and yeasts such as *Saccharomyces cerevisiae,* and *Schwannoiomyces castelii* (Konietzny *et al*., 2002). Microbial sources are more promising for the production of phytase on a commercial scale (Pandey *et al*., 2001). Because of its potential biotechnological applications, interest in the isolation of new bacterial isolates, producing novel and efficient phytase, is increasing (Shim and Oh, 2012).

Despite the recognized importance in biotechnology information on bacterial phytase and phytase-producing bacteria is clearly limited and major efforts are required to improve the knowledge of phytase present in bacteria and their use in Ethiopian. This investigation is important to isolate a phytase producing bacteria to improve the production of poultry feed. In the present study, selected Rhizospheresoil samples, fresh poultry excreta and Cattle shed samples were collected from locations for isolation of phytase producing bacteria.

1. 1.Significance of the study

Phytate are anti- nutritional factors that in feed reduce its quality by chelating proteins and essential minerals (phosphorous, calcium, iron, zinc, magnesium, manganese, copper and molybdenum) and prevent their absorption. Significance of the study is to improve the nutritional value of poultry feed, to reducing the cost of feeds reducing the supplementation of inorganic phosphate and increases the bioavailability of phosphorous in poultry and to maximize the poultry products by the supplementation of phytase with poultry feed. Phytase hydrolyzes the phosphate esters bond in phytic acid releasing phosphorus and removing the anti-nutritional chelating effects of phytic acid. Therefore, isolating and enriching phytase producing bacteria in the poultry feed in addition of enhancing the nutritional qualities and bioavailability of nutrients and mineral of the feed

1. 2. Objectives` of the study

1. 2. 1. General objective

- The main objective of this study was isolation and characterization of some phytase producing bacteria, purify the enzyme and identify the nutritional quality in the improvement of feeds of monogastric animals.

1. 2. 2. Specific objective

- The specific objectives of this study were to isolate (separate), screen and characterize potential phytase producing bacteria from Rhizospheresoil, fresh poultry excreta and cattle shed samples.

- To extract and purify crude phytase enzyme from the potential bacterial isolates using wheat bran fermentation medium.

- To optimize cultivation conditions (carbon sources, nitrogen sources, temperature, pH, incubation period, and inoculums size) for phytase-producing isolates using OVAT (One Variable at a Time).

- To analyze proximate composition of poultry feed before and after addition of experimental phytase enzyme.

2. Literature Review

2. 1. Phytase

Phytase (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate phosphohydrolase, EC: 3.1.3.8) is a special group of enzyme which catalyzes the stepwise exclusion of phosphates from salt phytate(phytic acid) (Lei *et al.* 2013). Phytase is an enzyme that can be breaking down the complex organic forms of phosphorous such as phytate into simpler forms by sequential hydrolysis of phosphate ester bonds and liberate the usable form of inorganic phosphates it is a natural enzyme frequently used for hydrolyze and increasing the food quality of phytate free containing minerals and nutrients compounds (Shivange *et al.*, 2010).

Phytase have potential applications in the food and feed industries (Alves *et al*., 2016). In recent years, phytase have paying attention of researchers and entrepreneurs in the areas of nutrition, environmental defense and biotechnology (Alves *et al*., 2016). Microorganisms are the main sources of phytase that is mainly used in animal feed industry to eliminate the anti-nutritional properties of phytate (Kim *et al*., 2006).

Supplementation of diets with microbial phytase enhances the bioavailability of phosphorus and micronutrients in addition reducing the aquatic phosphorus pollution in the areas of intensive livestock production and thereby combating environmental phosphorus pollution (Kumar *et al*. 2015). Phytase has great economic impact in animal feed market and according to the report of first phytase (summit in 2010) phytase share 60% of total feed enzyme market with a global phytase market of $350 million annually (Reddy *et al*., 2015).

2.2. Phytic acid (Phytate) and it's the anti-nutritional function

Phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexa-kishydrogenphosphate)is a common compound of plant-derived foods and constitute 1–5% of edible cornflakes, pollen, cereals, legumes, oil seeds and nuts in weight and plays an anti-nutritive role (Vohra, et. al., 2003 and Singh, et.al., 2013).It is the mainstorage space form of phosphorus in cereals, legumes, oil seeds and nuts(Chunshan *et al.*, 2001) and also phytic acid is the mixture of calcium –magnesium salts of inositol hexa phosphophoric acid considered to be an anti-nutritional part of human and animal diet because:

1. Negatively charged phytic acid chelates with positively charged divalent cations as Fe^{+2}, Ca^{+2}, Mg^{+2}, and Zn^{+2} and thus interferes with the assimilation of important trace metals. This is partially attributed to the wide spreading human nutritional deficiencies of calcium, iron and zinc in developing countries, where the staple foods of plant origin.
2. It binds to proteins and makes them more resistant to proteolysis digestion.
3. Phytate phosphorus is poorly available to monogastric (because the absence of phytase).

Monogastric animals are unable to utilize phytate phosphorus either due to lack of or insufficient amount of phytate degrading enzyme for example chickens, swine and humans are monogastric, thus inorganic phosphorus is supplemented in feed for poultry, fish and swine to meet their nutritional requirement for phosphorus, after digestion the unutilized phytate phosphorus from plant feeds is excreted, becoming an environmental pollutants in areas of intensive animal livestock (Soni,*et al* 2009).

Phosphorous is present in the form of phytic acid in cereals and legumes of animal feeds (Soni, *et al* 2009). In order to enhance the phosphate assimilation ability of simple-stomached animal, the significant metals such as calcium, zinc, and magnesium and iron reducing their bioavailability, phytase were added in animal feeds (Avinashtungala, 2013).

In addition to making the phosphorous unavailable, phytate binds to divalent cations (Ca^{+2}, Cu^{+2}, Mg+2, Fe^{+2}, Mn^{+2} and Zn^{+2}) preventing the absorption of these nutrients in the animal guts (Zuo *et al*., 2010).In animal feed industries, the feed is usually supplemented with inorganic phosphate to meet the phosphorous needs for proper growth and development of animals. However, the anti-nutritional effects of phytate remain unaffected (Sandberg *et al.*, 2002). Excretion of indigestible phytate, resulting in a large amount of phosphorous in manure, leads to redistribution of phosphorous in the soil (Turner*et al.*, 2004).

2. 3Phytase producing microbes

Recent research has shown that microbial phytase are most promising for a biotechnological application (Pandey, *et al*., 2001) although phytase from bacteria, yeast and fungi have been characterized (Konietzny, *et al.,* 2002 and Pandey, *et al*., 2001). Commercial production currently focuses on the soil fungus and bacteria because to be paid to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency (Kim *et al.*, 2003). Phytase detected from bacteria, such as *Pseudomonas sp*. (Lim *et al*., 2007), *Bacillus sp.* (Sajidan, *et al*., 2004), *Citrobacter braakii*(Kim *et al*., 2003),*Enterobacter*(Lim *et al.,* 2007).

Commercial phytase from bacteria and fungi are *E. coli* and *Peniphora lycii*,*Aspergillus niger* and *Aspergillus ficum* respectively (Selle and Ravindran, 2007). Furthermore, phytase from several sources may have various characteristics, such as resistance to degradation in GIT (gastro intestinal track), thermal stability and the suitable pH of the optimal enzymatic activity (Onyango *et al*., 2004). These characteristics can influence the phosphorus release, so it is important to indicate its effects in the enzyme efficacy (Onyango *et al*., 2004). Bacterial phytase are a real alternative to the fungal enzymes (Kim *et al*., 2003).Bacterial phytase that able to put down phytic acid can be isolated from a range of terrestrial and aquatic environments (Lim *et al*., 2007).

2.4. Regulation of phytase production

Phytase is an inducible biological catalyst and its appearance is subjected to a complex parameter but phytase formation is not controlled uniformly in different bacteria (Liu, et al., 1998). Phytase production was studied in detail only in *Escherichia coli* (Touati, *et al*., 1987) and *Raoultella terrigena* (Zamudio, *et al*., 2002).In non-limiting media the arrangement of the Escherichia coli and the *Raoultella terrigena* bacteria phytase were turned off in exponentially growing and in progress as soon as the cultures entered the stationary phase. Because the synthesis of the phytase happening immediately the growth time began to decrease, it was optional that either a nutrient or an energy constraint occur in the stationary phase at the origin of its instruction. A regulatory inhibition of phytase arrangement by inorganic phosphate levels was normally observed in all microbial phytase producers, together with moulds and yeast with the exclusion of *Raoultella terrigena* and the rumen bacteria (Yanke, *et at*., 1998).

In *Escherichia coli*, the main response to the constraint of a specific nutrient was shown to be an activation of a sureset of genes that permit a better uptake of the nutrient present in low concentration or the use of other substances that fit into the similar class of nutrients (Konietzny, *et al*., 2004).These nutrient-specific systems include the cyclic activator metabolism protein (cAMP) and its receptor the catabolism activator protein for the use of option carbon sources, the NtrB/NtrC/σ54 area that is induced below nitrogen constraint, and the PhoB/PhoR area that is in duce under phosphorus restraint (Hengge, 1996). However, the environment is completely weary for an essential nutrient; the cells go in to the stationary phase. The configuration of more than a few dozen proteins is enthused throughout change into stationary phase and a center set of proteins is induced in spite of the class of nutrient for which the cells are starved (Konietzny, et al., 2004).

The expression of the phytase-encoding gene (appA) was shown to be strongly dependent on the rpoS-encoded sigma factor (σS) (Konietzny, et al., 2004).Especially sigma factor reliant genes are keeping pace by several promoters and simply one of them is controlled by sigma factor (σS).Thus, not all genes identified as sigma factor controlled are completely dependent on sigma factor for expression.

In minimum media, starvation for phosphate, but not for glucose or ammonia, resulted in a physically powerful inspiration (stimulation) of rpoS expression followed by an increase in phytase activity. Moreover phytase expression depends on the nature of the carbon source used for growth, Glucose has widely used for improve phytase production in cause of catabolism repression. Combination of the phytase in *Escherichia coli* and *Raoultella terrigena* has been reported to be negatively keeping up by cAMP (Zamudio, *et al*., 2002; Konietzny, *et al*., 2004).

2. 5 Application of phytase

2. 5. 1 Application of bacterial phytase in food and feed additives

Monogastric animal that include swine, pig, fish and poultry are incapable to humiliate phytate in food. They have no phytase in their digestive tract. Undigested phytate in the small intestine negatively affects the absorption of zinc, calcium, magnesium and iron. It also reduces the digest ability of dietary protein and inhibits digestive enzymes (Jorquera *et al*., 2008).Thus, inorganic phosphate is frequently add to the feed to improve the growth of these animals however phosphorous up take by mono gastric animals is inefficient (Tumer, *et al*., 2002). Of this, 70% of the total phosphorous in feed is released in excreta (Tumer, *etal*., 2002).

Bacterial phytase can be exacting interest as feed additives. Phytase from *E. coil* and *Citrobacter braakii* are extra resistance to pepsin and pancreatic than marketable *Aspergillus phytase*

(Konietzny and Greine, 2004).Several studies have desired the effectiveness of supplemental E. coil-derived phytase, highly stable under acidic conditions, even at pH 2.0 and in improving use of phosphors by poultry and swine consequence in better feed intake and body mass increase (Anandham, *et al*., 2007).

Currently phytase are used mainly in animal feed additives for the diets of monogastric animals(Sandberg and Andlid, 2002).There is great potential application area of phytase in processing and manufacturing of food for human and animal consumption (Sandberg and Andlid, 2002Investigations in this area paying attention on better mineral absorption or technical improvement of food processing. The high phytic acid (salt phytate) content in cereal and legume-base diets contributes to inhibition of mineral incorporation (Sandberg and Andlid, 2002).

2.5.2 In vivo function of bacterial phytase

The complex mode of regulation does not shed much light on the role of bacterial phytase. The stationary phase induction suggests that phytase is not required for balanced growth, and that this enzyme may be synthesized in response to a nutrient or energy limitation (Konietzny, *et al*., 2004).Contrast to different other bacteria; anaerobic rumen bacteria are capable of tolerating a high level of phosphate without any negative impact on phytase production. This unique ability leads to a more efficient phytate hydrolysis in the rumen, even under the high phosphate levels in the rumen fluid of ruminants fed concentrated feed (Konietzny, *et al*., 2004).The phosphate generated by splitting of phytate is utilized by both the microbial flora and ruminant host. Because phytase formation was observed when bacterial cells had to adapt to environmental fluctuations imposed before the onset of growth or when actively growing cells are stressed, it was suggests that phytase could be involved in a signal transduction mechanism of metabolic regulation (Zamudio,*et al*., 2002).Lactic acid bacteria are adapted to environments rich in nutrients and energy, explain why there is, with the exception of sourdough bacteria, give clear evidence for lactic acid bacteria with phytase-producing ability and Raises the issue of a possible role in providing the cell by means of phosphate hydrolyzed from phytate (Ireland,*et al*., 2002).

Table 1 Application of phytas in poultry

No.	Application	Reference
1	Enhance broiler body weight gain and performance	Scholey *et al*., 2018; Broch *et al*., 2018 Hamdi *et al*., 2018
2	improve egg production	Gao *et al*., 2013; Cabuk *et al*., 2004
3	Enhance egg quality	Gao *et al*., 2013; Cabuk *et al*., 2004
4	fillet(bone) mineralization	Scholey *et al*., 2018; Hamdi *et al*., 2018; Li *et al*., 2018
5	Improve illegal digestibility performance of phosphorous, Calcium, magnesium, zinc or sodium in broiler chicken diets	Moss *et al*., 2018; Kim *et al*., 2017; Hamdi *et al*., 2018
6	Reduce liver weight	Viveros et al., 2002
7	Improve digestibility of protein and amino acid (Val, Ile, nonessential amino acid and total amino acid	Moss *et al*., 2018; Sa *et al*., 2013
8	Enhance apparent metabolize energy (AME)	Moss *et al*, 2017
9	Improve immunity against new castle virus	Ghosh *et al*., 2016
10	Decrease *E.coli* count and enhanced villous length	Roofchaei *et al*., 2017
11	Improved feed conversion ratio (FCR) or feed intake	Bradbury *et al*.,2017; Moss *et al*., 2017;Lee *et al*, 2017

2.6. Factors Affecting Phytase Production in microorganisms

2.6.1Temperatures

For an organism temperature is one of the important factors that affect the growth of microorganism and produce desired metabolites (Pandey *et al*., 2001 and Spier et al., 2008). Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this is usually varied from one organism to another

(Banargee and Bhattacharya, 1992; Kumar and Takagi, 1999).So, optimal temperature for the production of phytase from various sources varies, the production of phytase increased with increase the fermentation temperature level between 30 to 80°C (Wang *et al.*, 2004).The highest productions of phytase by *Klebsiella* spp.at 37ºC has also been reported by more than a few researchers as the optimum temperature (Aziz *et al.*, 2015;Wang *et al.*, 2004). The cell free supernatant of culture has the phytase activity that exhibited temperature optimal at 37ºC (Wang et al., 2004) and constant at broad variety of temperature (30 up to 80ºC). Phytase from some other microbes has found at 37ºC to the best function (Aly, 2015 and El-Toukhy, 2013). As the main future application of phytase is the digestive aid in animal feed, it needs to be thermo tolerant, so that phytase can resist high temperature during pelleting process of food especially for monogastric animals (Arpana *et al.*, 2011). Low and high temperatures may affect the metabolism of the microorganism and hence the enzyme production. At higher temperature the enzyme production decreases which might be due to growth reduction and enzyme inactivation or suppression of cell viability (Ellaiah *et al.*, 2002). In contrast, low temperature values may reduce the metabolism of the microorganism and consequently the enzyme synthesis (Mazutti *et al.*, 2007).

2.6. 2pH

Another most important factor is pH of the medium for enzyme production and fermentation process as it affects growth rates and production rate (Demirkan, 2014). Both effects are influenced by the breakdown of substrates and the transport of both substrate and product through cell wall. (Demirkan, 2014). Increased un-dissociated phytase production in agreement with decreasing pH due to phytase production is considered to inhibit the fermentation of several enzyme producers (Wang *et al.*, 2004) the nature of pH environment importance for requirement ofthe culture media for the grown of organism (Demirkan, 2014). Various researchers studied the effect of pH in the range of 4.0–8.0 for their effect of phytase production by the selected isolate grown in the production medium, at least 80% of phytase activity residual (Demirkan, 2014). At pH 4.0 highest activity it indicates acidic in nature. Thus, the pH stability profile of phytase from *Klebsiella* sp. and this is used for monogastric animals feeds in the digestive aid (Elkhalil *et al.*, 2007; Escobin-Mopera *et al.*, 2012 and Sajidan *et al.*, 2004). The Maximum phytase production was reported at pH 6.0 from *Klebsiella pneumoniae* SCTb2 (Das *et al.*, 2013).

2.6. 3 Carbon source

A number of different substrates have been used to fermentative production of phytase by phytase producing bacteria (Kumar, *et al*., 2013). A wide variety of carbon sources including glucose, sucrose, fructose, starch and lactose are capable of supporting the production of phytase bacteria (Demirkan, 2014). The *Bacillus megaterium* has produced higher quantity of phytase from fructose, but in many other phytase enzymes in the production medium is glucose as a general carbon source for the better growth and production (Kumar, *et al*., 2013).

2.6.4 Nitrogen source

Different sources of nitrogen including yeast extract, peptone, urea, and ammonium sulphate, sodium nitrate ammonium nitrate, and urea are used for maximum phytase production in bacteria (Demirkan, 2014). Supplementation with a nitrogen source resulted in a better phytase production compared to the control without supplementation (Demirkan, 2014). Supplementation with sodium nitrate and ammonium sulphate also resulted in a significant increase in phytase production compared to the un supplemented controls this is in agreement with previous studies using *A. niger* van Tieghem as the fermentation organism and *Sporotrichum thermophile* Apinis (Vats, 2002; and Bijender & Satyanarayana,2008).

2.6.5 Incubation time

Incubation period has great effect on the enzyme production and its activity, the enzyme production from microorganism is directly correlated to the period of incubation (Smitt *et al*., 1996).The Incubation time varies from 12 hrs to 80 hrs of the fermentation medium(Kumar, *et al*., 2013).The cells may reach at the optimum Fermentation time or stationary phase increases the enzyme production but beyond the optimum or the cells may reach the decline phase and displayed low phytase synthesis(Kumar, *et al*., 2013).

2.6.6 Inoculum size

Inoculums size plays an important role in fermentation process, a suitable inoculums size sufficient amount of nutrient and oxygen accessible for growth of bacterial (Demirkan, 2014). Size of inoculums is an important biological factor for the production of enzyme. Hence, a balance between the biomass and available materials should be for maximum enzyme production (Demirkan, 2014).the size of inoculums varies from 2-10% (v/v) of the fermentation medium. Normally for bacterial fermentations inoculums size varies from 2-5% (Demirkan, 2014). Lower inoculums level results in a lower number of cells in the production medium. This requires a longer time to grow to an optimum number to utilize the substrate and form the desired product (Kashyap *et al*., 2002). Phytase yield and biomass formation had parallel however, phytase activity decreased at lower and higher inoculums size (Gulati, 2007). In contrast, higher inoculums sizes produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis (Baysal *et al*., 2003).

3. Materials and Methods

3. 1 Description of study area

The study was conducted to characterize phytase producing bacteria from May 20, 2018 up to March 8. 2019. The experiment was conducted in Microbial Biotechnology Laboratory at Addis Ababa Science and Technology University (AASTU).

3.2 Sample collection

Samples used as basis of phytase producing bacteria were collected randomly block design (X-fashion) from different sites within the premises of Addis Ababa Science and Technology University(AASTU) parameter, Total 60 samples were gathered. Those are: 20 rhizospheric soil samples from common cereal crops (Maize, Wheat, Teff, and Sorghum) and 30 samples from legumes (Chickpea, Cowpea, Field pea, Grass pea, Faba bean, and Lentil). 5 fresh poultry excreta samples were obtained from the area of Addis Ababa. 5 Cattle shed samples were gathered from around Debre Zeit. In this study, sample collection was performed aseptically usi444ng sterile spatula and plastic bottle. Then it was transported in Ice box to a Microbial Biotechnology Laboratory, AASTU. After arrival to the laboratory, samples were stored at 4^{o}C until used.

3.3 Preparationofawheat bran extracts medium

Twenty five kilo gram (25kg) wheat bran was collected from Charily Feed Factory Addis Ababa, Ethiopia. A mass of 1 kg of oven dried and milled wheat bran was suspended in a solution containing 10 L of distilled water and 50 mL of H_2SO_4 and then autoclaved at 121^{o}C for 20 minutes. The liquid fractions were separated by filtration (47 mm pore size), and pH was adjusted to 6.5 using 1N of NaOH solution and/or HCL. Finally the wheat bran extract was obtained by the removal of water at 50°C under reduced pressure as in indicated by Gao and Xiao (2009).

3.4 Isolation and screening of phytase producing bacteria

Ten gram of each sample was separately suspended in 90 ml of sterile distilled water and each samples suspension was serially diluted (10^{-1}up to 10^{-6} fold). Luria agar medium (yeast extract 5g/L, peptone 10g/L, NaCl 10g/L, Agar 20g/L; distilled water 1000 ml) was prepared. The pH value of the medium was adjusted at pH 7 using 1N of HCl and 1N NaOH.100µl of each sample suspension was inoculated and spread over it. Plates were cultured aerobically at 28°Cfor 72 hours. Suspected isolates were picked up and sub-cultured to purify all isolates (Powar and Jagannathan, 1982).

All isolates were screened by re-culturing each of the single colonies on Wheat Bran Extract Agar Medium(0.04% of $NH4)_2SO_4$, 0.02% of $MgSO_4.7H_2O$, 0.1% of casein, 0.05% of KH_2PO_4, 0.04% of K_2HPO_4 and 2% of agar with 1000 ml of Wheat Bran Extract) for phytase production. The pH was adjusted to 6.5 and incubated at 37°C for72 hours aerobically. Colonies which form a clear zone was considered as potential phytase producer (Stephen and Jisha, 2008). The halo (Z) diameters and colony (C) diameters were measured using ruler. The hydrolysis efficiency of all the isolates was determined by the formula Z-C/ C (Stephen and Jisha, 208). Isolates above 50% efficiency were selected and transferred into nutrient agar slants, and stored at 4^oC for further activities (Stephen and Jisha, 2008).

3.5. Characterization and Identification of the Selected Isolates

3.5.1Cultural and morphological characteristics

Colony characteristics such as size, color, shape, margin, texture, and elevation were studied after growing the isolates on phytase screening agar medium containing 1% starch for 24 hrs at 37°C (Ashwini *et al.,* 2011; Tom-Sinoy *et al.,* 2011); Identification of the selected bacterial isolates were done using data obtained from morphological, biochemical characterization and the isolates were further identified to the genus level according to Bergey’s Manual of Determinative Bacteriology.

3.5.2. Gram stain

Gram staining method was performed using fresh cultures of 24 hrs old culture following standard procedures (Abeba Haile, 2004). It is the study of microscopic look of the isolates. A small portion of colonies smeared on microscopic slide and heat fixed. Heat fixed slide was flooded with crystal violet for about 60 seconds. The slide was rinsed several times under gently running tap water and stained with iodine solution for a minute. The slide was rinsed under gently running tap water and air dried. The air dried slide was immersed in 95% ethanol for 5 second and rinsed under tap water several times. Thereafter, the rinsed slide was counter stained with saffranin for about a minute and rinsed under gently running tap water. The slide was either air or blot dried to be observed under the bright field compound microscope (Brand and origin) using 100x (oil immersion) objective. Gram positive cells appeared as blue violet but Gram negative bacteria took the appearance of the counter-stain.

3.5.3. Biochemical characterization of the selected isolates

The biochemical characterization of the selected isolate were performed using test for catalase production, casein hydrolysis, urease production, starch hydrolysis, H_2S production and carbohydrate fermentation patterns for 12 different sugars (Avinash *et al*., 2013).

3. 5. 3. 1.Catalase test

Catalase production was detected by adding 1 ml of 3% H_2O_2 to 24 hrs old starch agar slant culture using un-inoculated medium as a negative control. Formation of bubbles confirms a positive test for catalase while its absence indicates negative result (Abeba Haile, 2004).

3. 5. 3. 2. Casein hydrolysis test

For the Casein hydrolysis test the potent isolates from24 hrs old cultures were streaked on casein containing medium (1% casein and 2.8% nutrient agar) and incubated for 24 hrs and at 37ºC, after 24 hrs incubation, trichloro acetic acid (TCA) was added over the cultures. If observe the clear hydrolysis zone formed by the colony the organism is positive for casein hydrolysis (Vishwanatha *et al*. 2010).

3. 5. 3. 3. Urease production test

From overnight culture each isolate 200 ml of culture broth was inoculated into 10 ml of urea broth media with pH indicator phenol red (0.1g Yeast extract, 0.09g mono-potassium phosphate, 0.095g Disodium phosphate, 20.0g Urea and 0.01g phenol red in 1000ml distilled water, pH 6.9) and incubated at 37ºC for twenty four hours up to forty eighty hours. After twenty four hours of incubation the observation was complete if urea was hydrolyzed, ammonia accumulates in the medium and makes it alkaline. The color of indicator to change from orange-red to deep pink or purplish red and it was a positive test for urea hydrolysis. Failure to developing of a deep pink color was a negative test (Abeba Haile, 2004).

3. 5.3. 4. Starch hydrolysis test

Culture of each isolate taken from 24 hrs old growth was streaked on starch agar media and incubated for twenty four hours at 37°C. After 24 hrs, each of the line streaks on the starch agar plate was flooded with Gram’s iodine reagent if the Presence of a clear surrounding area in the streaked line indicated the hydrolysis of starch, and so positive for the test (Abeba Haile, 2004).

3. 5. 3. 5.Hydrogen sulfide (H2S) production test

Triple Sugar Iron (TSI) agar slants containing (1% lactose, 1% sucrose, 0.1% glucose, 2% peptone,0.5% NaCl, 0.3% beef extract, 0.03% ferric citrate, 0.03% Sodium thiosulfate, 0.0024% phenol red, and 1.2 % agar) with pH 6.5 was inoculated with 10 ml culture broth by streaking the slant surface using a zig-zag line model and stabbing the butt using inoculating needle. The tubes were incubated for 24 hrs at 37ºC. H_2Sproduction was detected by blackening of the agar slant

3. 5. 3. 6. Carbohydrate fermentation tests

For a carbohydrate fermentation test 10 ml of basal medium (0.1% peptone, 0.02% yeast extract, 0.3% NaCl, 0.05% KH_2PO_4, 0.01% MgSO4 and 0.01% CaCl) containing 0.1% of one of the 12 carbohydrates (glucose, galactose, sucrose, maltose, manitol, cellulose, dextrose, lactose, Arabinose, sorbitol, xylose, inositole,) and 0.0025% phenol red as pH indicator. 200 µl culture broths were inoculated at 37°C for 48 hrs. The observation after 48 hrs of incubation, change in the color of the medium from red to yellow indicates a positive result for the test (a positive test for acid production from the fermentation of the carbohydrate tested)

3. 5.4 Molecular characterization and sequencing

DNA was extracted by proteinase K digestion in a lysis buffer via phenol-chloroform extraction method (Janarthanan and Vincent, 2007).To extract Genomic DNA the putative bacterial isolates, RS1, RS8, RS10 and RS15 were cultured in Wheat Bran Extract broth and incubated at 37°C for 24 hrs in an orbital shaker at 200 rpm. From each culture grown 2 ml was centrifuged at 10,000 rpm for 10 min at 4^{o}Cand the supernatant were transfer to in1.5ml Eppendorf tube and added 600µllyses solution (100mM NaCl, 10mM Tris HCland 0.5 SDS at pH 8) and 40µl of proteinase K incubate at 60°C for 1hrs. Add an equal volume of buffer –saturated phenol to the DNA solution and centrifuged. Carefully remove the aqueous layer from each sample and place in to anew tube. Add an equal volume of a buffer –saturated phenol: chloroform (1:1) mixture to the aqueous layer. Centrifuged and add an equal volume of chloroform the aqueous layer to remove traces of the phenol. Repeat the chloroform steps twice. finally add an equal volume of isopropyl alcohol, 70% ethanol to the aqueous layer, centrifuged as above, discard supernatant and wash the pellet with 70% ethanol, genomic DNA was extracted and Later on, bands of tested isolates were visualized using 0.8% (w/v) agarose gel electrophoresis.

PCR amplification was carried out using 16S rDNA with universal primers 8f[(Forward primer: 5'AGAGTTTGATCCTGGCTCAG3')1492r(Reverseprimer:5'GGTTACCTTGTTACGACTT3')]in an Eppendorf Thermo cycler. The PCR product was checked by agarose gel electrophoresis. The gel was visualized under UV transilluminat or and photographed. The PCR amplification was carried out in a PCR thermal cycler. The PCR reaction mixtures(Master mix 10µl,nuclease free water 7µl, 1µl template DNA, 1 µl forward primers and 1 µl reverse primers to bring the total volume to 20 µl).PCR was performed in a thermal cycler (Eppendorf) using cycling conditions that consisted of an initial denaturation at 95°C for 10 min. and then 35 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 30 s and primer extension at 72°C for 60 s followed by a final extension at 72°C for 7 min. A negative control was also loaded to eliminate the possibility of reagent contamination. PCR products stained with ethidium bromide were analyzed using agarose (0.8%) gel electrophoresis and visualized under gel documentation system.

The quality PCR products were sentto Leibiz institute DSMZ (Deutsche Sammlung von mikro organismen zellkulturen) Braunschweig, Germany for sequencing. Sequenced data were edited using Bio Edit Sequence Alignment Editor (Version 7.0.5.3) and Mega x version 10.1.5 software used for sequences alignments analysis aligned and analyzed pylogentices tree construction respectively for finding the closest homolog using National Centre for Biotechnology Information (NCBI) Gen Bank database (Kumar, *et al* 2018). (http:// www.ncni.nlm.nih.gov).

3.6. Crude phytase enzyme extraction (Phytase Recovery)

To obtain crude enzyme the potent isolates were subjected to fermentation in a Wheat Bran Extract Broth Medium as g/l $(NH4)_2SO_4$0.04%, $MgSO_4.7H_2O$-0.02%, casein-0.1%, $KH_2PO_4$0.05%, K_2HPO_4-0.04%).The pH of the medium was adjusted at 6.5 before sterilization. The inoculated medium was incubated on an orbital shaker incubator (200 rpm) at 37^0C for 3 days. After 3 days of fermentation, the fermented broths from the flasks50 ml was transferred in to centrifuge tubes and centrifuged at 6000 rpm for 30 minutes at room temperature. The supernatants were transferred into clean test tubes and used as crude enzyme solution (Sreedevi and Reddy, 2013).

3.7. Phytase enzyme purification

Purification of extracted crud phytase enzyme was done by ammonium sulphate precipitation followed by dialysis. Cell free extract of 50 ml from each isolate was saturated with ammonium sulphate up to 20-60%. The content was incubated overnight and centrifuged at 10,000 rpm for 10 min. Supernatant from each isolates was collected and checked for enzyme activity; pellet were collected for further analysis. The enzyme mixture (pellet) from each isolate was transferred to dialysis tube and immersed in 2mM Tris-HCl buffer at pH- 7 at 4°C for 24 hrs for phytase purify.

3. 8. Phytase enzyme activity

Ferrous sulphate molybdenum blue technique was used to measured Phytase enzyme activity it was quantification of enzyme activity (Selvamohan *et al.*, 2012).To get cell free supernatant (CFS) from each culture grown in wheat bran a culture of 2 ml was centrifuged at 10,000 rpm for 10 min at 4^oC . From each isolate0.2 ml (200µl) of the CFS was mixed with 0.16 ml(160µl) of substrate solution contain 0.1 M tris-HCl, 2 mM sodium phytate and 2mM $CaCl_2$. The mix was incubated at 37°C for 30 minutes. Reactions were stopped by the addition of 2 ml (2000µl) from freshly prepared 5% of tri chloro acetic acid (TCA) followed by the addition of 2000 µl of coloring reagent. Coloring reagent was freshly prepared by mixing 4 volumes of 1.5% (w/v) ammonium molybdate with 5.5 % (v/v) sulfuric acid and 1 volume of 2.7% (w/v) ferrous sulfate solution (Supreeth*et al*, .2015). After the addition of coloring reagent, absorbance was taken at 700 nm. To determine the quantity of inorganic phosphorous released the absorbance were compared to the standard curve, prepared by using different concentrations of di potassium hydrogen phosphate (K_2HPO_4) as a source of inorganic phosphorous. Standard curve used to estimate the conce44ntration of known phytase sample. Phytase activities were measured in terms of enzyme units from the amount of inorganic phosphorous released. The phytase activities (IU/ml of CFS) were defined because the micromoles of inorganic phosphate liberated in one minute.

Phytase activity IU/ml = K*OD *f /S*M*30

K is slope of standard curve, OD is optical density is dilution multiple, S is determination amount of sample (0.2 ml) and M is sample weight (0.06).

3. 9 Optimization of phytase production

3. 9. 1 Effect of nitrogen sources

Wheat bran extract medium was prepared in bulk and discharged into five separate 250 ml flasks and then supplemented separately with a different nitrogen source at 1% (w/w). The nitrogen sources used were ammonium nitrate, sodium nitrate, ammonium sulphate, peptone and urea. Each wheat bran extract medium in separate flask with different nitrogen supplement was inoculated separately with 10% of 24 hr old inoculum and agitated in a shaker incubator (200 rpm) at 37^0C for 3 days. After 3 days, the fermented broth from each flask was centrifuged and the supernatant was collected for examination of phytase activity (Sreedevi and Reddy, 2012).

3. 9. 2. Effect of carbon sources

Wheat bran extract medium was prepared in bulk and discharged into four separate 250 ml flasks and then supplemented separately with a different carbon source at 1% (w/w). The carbon sources used were glucose, sucrose, galactose and starch at 1% (w/w). Each wheat bran extract medium in separate flask with different carbon supplement was separately inoculated with 10% of 24 hr old inoculum and reserved in a shaker incubator (200 rpm) at 37^0C for three days. After

3 days, the fermented broth from each flask was centrifuged and then the supernatant collected and examined for phytase activity as indicated by (Sreedevi and Reddy, 2012).

3. 9. 3. Effects of incubation period

To optimize incubation period for the maximum phytase production by the selected experimental isolates were determined by growing each isolate separately in wheat bran extract medium at a temperature of 37°C and pH 6.5. The experiments were carried out individually for 24, 48, 72 and 96 hrs. The enzyme assays were carried out at each time interval (Sreedevi and Reddy, 2012).

3. 9. 4. Effects of inoculums size

The wheat bran extract medium was prepared and transferred into six separate 250 ml flasks. Phytase producing isolates were inoculated (10% of 24 hr old inoculum)in to Wheat Bran Extract Medium at different inoculum levels (100 µl,200 µl, 400µl, 600µl, 800µl and 1000µl)respectively. The optical density (OD) of enzyme activity was measured after 24 hr of incubation (Sreedevi and Reddy, 2012).

3. 9. 5 Effects of pH

The wheat bran extract medium was prepared into seven separate 250 ml flasks and the pH in each flask was adjusted to 3, 4, 5, 6, 7, 8, and 9 pH using 1NHCl and 1N of NaOH. Then each flask was separately inoculated with 10% of 24 hr old inoculum and kept in a shaker incubator at 200 rpm for 3 days. At the end of 3 days the fermented broth was collected, and centrifugeted, then supernatants were collected and phytase activity was estimated (Sreedevi and Reddy, 2012).

3. 9. 6 Effects of Temperature

The wheat bran extract mediums were prepared into seven separate 250 ml flasks and each medium of flask was inoculated with 10% of 24 hr old inoculums. The effect of temperature for phytase production was studied by incubating 10% of 24 hr old inoculums (culture medium) at different temperatures 35, 40, 45, 50, 55 and 60°C along with arbitrary control at 37°C. At the end of 3 days the fermented broth was collected, centrifuged, the supernatant collected and the enzyme assay were carried out (Sreedevi and Reddy, 2012).

3.10. Proximate composition of commercial poultry feed before and after addition of phytase enzyme analysis

Poultry feed from commercial(well-mixed) and poultry feed from commercial with experimental phytase were sent to Bless Agri food Laboratory Services P.L.C and Ethiopian Public Health Institute for the proximate composition (quality) analysis of crude enzyme, phosphorus, calcium, sodium, and potassium.

3.10.1 Determination of crude enzyme

The macro Kjeldahl method as described by AOAC (2016) was used to determine the crude enzyme content. From each sample, 2 g was introduced into the digestion flask. 10 g of copper sulphate and sodium sulphate in the ratio of 5:1 and 25 ml of concentrated sulphuric acid was added to the digestion flask. The flask was placed in a digestion block in fume cupboard and heated until frothing ceased given a clear and light blue coloration. The mixture was allowed to cool and diluted with distilled water until it reaches 25 ml of volumetric flask. Then after, 10 ml of the mixture was poured into the distillation apparatus and 10 ml of 40% sodium hydroxide was added. The released ammonia by boric acid was allowed to continue until 10 ml of boric acid is treated with 0.02 M of hydrochloric acid and the green color change to purple. Finally, the percentage of nitrogen in the sample was determined and calculated as the following:

$$\%\text{Crude enzyme} \quad = \frac{10 \times 1000 \times \text{sample weight (2g)}}{\%\text{Nitrogen} \times 6.25}$$

$$\%N = \frac{(S - B) x N x\ 0.014 x D x 100}{\text{Wt. of the sample} x V}$$

Where

S = Sample titration reading

B = Blank titration reading

N = Normality of HCl

D = Dilution of sample after digestion

V = Volume taken for distillation

0.014 = Millis equivalent weight of nitrogen

3.10. 2 Determination of minerals

Mineral contents of poultry feed from commercial before and after addition of experimental phytase enzymes were determined by atomic absorption spectrometry for calcium, flame photometry for sodium, and potassium and spectrophotometer for the phosphorus determination according to the methods of Association of Official Analytical Chemists (2016).From each sample wet digestion sample were prepared for the mineral determination. Samples of1 g samples were introduced in to the digestion glass tube. 12ml of $HNO_3$was added to the samples and mixture was kept for overnight at room temperature. Then 4.0 ml perchloric acid ($HClO_4$) was added to the mixture and kept in the fumes block for digestion. The temperature was started from 50ºC and increasing up to 250-300^{o}C the digestion completed in about 70- 85 min as indicated by the appearance of white fumes. The mixture was left to cool down and the contents of the tubes were transferred to 100 ml volumetric flasks and the volumes of the contents were made to 100 ml with distilled water. The wet digested solution was transferred to plastic bottles labeled accurately, stored the digest at 4ºC for the analysis.

Different electrode lamps were used for each mineral. The equipment was run for standard solutions of calcium before and during determination to check that it was working properly. Dilution factor for phosphorus is 2500 and Dilution factor calcium, potassium and sodium, was 100.The concentrations of minerals recorded in terms of parts per million (ppm) were converted to milligrams (mg) of the minerals by multiplying the ppm with dilution factor and dividing by1000, as follows:

$$MW = \frac{\text{absorbency (ppm) x dry wt. x D}}{\text{Wt. of sample x 1000}}$$

3.10.2.1 Determination of phosphorus (P)

3.10.2.1.2Preparation of the mix reagent

Twelve gram of the ammonium molybdate was taken and mixed with 250 ml-distilled water in a beaker (solution A), 0.2908 gm antimony potassium tartarate was taken and dissolved in 500 ml H SO (5N) solution in a 2 4 volumetric flask. Enough distilled water was added to make the solution up to 1000 ml (solution B), the two solutions (A and B) were mixed in a 2000 ml volumetric flask to get mix reagent. The volume of the mix reagent was made up to 2000 ml by addition distilled water, 0.739 gram of ascorbic acid was mixed with 140 ml of the mix reagent in a beaker and left until dissolved to make color reagent and1ml of wet digested duplicate food sample was taken in a plastic bottle labeled properly and to it was added 4.0ml distilled water to make a diluted volume of 5.0ml. Five milliliters (5.0ml) of color reagent was added to this volume and the total volume of this mixture (final solution) was made up to 25.0ml. The dilution factor of this solution was 2500ml. After some time, the color of this final solution turned blue. Sample from final blue solution was taken in a cuvett and introduced to spectrophotometer. The reading of the phosphorus was recorded in ppm. The calculations for the total mineral intake involved the same procedure as given in Atomic Absorption Spectrophotometer (AAS).The concentrations of minerals recorded in terms of parts per million $1/10^6$(ppm)and converted to milligrams (mg) of the minerals by multiplying the ppm with dilution factor and dividing by 1000.

3. 11. Data Analysis

All the experiments were carried out independently in triplicate. The data represented were in the form of mean± Standard Error(SE) by using SPSS (Statistical Package for Social Sciences), version 23 software, micro soft excel 2010, Bio Edit version7.0.5.3andMega xversion 10.1.5 software used for sequences alignments and pylogentices tree analysis respectively. Results were presented by using tables and graphs.

4. Results

4.1. Screening of purified isolates for phytase production

From three different sample sources such as Rhizosphere soil, Cattle shed and Fresh poultry excreta samples a total of 169 isolates were selected for phytase production out of which 36 (100%) isolates showed positive for phytase production (Table2, Figure 1).

Table 2 Total number of phytase produce isolates

No	Sample sources	Number of isolates tested	
		Isolates	**Positives**
01	Rhizospheresoil samples	72(42.6%)	18(50%)
02	Cattle shed samples	55(32.54%)	10(27.78%)
03	Fresh poultry excreta samples	42(24.85%)	8(22.22%)
Total		169(100%)	36(100%)

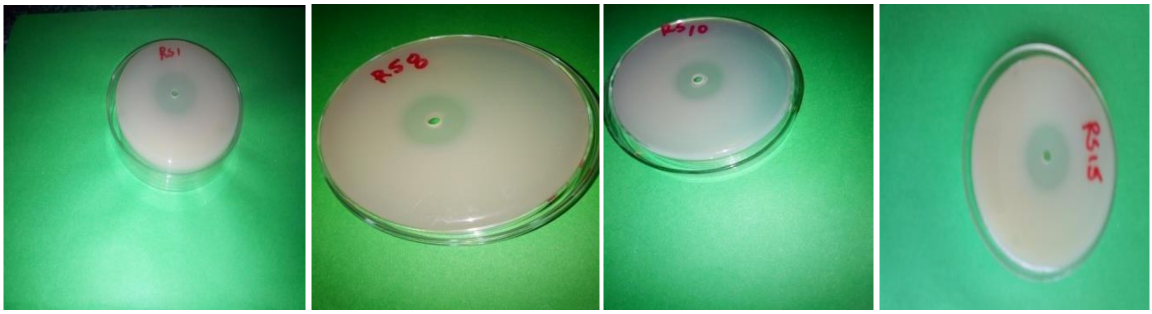
The clear zone of hydrolysis produced by the isolates found positive for a phytase activity on wheat bran extract medium found ranging from the least 10mm to the largest 32 mm (Table 3). From the 36 positive isolates, 16 isolates that produced large clear zone of hydrolysis from 10-32 mm) were tested further. From the 16 isolates, 4 potential isolate those produced clear zone (30-32 mm) were for this study. The remains 12 isolates were inefficient capacity (<50%) then kept at 4^0C (Table-3).

Table 3. The efficiency of phytase producing bacterial isolates

Samples sources	**Positive isolates**	**Holo diameter (mm)**	**Colony diameter (mm)**	**Efficiency of hydrolysis Z-C/C (%)**	**Remarks**
Rhizospheres oil samples	Rs 1	31	5	52	Selected for further
	Rs 8	32	5	54	Selected for further
	Rs9	18	5	26	Not selected
	Rs 10	30	5	50	Selected for further
	RS 15	30	5	50	Selected for further
	Rs 16	27	5	44	Not selected for further
	Rs 30	28	5	46	Not selected for further
	Rs 32	21	5	32	Not selected for further
	Rs33	23	5	36	Not selected for further
	RS36	25		40	Not selected for further
Cattle shed Samples	C3	14	5	18	Not selected for further
	C12	10	5	10	Not selected for further
Fresh Poultry excreta samples	FPE a	26	5	42	Not selected for further
	FPE 1	23	5	36	Not selected for further
	FPE 10	16	5	22	Not selected for further
	FPE 14	18	5	26	Not selected for further

Key: RS was isolated from Rhizospheresoil samples, C was isolated from Cattle shed samples, and FPE was isolated from Fresh Poultry excreta samples.

The detection of these four selected isolates obtained from Rhizospheresoil samples using wheat bran extract agar media is shown in (Figure 1).



RS1 isolate RS8 isolate RS10isolate RS15 isolate

Figure 1. The clear zone hydrolysis efficiency of phytase producing isolates

4.2. Characterization of the selected phytase producing bacteria isolates

Cultural bacteria isolates were characterized. Colony Size, Shape, Color, Margin and Elevation were observed on solid media (Table 4).the isolates RS1 and RS8 were found in the size of colony, shape and color indicter that small, circular and white, respectively and the isolates RS10 and RS15were found in the size of colony, shape and color indicter that Medium, Irregular and Blond respectively. However, the Margin and Elevation of the isolates RS1 and RS8 were show that entire and flat fish respectively and the isolates RS10 were show that Filaments and Flat respectively were as RS15 show that undulate and Flat respectively. Gram staining, cell-shape, and arrangement of four selected isolates were characterized morphologically (Table 4).

Biochemical test results obtained from four bacterial isolates (RS1, RS8, RS10 and RS15) were shown in (Table 4). All the four selected phytase producing bacteria were found negative for casein, starch, H_2S production and glucose fermentation. However, Isolates RS8, RS10, and RS15 were found catalase producers, whereas RS1 was not, Except RS8, the other three isolates (RS1, RS10 and RS15) were shown to be urease negative. Selected bacterial isolates RS1, RS8, RS10 RS15 were further identified to the genus level by Bergey’s Manual of Determinative Bacteriology.

Table 4 Cultural, morphological and biochemical test characteristics of the selected isolates

No	**Cultural characteristics**	**RS1**	**RS8**	**RS10**	**RS15**
01	Size	Small	Small	Medium	Medium
02	Shape	Circular	Circular	Irregular	Irregular
03	Color	White	White	Blond	Blond
04	Margin	Entire	Entire	Filaments	Undulate
05	Elevation	Flat fish	Flat fish	Flat	Flat
	Morphology characters				
06	Gram Staining	Negative	Negative	Negative	Negative,
07	Cell- shape	Rod	Rod	Rod	Rod
08	Cell arrangement	Diplobacillus	Steptobacillus	Cocobacillus	Bacillus
	Biochemical tests				
09	Catalase production	-	+	+	+
10	Casein hydrolysis	-	-	-	-
11	Urease production	-	+	-	-
12	Starch hydrolysis	-	-	-	-
13	Hydrogen sulfide production	-	-	-	-
14	Carbohydrate fermentation	-	-	-	-
15	Suggested genus	Enterobacter sp	*Klebsiella pneumonia sp*	Pseudomonas sp	Escherichia coli sp

Key: (-) indicates negative test and (+) indicates positive test.

4.2.1. PCR amplification of the 16S rDNA from the selected isolates

Amplification of DNA isolated from four pure selected bacterial isolates RS1, RS8, RS10 and RS15 with universal primers for 16S rDNA, generate 16s rRNA PCR product and its PCR product sizes were around 1500 bp (Figure 2). This indicates that the primers used to amplify 16S rDNA weretargeted so that it targets the required conserved DNA region (Figure 2).

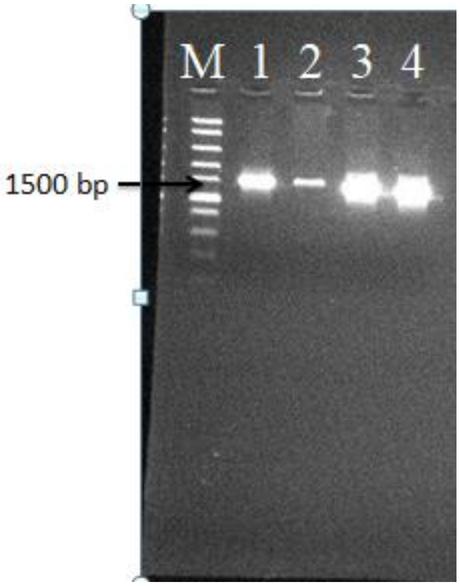


Figure 2. Amplified 16S rDNA of the four bacterial isolates and the control run in 0.8% (w/v) of Agarose Gel: M- 1kb DNA ladder, 1-4 showed 16S rDNA regions of RS1, RS8, RS10 and RS 15 respectively.

4.2.2. Molecular characterization and sequencing

16S rRNA partial gene sequences were performed for selected bacterial isolates (RS1, RS8, RS10 and RS15 (Table 5).BLAST analysis of the partial 16S rRNA gene sequences for the four bacterial isolates with the highest phytase activity are presented. The bacterial selected isolates of 16S rRNA sequence RS1, RS8, RS10 and RS15 were 100%, 99.59%,99.65% 99.66% respectively and gene bank similarity with Enterobacter asburia strain CPS B-2, *Klebsiella pneumoniae strain Sihong_846_1, Pseudomonas putida strain Xuyi* and *Escherichia coli* 25-Ec-C-116 plasmid strain respectively and the total score 2488, 1321, 1554 and 1611 Spp., size (bp) 16S ribosomal RNA gene length. However, the bacterial selected isolates of 16S rRNA sequence RS1, RS8, RS10 and RS15 were found the Gen Bank Accession number KF516258.1, MN314319.1, MN314434.1 and LC501697.1 respectively.

Table 5 Molecular characterization of Selected bacterial isolates

No	**isolates**	**Similarity**	**Species size (bp)**	**Identity**	**Accession Number**
01	RS1	Enterobacter asburia strain CPS B-2	2488	100%	KF516258.1
02	RS8	*Klebsiella pneumoniae strain Sihong_846_1*	1321	99.45%	MN314319.1
03	RS10	Pseudomonas putida strain Xuyi_351_2	1554	99.65%	MN314434.1
04	RS15	Escherichia coli 25-Ec-C-116 plasmid p25C116-2, sequence_id: contig13	1611	99.66%	LC501697.1

Phylogenetic analysis of 16S rRNA partial gene sequences was retrieved by LAST search from NCBI Gen Bank Database. The homology nucleotides were presented in Dendrogram (Figure 3) Likeness and homology of the adjacent sequences have been shown via the robustness tree determined by the analysis of 1000 time's bootstrap values between the intermediate.

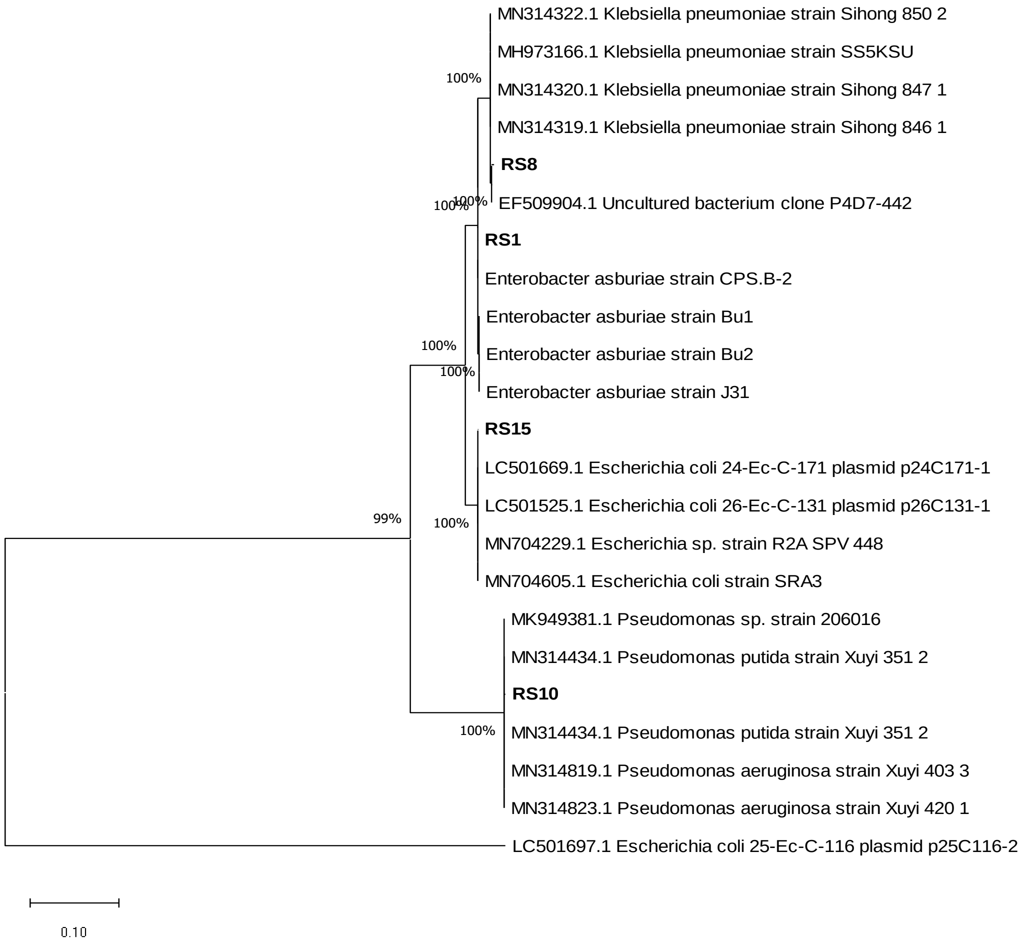


Figure 3. Phylogenetic position of the bacterial strain selected isolates with other directly related bacterial strains retrieved from NCBI Gene Bank. ween

4.3. Crude and purified phytase enzyme activity

During sub-merged fermentation, isolated bacteria's were produce high amount of total crude enzyme, crude enzyme activity and purified enzyme activities (Table 6). The isolates RS15 and RS10 were found producing the maximum (0.071and 0.058 IU/ml) and minimum (0.049 and 0.019 IU/ml) amount crude enzyme activity and purified enzyme activities, respectively of the purified enzyme (Table 8) as seen in the Appendix Table 1and 2. However, theisolatesRS10 and RS15 was shown as 1.09(g/100g) and 0.78 (g/100g) the amount total crude enzyme (Table 8).

Table 6 Activity of phytase

No	Name bacterial species	Total Crud enzyme (g/100g)	Crud enzyme activity (IU/ml)	Purify enzyme activity (IU/ml)
01	*Enterobacter asburiae*(RS1)	1.00	0.061	0.029
02	*Klebsiella pneumonia(RS8)*	0.72	0.054	0.027
03	*Pseudomonas aeruginosae (RS10)*	1.09	0.049	0.019
04	*Escherichia coli* (RS15)	0.78	0.071	0.058

4. 4. Optimization for phytase production

4. 4.1 Effect of nitrogen sources

The effect of nitrogen source on phytase production indicated that the four bacterial isolates (RS1, RS8, RS10 and RS15) (Figure4).The isolates RS1andRS8 were found to produce the maximum0.046IU/ml and 0.056IU/ml enzyme activity at 1% Ammonium sulphate. The isolates RS10and RS15were shows to have phytase activity of 0.04 IU/ml and 0.043 IU/ml in media supplemented with 1% urea, respectively.

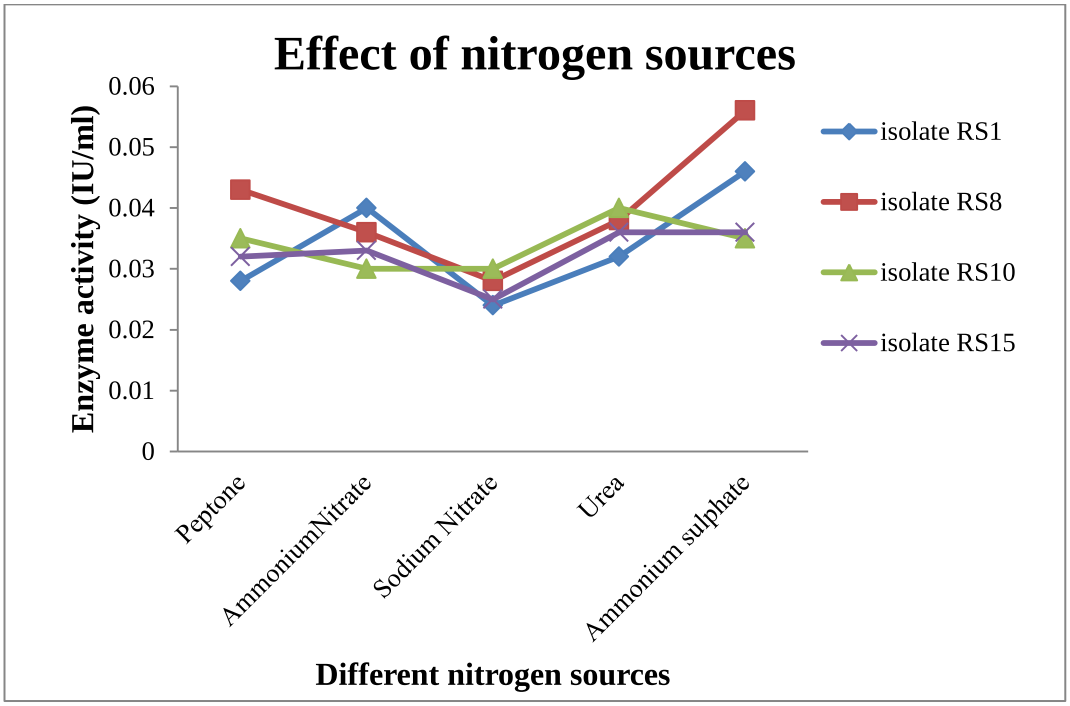


Figure 4. The effect of Nitrogen sources on phytase production

4. 4 2. Effect of carbon sources

Bacterial isolates (RS1, RS8, RS10, and RS15) were shown the effect of different carbon source (sucrose, starch, Galactose and glucose)at1% during submerged fermentation (Figure 5) and in the AppendixTable 3.Isolates(RS1, RS8, RS10, and RS15) wereproduce show high amount of phytase activity at1% Galactose0.027 IU/ml, 0.042 IU/ml 0.023 IU/ml and 0.026IU/ml respectively (figure 5).

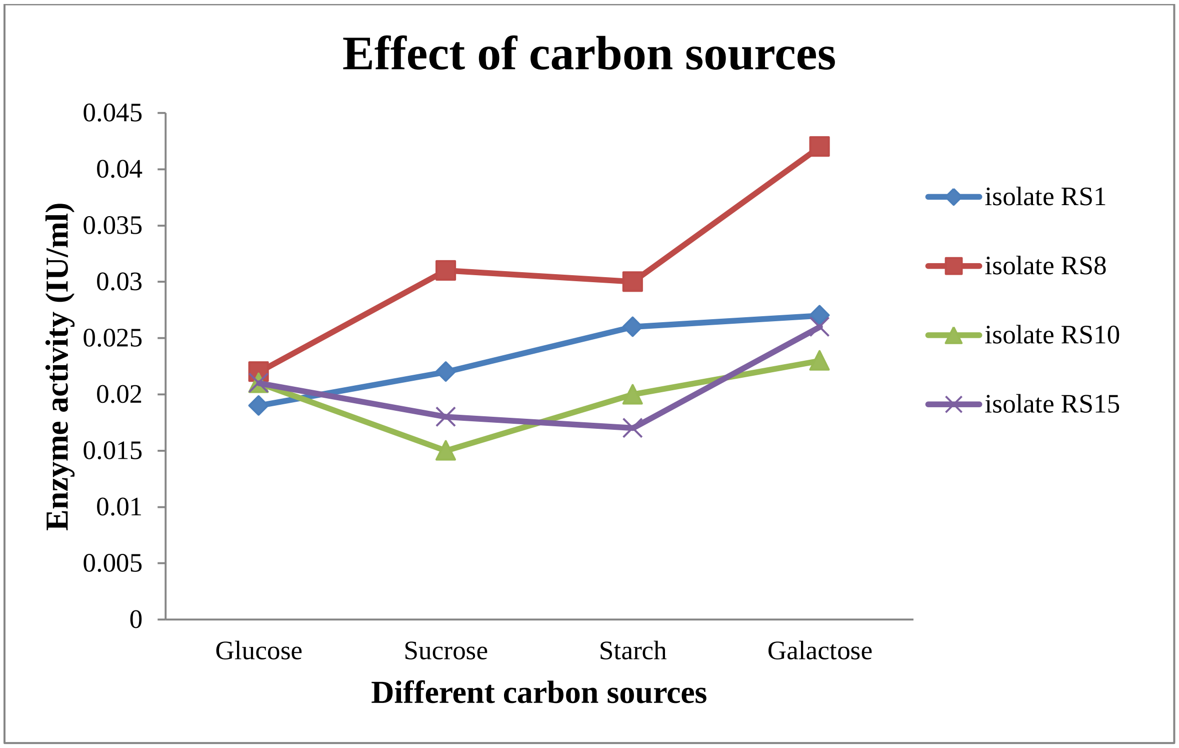
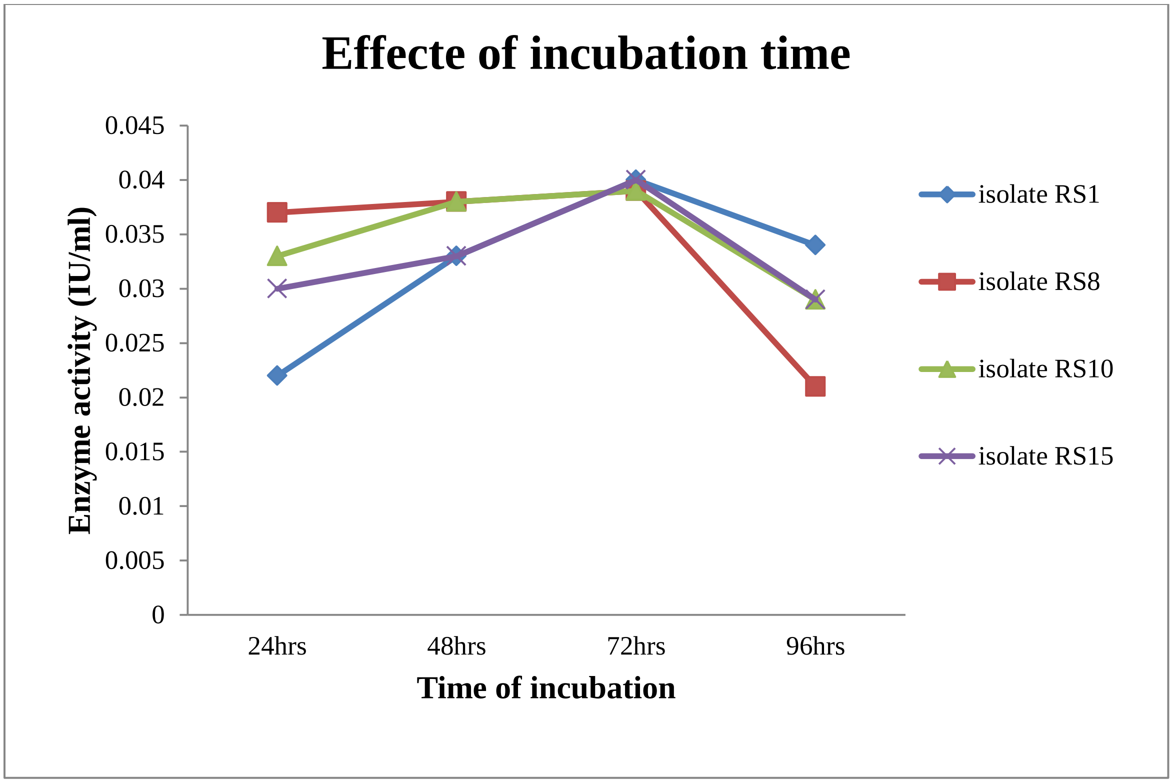


Figure 5. The effect of carbon sources on phytase production

4. 4 3.Effect of incubation time

The results showed that all isolates (RS1, RS8, RS10 and RS15) the production increased with reached maximum0.04, 0.039, 0.039 and 0.04IU/ml at 72hrs, respectively (Figure 6). As indicated Appendix Table 5. The isolates (RS1, RS8, RS10, and RS15) were phytase production was reduced to 0.022, 0.021, 0.029 and 0.029IU/ml. at 96 hrs respectively (Figure 6)



.

Figure 6. The effect of Time on phytase production

4.4 4. Effect of inoculums size

The effect of inoculums (100µlup to 1000µl v/v) on the production of phytase by the four selected isolates was shown in Figure 7. The data indicated that as the inoculums size increases, the production of phytase by all the selected isolates under the given condition (temperature, agitation, pH, and medium used) was increasing up to 800µl volume then decline after that lone The isolate RS8 was shown a maximum amount of phytase production almost in all inoculum size followed by RS1, RS15 and RS10.The phytase productions reduce at 1000µl by all isolates.

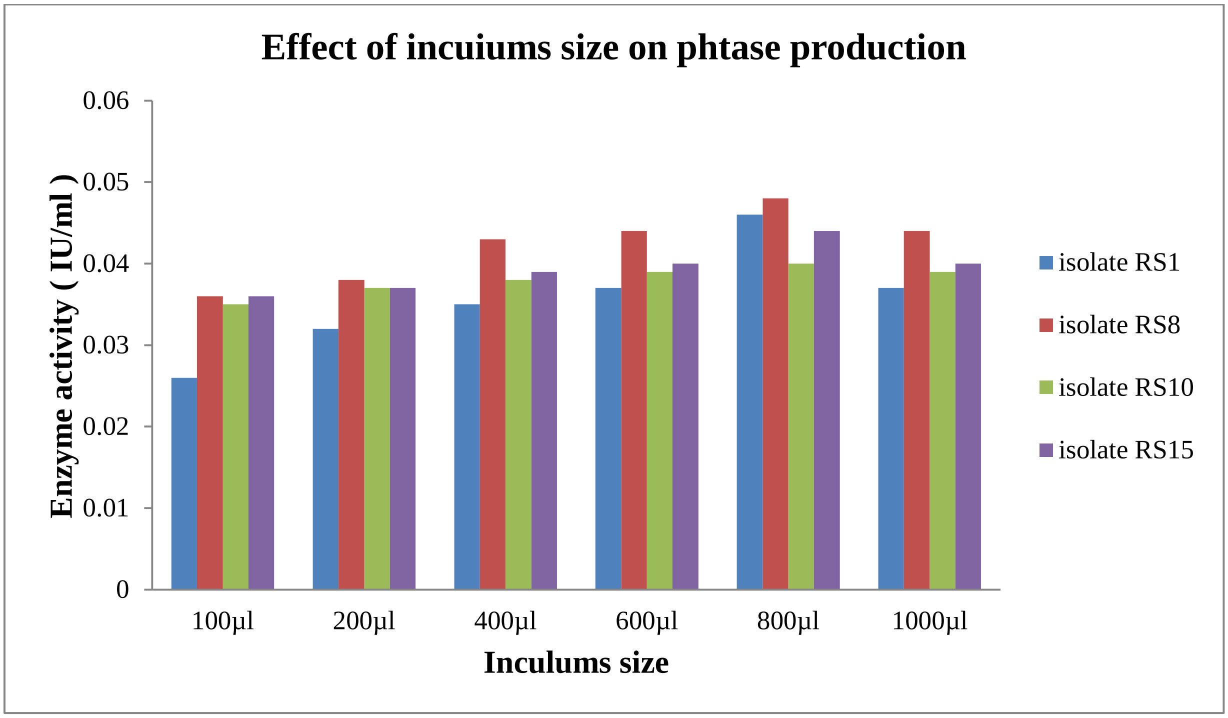


Figure 7. The effect of inoculums size on phytase production

4. 4. 5. Stability of phytase at different pH

The results showed that in every one isolates (RS1, RS8, RS10 and RS15) the phytase activity gradually increased and reached maximum (0.046 IU/ml, 0.049 IU/ml, 0.049 IU/ml and 0.047IU/ml respectively at pH 6 (Figure 8). All the isolates showed a wide variety of pH outline (3-9) for phytase production. Enzyme production showed a gradual increase from pH 3 to pH 6 but easily decreased from pH 7 to pH 9. For all the isolates, there was a pointed increase in enzyme activity as the pH approached 6 but decreased as the pH to alkaline values.

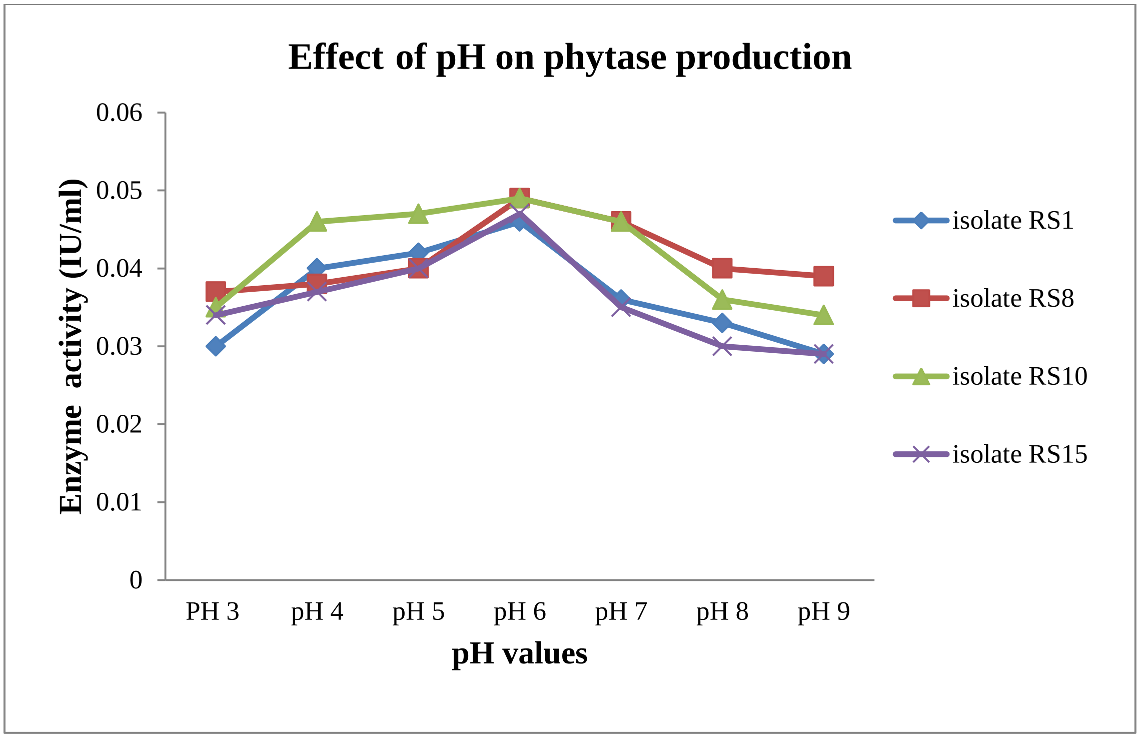


Figure 8. The effect of pH on phytase production

4. 4.6. Stability of phytase at different temperature

Temperature effect on the phytase production by the four selected isolates was shown in (Figure 9). All the four selected isolates showed that also as the temperature of incubation increase from 30^0C to 50^0C the production of phytase shown increased. The production of phytase shown declined at 55^0C of incubation temperature. The result indicated that RS15 the maximum amount of the enzyme at all incubation temperature except at 55^0Cand these were observed to be followed by RS.

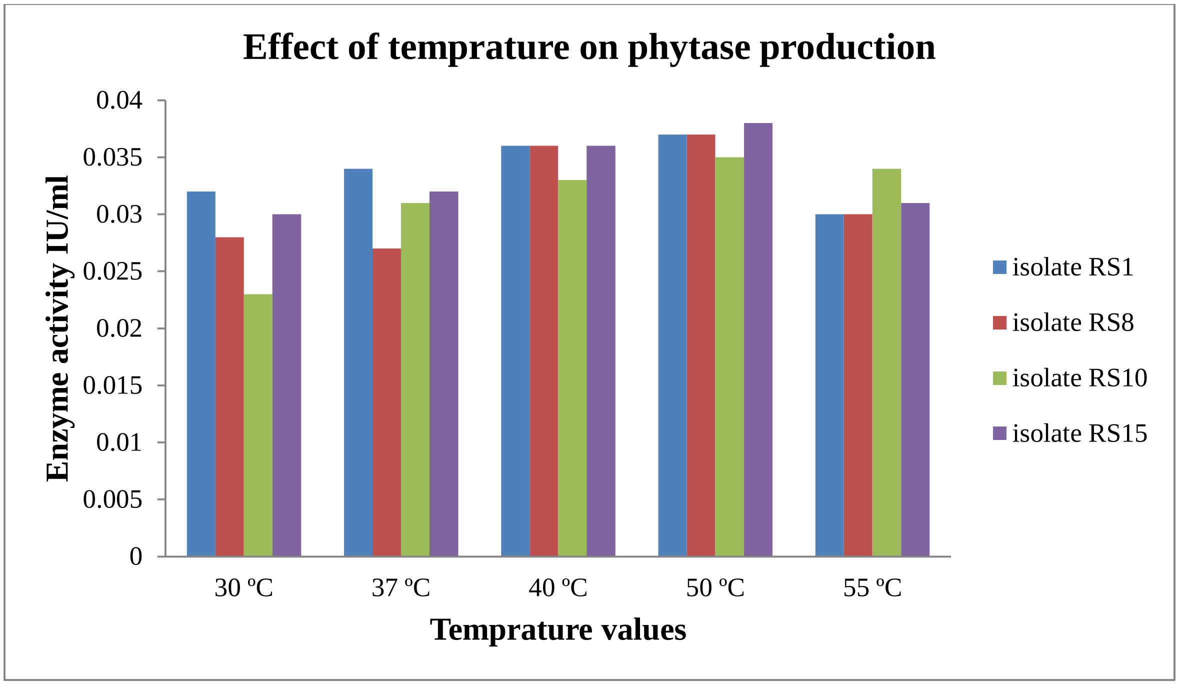


Figure 9. The effect of temperature on phytase production

4. 4.7 Proximate composition of poultry feed before and after addition of experimental phytase enzyme

The results of proximate analysis of commercial poultry feed (PF) as control and poultry feed with phytase enzymes (E1, E2, E3 and E4)(Table 7). The result of proximate compositions analysis of poultry feed with phytase enzymes (E1, E2, E and E4) from selected bacterial isolates (RS1, RS2, RS3 and RS4) improve bioavailability of phosphorus, calcium, potassium and sodium were presented.

Table 7 Analysis of poultry feed

	Isolates	**P Mg/100g**	**Ca Mg/100g**	**K Mg/100g**	**Na Mg/100g**
Before (only feed)	PF	80.15±0.016	142±0.081	514.99±0.41	317.78±0.02
After[feed with phytase (500U/kg)]	E1	81.75±0.02	497±0.81	537.25±0.008	326.41±0.008
	E2	80.85±0.008	186±0.008	531.24±0.008	323.22±0.012
	E3	82.13±0.008	271±0.81	537.73±0.008	319.8±0.081
	E4	83.85±0.008	244±0.81	533.59±0.016	322.19±0.012

Key: PF (Poultry feed), E1 (Enzyme one from RS1), E2 (Enzyme two from RS8), E3 (Enzyme three from RS10) and E4 (Enzyme four from RS15).

Statistical analysis showed that all treatments were significant difference at P< 0.05.

5. Discussion

Phytase producing bacterial isolates were selected based on their activity of phytase production and their clear zone hydrolysis of around the colony. This potential isolates were showed great variation in the sizes of the clear zone of hydrolysis. They were exhibited on wheat bran extract agar plates ranging from the least 10mm to the largest 32 mm. This study was agreed with the earlier studies that already reported clear zone of phytate hydrolysis ranged from > 6mm (Aziz, *et al*.2015).

Morphological, biochemical, Cultural and molecular Characterizations also studied for the selected isolates (RS1, RS8, RS10 and RS15) they were alone gram-negative, rod cell shapes and *Diplo bacillus*, *Stepto bacillus*, *Coco bacilluss* and *Bacillus* respectively based on Cell arrangement. Except isolates RS1, the rest of the other isolates positive in catalase test andRS1, RS10 and RS15 were show urease negative, whereas isolate RS8 was show urease Positive. All the selected isolates were negative results in H_2S production, starch hydrolysis, casein hydrolysis and Carbohydrate fermentation (glucose)According to Bergey’s Manual of Determinative Bacteriology selected bacterial isolates RS1, RS8, RS10 RS15 were further identified to the genus level *Enterobacter, Klebsiella pneumoniae, Pseudomonas* and *Escherichia coli sp.* respectively.

The 16S rRNA gene-based approach was used as a tool for identification unknown fungal species which are able to produce phytase enzyme. Sequence nucleotide analysis of conserved 16S rRNA region helps to find out the sequence similarity of different eukaryotic species based on either pair wise or multiple sequence analysis (hailu, *et al* 2017). A 16S rRNA the selected isolates alone belongs to (*Enterobacter asburiae* (RS1), *Klebsiella pneumonia (*RS8), *Pseudomonas aeruginosae* (RS10) and *Escherichia coli*(RS15) were show 100%, 99.45%, 99.65% and 99.66% respectively similarity with *Enterobacter asburiae strain CPS B-2*, *Klebsiella pneumoniae strain Sihong_846_1*, *Pseudomonas putida strain Xuyi* and *Escherichia coli* 25-Ec-C-116 plasmid strain, respectively verified in the database. The microbes identification based on 16S rRNA partial gene sequence focuses on key parts of the genetic makeup of bacterial (hailu, *et al* 2017).

The 16S rRNA partial gene sequence of nucleotide homology was inferred by BLAST search in the NCBI Gen Bank database. This investigation involving 5 BLAST sequences of similar homolog's which were found in Gen Bank revealed that the four samples (RS1, RS8, RS10 and RS15) have 100%, 99.45%, 99.65% and 99.66% respectively sequence similarity or identity with other species and phylogenetic family member of the four identified bacterial isolates with other closely related type strains retrieved from the NCBI database are accessible in the dendrogram (Fig 3). The 16S rDNA is a good candidate for finding consensus conserved regions suitable for genus or higher taxonomic level detections because of its relatively slow rate of molecular evolution.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). Phylogenetic tree based on 16S rRNA gene sequence using maximum likelihood tree method a powerful tool for deducing evolutionary and phylogenetic relationships among organisms was decisive in their identification (Sharma,et al 2017).

The tree with the highest log likelihood is shown. The percentage of trees in which the linked taxa clustered together is revealed next to the branches. This analysis involved 18 nucleotide sequences. A total of 728 positions in the final dataset. There Evolutionary analyses were conducted in Mega x software (Kumar, et al., 2018).

The nitrogen sources are secondary energy sources for the organisms, which play an important role in the growth of the organisms and the production (Kumar, *et al.*, 2013). In this study the effect of different nitrogen sources (ammonium sulphate, ammonium nitrate peptone sodium nitrate and urea) on phytase production show that the selected bacterial isolates (RS1, RS8, RS10, and RS15) (Figure4). The isolates RS1 and RS8were produced the maximum phytase activity of 0.046IU/ml and 0.056IU/ml better enzyme activity was recorded at 1% of Ammonium sulphate at 37°C, pH 6.5, 200 rpm and at 72hrs.More than a few studies had revealed that maximum phytase production was found when ammonium sulphate and peptone were used as a nitrogen source (Shah *et al.*, 2009: Gulati *et al.,* 2007). Ammonium sulphate induced phytase production more than ammonium nitrate (Shah *et al.*, 2009: Gulati *et al.*, 2007). In fact these nitrogen sources are the most common to effectively induce phytase enzyme production by bacteria (Choi *et al.*, 2001).

Carbohydrates (glucose, sucrose, galactose and starch) are essence energy source for the growth of organisms (Kumar, *et al*., 2013). In many other enzymes, the production is carried out by medium amended with glucose as a general carbon source for better growth and production. But the isolates have produced higher quantity of phytase from Fructose. All isolates (RS1, RS8, RS10, and RS15) were indicated that the maximum phytase activities 0.027, 0.042, 0.023 and 0.026 IU/ml were record at1% galactose utilization respectively at studies 72hrs, at 200 rpm, at 37°C and pH at 6.5. The result of this study was consistent with others (Tungala et al., 2013; Kumar, *et al*., 2013).

The maximum phytase activity was record at 72hrs, 200 rpm, 37°C and pH of 6.5 and at 96 hrs phytase activities was reduced. All isolates (RS1, RS8, RS10, and RS15) were shows that the maximum phytase activity 0.04, 0.039, 0.039, and 0.04IU/ml at 72 hrs respectively and the isolates (RS1, RS8, RS10, and RS15) were phytase activity was decreases to 0.022, 0.021, 0.029 and 0.029IU/ml respectively (Figure 6).The decrease of phytase production might be due to the reduction of nutrients, or the death phase of the organism or due to the buildup of by-products such as toxins, inhibitors and proteolysis activities in the medium. These by-products are reduced the growth of organisms and enzyme arrangement. This result showed a similarity to phytase from *Pseudomonas fluorescen* (Tungala *et al*., 2013).

The effect of inoculum size of the selected phytase producing bacterial isolates, (RS8, RS1, RS10 and RS15) were studied; maximum phytase production was recorded at inoculum size of 800µl) 72hrs, 37°C and pH of 6.5. In the present investigation the isolates grown in wheat bran extract culture medium with different inoculum size. The isolates (RS1, RS8, RS10 and RS15) were show that the maximum phytase productions 0.046, 0.048, 0.04 and 0.044 IU/ml respectively (Figure 7).At low inoculums size, growth of the organism might be reduced resulting in insufficient biomass and long-drawn-out time for the organism to enter the stationary phase (Sreedevi and Reddy, 2012).This increases the time needed for consuming substrate and synthesizing the desired product (enzyme). Alternatively at high concentration of inoculums, the bacteria grow rapidly and the nutrients present in the media become insufficient to support the increased number of bacteria and making it very difficult for them to synthesize the desired product (Sreedevi and Reddy, 2012).In addition, higher inoculums size may result in a rapid over population of the bacteria and may cause problem of aeration, rapid pH change of the medium. This may affect the phytase activity of the bacterial. Thus, the production of phytase was affected at lower inoculums size this result showed a similarity to phytase from *Bacillus* sp.C43 (Sreedevi and Reddy, 2012).

The effect of different pH value 3 up to 9 on the production of phytase in four selected bacterial isolates alone shown in (Figure 8).All isolates results showed that in the phytase activity increased and reached the maximum at pH 6 (0.046, 0.049, 0.049, and 0.047IU/ml) respectively at 72hrs, 37°C and pH of 6.5. In the present study, this result showed a similar to phytase from *Pseudomonas* sp. aazad, *Bacillus* spp. and *Klebsiella* spp. (Singh *et al*., 2017). Due to that the isolates favor neutral pH for optimum growth (Singh *et al*., 2017; tungala *et al*., 2013). All isolates at pH 9 decrease the production of phytase relative to the rest of the pH values. A Similar study also revealed that phytase from *E. coli* ATCC, reduce phytase production at pH 9 (Singh *et al*., 2017).

The effect of different temperatures values (30, 37,40, 50 and 55°C) on the phytase production of four selected bacterial isolates were studied at 72hrs, 37°C and pH of 6.5.All isolates) were showed that the optimum temperature activity of phytase recorded at 50°C from the rest of the other temperatures value and the phytase activity reduced at 55°C. The result of this study similar to phytase from *Pseudomonas* sp. strain aazad (Singh *et al*., 2017; Farouk*et al*., 2012, Elkhalil *et al*., 2011). *Aspergillus japonicus* BCC18313 (TR86), phytase from *A. niger* BCC18081 (TR170) and *E. parvum* BCC17694. **(**Fugthong*et al*., 2010; Promdonkoy *et al*., 2009) and also some investigators results showed on optimal temperature from different microbial sources agreement with our results (Seo *et al*., 2005).Similar studies also stated that phytase isolated from *Aeromonas*sp. LIK 1–5 and*Citrobacter braakii* YH-15 the optimumtemperature determined at50°C and the activity decreased after 55°C (Kim *et al*., 2003).

Proximate composition analysis of (phosphorus, calcium, potassium and sodium) before poultry feed used as control for the poultry feed with phytase enzymes (E1, E2, E3 and E4) (Table 9). Phytase as a supplement the poultry feed increases compared to the control. The result show that proximate composition analysis after supplementation of phytase with commercial poultry feed to release phytate-bound phosphorus and improve bioavailability of phosphorus, calcium, potassium and sodium. The result of this study was in agreement with the previous study of the percent of commercial phosphorus, calcium and phytase were added in to the poultry feeds for the proportion of feed ingredients and increase feed efficiency (Sukumar and Jalaudee, 2003) and improved feed efficiency without affecting feed intake (Ponnuvel *et al*., 2015). The Ca-phytate complexes are not easily hydrolyzed by the phytase, resulting in unavailability of phytate P and bound Ca to poultry. Poultry feed containing calcium can have a great impact on phytase efficacy and phytate P utilization (Jorquera *et al*., 2008). A phytase will not be competitive if it cannot be produced in high yield and purity by a relatively inexpensive system. Recently, economically competitive expression and/or secretion systems for microorganisms have been developed (Konietzny *et al*., 2004). A different strategy to overcome the problems using phytase as a feed additive such as cost, inactivation at the high temperatures required for pelleting feed and loss of activity during storage.

6. Conclusions

- Phytase obtained from RS1, RS8, RS10 and RS15 are phytate-degrading enzyme and suitable for the purpose of poultry feeds.
- The optimum phytase activity of the selected bacterial isolates[0.042IU/ml from RS8 at 1% galactose (a carbon source),0.056IU/ml from RS8at 1% ammonium sulphate (a nitrogen source, 0.038 IU/ml from RS15 at50°C(temperature),0.049 IU/ml from RS8 andRS10at pH 6, 0.04 IU/ml from RS1 and RS15at72 hr (incubation time)and 0.048 IU/ml from RS8at 800µl(size of inoculum)]obtain under cultivation conditions agitated at 200 rpm, 37°C, and pH 6.5 after 72 hr of incubation time.
- Based on the results, all isolates alone possible to increase the phytase production in cultures medium (wheat bran extract medium) by supplementing with 1% galactose and ammonium sulphate, temperature (50°C), pH (6), incubation time (72 hr) andsize of inoculum (at 800µl).
- Maximum phytase productions are recorded in the presence of galactose as carbon source and ammonium sulphate as nitrogen source. The production of phytase by these isolates was shown improving nutritional status of the feed.
- Finally, these isolates could be a potential candidate for the production of phytase and applicable in poultry feed industries.

7. Recommendations

Based on the result of this study, the following recommendations were made:

- For the bacterial cultures cultivating in liquid media, continuous shaking of the growth media is essential. So, fermentation of cultures in shaker incubator would improve the performance of the isolates for production of phytase.
- Further work is needed regards too optimization methods such as factorial design/by combining factors could give best interactions of the factors for scale up the production phytase.
- Further study should be conducted for different factors like agitation, different other carbon source and nitrogen source in combination and separately to study their effect on the production of phytase. This will improve the production of phytase in industrial level.
- Strain improvement such as classical and genetic engineering of the phytase producing bacterial isolates should be done in order to increase poultry production for industrial level

8. References

Abeba Haile (2004). Amylase Production from Bacterial Isolates of Forest Soil, Selected Agricultural Waste and Kitchen Waste. Thesis, M.Sc. Haramaya University, Haramaya.

Alves, N. M., Guimarães, L. H. S., Piccoli, R.H. and Cardoso, P.G. (2016). Production and Partial Characterization of an Extracellular Phytase Produced by *Muscodor sp*. under Submerged Fermentation. *J. Adva. Microbiolo.***6:** 23-32.Skip

Aly, M. M., Tork, S., Al-Garni, S. M.and Kabli, S. A. (2015). Production and Characterization of Phytasefrom Streptomyces luteogriseus R10 Isolated from Decaying Wood Samples. *Int. J. Agri. and Biolo.***17**: 515-522. Skip

Anandham, R.K. H., Choi, P.I., Gandhi, W .J., Yim, S. J., Park K.A., Kim, M. and Madhaiya, T.M.S. (2007). Evaluation of shelf life and rock phosphate solublastion of Burkholderial, sp.in nutrient ammended clay, rice bran and rock phosphate based granular formulation. *J. world. Microbiol. Biotechnolo.* **23:** 1121-1129.

AOAC (2016). Association of Official Analytical Chemists. AOAC Press,Washington DC.

Arpana, M., Gulab, S., Varsha, G., Anita, Y., KamalRai, A., SanjeevKumar, G.and NeerajKumar, A. (2011). Isolation and biochemical characterization of acido-thermophilic extracellular phytase producing bacterial strain for potential application in poultry feed. *J. undishapur J. Microbiolo*. **4**: 273-282.

Ashwini, K., Kumar, G., Karthik, L. and Rao, B.K.V. (2011). Optimization, production and partial purification of extracellular α- amylase from *Bacillus* sp/ Marini. *J. Archives. Applied. Sci. Resea.***3:** 33-42.

Avinash, t. k., narayanan, A. and muthuraman, M. S. (2013). Isolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production. *Int. J. Pharma. and Pharmaceuti. Sci.* **5**: 975-1491.

Aziz, G., Nawaz, M., Anjum, A. A., Yaqub, T., Mansur-ud-din, A., Nazir, J., Khan, S. U. and Aziz, K.(2015). Isolation and characterization of phytase producing bacterial isolates from soil. *J. Animal* and *Plant Sci.* **25**: 771-776.

Banargee, R. and Bhattacharya, B.C.(1992). Extracellular alkaline protease of newly isolated *Rhizopus oryzae*. *J. Biotechnol.* **14**: 301-304.

Baysal, Z., Uyar, F. and Aytekin, C. (2003). Solid srtate fermentation for production of alpha amylase by a thermotolerant *B. subtilis* from hot spring water, *J. Process. Biochem.* **38**: 1665-1668.

Bijender, S. and Satyanarayana. (2008).Phytase production by a thermophilic mould Sporotrichum thermophile in solid state fermentation and its potential applications, *J. BioresourTechnol.***99**: :2824-2830.

Birhanu, H., Tehetna A, Yohannes Hand. Awot T (2015) Assessment of BioSecurity Condition in Small Scale Poultry Production System in and Around Mekelle, Ethiopia. European *J. Biol. Sci.***7**: 99-102.

Bradbury, E.J., Wilkinson, S.J., Cronin, G.M., Thomson, P., Walk, C.L.and Cowieson, A.J. (2017). Evaluation of the effect of a highly soluble calcium source in broiler diets supplemented with phytase on performance, nutrient digestibility, foot ash, mobility and leg weakness. Anim Prod Sci, **57**: 2016-2026. doi: 10.1071/AN16142

Broch, J., Nunes, R. V., Eyng, C., Pesti, G. M., de Souza, C., Sangalli, G. G.and Teixeira, L. (2018). Effect of dietary phytase super dosing on broiler performance. Anim Feed Sci Technol doi: 10.1016/j.anifeedsci.2018.06.001

Cabuk,M., Bozkurt, M., Kyrkpynar, F.and Ozkul, H. (2004). Effect of phytase supplementation of diets with different levels of phosphorus on performance and egg quality of laying hens in hot climatic conditions. A*fr. J. Anim Sci.*,**34**: 13-17.doi: 10.4314/sajas.v34i1.3804

Choi,Y.M, Suh, H.J.and Kim J.M. (2001). Purification and properties of extracellular phytase from *Bacillus sp*. KHU-10. *J. Prot. Chem.***20**: 287-292.

Chunshan, Q., Linghua, Z., Yunj, W. and Yoshiyuki, O. (2001). Production of phytase in slow phosphate medium by a novel yeast candida krusei. *J. Bio.sci. Bioengin.* **92**:154-160.

Dana, N., Vander, w. L, Dessie, T., Johan, A. and Van Arendonk. J. (2010). Production and traits preferences of poultry producers of Ethiopia. *Trop Anim Health Prod.* **42**: 1519-1529.

Das, K., Bandyopadhyay, D.and Sen, S. K. (2013). Optimizations of fermentation conditions for phytase production by the novel isolate *Klebsiella sp*. *J. Bioscan.***8**: 1315-1320.

Demirkan, E. Baygın, E. and Usta, A. (2014).Screening of phytate hydrolysis Bacillus sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. *J. Biochem.* **39**: 206–214.

Elkhalil, E., Männer, K., Borriss, R, and Simon, O. (2007). In vitro and in vivo characteristics of bacterial phytase and their efficacy in broiler chickens. *J. British. Poultry Sci.***48**:64-70. doi:http://dx.doi.org/10.1080/00071660601148195.

El-Toukhy, N. M., Youssef, A. S., and Mikhail, M. G. (2013).isolation, purification and characterization of phytase from *Bacillus subtilis* MJA. *Afri. J. Biotechnolo.***12**: 2957-2967.

Escobin-Mopera, L., Ohtani, M., Sekiguchi, S., Sone, T., Abe, A., Tanaka, M. and Asano, K. (2012). Purification and characterization of phytase from *Klebsiella pneumoniae* 9-3B. J. Biosci. Bioeng.**113:** 562-567.

FAO (2010). Poultry Meat and Eggs vialedelleterme di Caracalla, 00153 Rome, Italy. Pp. 5-7.

Farouk, A .F, Greiner, R and Hussin, A.S. M.(2012). Purification and properties of a phytate-degrading enzyme produced by *Enterobacter sakazakii* ASUIA279.*J. Biotechnol. Biodive*. **2**: 15-73.

Stephen, Joseph. and Jisha, M.S.,(2008). Phosphate Solubilisation efficiency. *J. Agri. and Enviro. Sci.,***4**: 110-112.

Fugthong, A., Boonyapakron, K., Sornlek, W., Tanapongpipat, S., Eurwilaichitr, L. and Pootanakit, K. (2010). Biochemical characterization and in vitro digestibility assay of *Eupenicillium Parvum* (BCC17694) Phytase Expressed in Pichia Pastoris. *Protein expression and purification*. **70**: 60−67.

Gao, Q.L and Xiao L.W. (2009).Selection of a Culture Medium for Reducing Costs and Enhancing Biomass and Intracellular Polysaccharide Production by A. blazei, Food Technol. Biotechnolo. **47**: 210–214

Ghosh, A., Mandal, G.P., Roy, A., Patra, A.K. (2016). Effect of supplementation of manganese with or without phytase on growth performance, carcass traits, muscle and tibia composition, and immunety in broiler chickens. Livest Sci, **191**: 80-85. doi: 10.1016/j.livsci.2016.07.014

Gulati, H.K., Chandh, B.S and Saini, H.S. (2007). Production and characterization of thermostable alkaline phytase from Bacillus laevolacticus isolated from rhizosphere soil. *J. Ind Microbiol Biotechnol*. **34:** 91-98.

Haftu , K (2016) Exotic Chicken Status, Production Performance and Constraints in Ethiopia: A Review. *J. Poultry Sci.***10**: 30-39.

hailu, W.H., pangastutiand,S.A. and greiner, R. 2017) Isolation, characterization and 18S rDNA sequence analysis of phytase producing fungi from Indonesia. *J. biodiver.***18:** 10-14.

Hamdi, M., Perez, J. F., Létourneau-Montminy, M. P., Franco-Rosselló, R., Aligue, R. and Solà-Oriol, D. (2018). the effects of microbial phytases and dietary calcium and phosphorus levels on the productive performance and bone mineralization of broilers. *Anim Feed Sci Technol*
doi: 10.1016/j.anifeedsci.2018.07.005

Hengge-Aronis, R. (1996). Regulation of gene expression during entry in to stationary phase. **In**: Neidhardt (ed). *Escherichia coli* and *Salmonella*Cellular and Molecular Biology, 2nd Edition, Vol. 1, ASM Press,Washington D.C., p.1497-1512.

Ireland, M.M.E., Karty, J. A., Quardokus, E.M.;, Reilly, J.P. and Brun, Y.V.(2002).Proteomic analysis of the *Caulobacter crescentus* stalk indicatescompetence for nutrient uptake *.J.Mol. Microbiol*., **45**: 1029-1041.

Janarthanan, S. and Vincent, S. (2007). Phenol chloroform extraction of DNA. In: Practical Biotechnology. **Pp**. 29-30.

Jorquera, M., Martinez, O., Maruyama, F., Marschner, p. and Mora, M. D. (2008). Current and future biotechnology applications bacterial phytase and phytase producing bacterial. *J. Enviro. Microbial.* **3**:182-191.

Kashyap, P., Sabu, A., Pandey, A., Szakas, G. and Soccol, C.R. (2002). Extracellular L-glutaminase production by Zygosaccharomyces rouxii under SSF. *J. Process. Biochem.* **38**: 307-312.

Kim, H. W., Kim, Y. O., Lee, J. H., Kim, K. K. and Kim, Y. J.(2003) Isolation and Characterization of a Phytase with Improved Properties from *Citrobacter Braakii.J.444Biotechnolo.***15:**1231–1234.

Kim, J.H., Han, G.P., Shin, J.E., Kil, D.Y. (2017).Effect of dietary calcium concentrations in phytase-containing diets on growth performance, bone mineralization, litter quality, and footpad dermatitis score in broiler chickens. Anim Feed Sci Technol, **229**: 13-18. doi:10.1016/j.anifeedsci.2017.04.008

Kimura,M. (**1980**). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Molecular Evolu.* **16**:111-120.

Konietzny U, Greiner R. (2002) Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. *J. Food Sci. and Tech.***37**: 791-812.

Konietzny, U. and Greiner, R. (2004). Bacterial phytase: potential application, in vivo function and regulatoin of its synthesis. *J. Microbiol.* **35:** 11-18.

Kumar, C.G. and Takagi, H. (1999). Microbial alkaline protease: from a bio industrial viewpoint. *J. Biotechnol. Adv.***17**: 561-594.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (**2018**). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. J. *Molecular. Biolog and Evolu.* **35**:1547-1549.

Kumar, V. Singh, D. Sangwan, P. and Gill, P. K (2015) Management of environmental phosphorus pollution using phytases: current challenges and future prospects. In: Applied Environmental Biotechnology: Present Scenario and Future Trends. Springer. pp 97-114.

Kumar,V., Singh, P. Jorquera, M. A. Sangwan, P. P. Kumar, V. A. K. and Agrawal. S. (2013). Isolation of phytase-producing bacteria from Himalayan soils and their effect on growth and phosphorus uptake of Indian mustard (Brassica juncea). *J. Microbiol. Biotechnol.***29:**1361–1369.

Lee, S. A., Nagalakshmi, D., Raju, M.V., Rao, S.V.R., Bedford, M.R. (20417). Effect of phytase superdosing, myo-inositol and available phosphorus concentrations on performance and bone mineralisation in broilers. Anim Nutr, **3**: 247-251. doi: 10.1016/j.aninu.2017.07.002

Lei, X. G., Weaver, J. D., Mullaney. E., Ullah, A. H., Azain, M. J (2013) Phytase, a new life for an “old” enzyme. *J. Annu Rev Anim Biosci.***1:** 283-309.

Lim, B. L., Yeung, P., Cheng, C. and Hill, J. E. (2007). Distribution and diversity of phytate mineralizing bacterial. J. inte. Sie. Mari. Envroin. **1:** 321-330.

Liu, B.-L., Rafiq, A., Tzeng, Y.-M. and Rob, A. (1998). The induction andcharacterization of phytase and beyond. *J. Enzyme Microbial Technol.,***22**: 415-424.

Matawork, M. (2016). Chicken Meat Production, Consumption and Constraints in Ethiopia. *J. Food Sci. Qual.* Managem. **54:** 2224-6088.

Mazutti, M., Ceni, G., DiLuccio, M. and Treichel, H (2007). Production of insulinase by solid state fermentation: effect of process parameters on production and preliminary characterization of enzyme preparations. Bioprocess Biosyst. Eng. **30**: 297-304.

Melkamu, B (2013) Effect of feeding different levels of dried tomato pomace on the performance of Rhode Island Red grower chicks. *Int J Livest Prod.***4**: 35-41.

Moss, A. F.,Chrystal, P.V., Dersjant-Li, Y., Selle, P. H. and Liu, S.Y. (2018). Responses in digestibilities of macrominerals, trace minerals and amino acids generated by exogenous phytase and xylanase in canola meal diets offered to broiler chickens. *Anim Feed Sci Technol.* **240**:22-30. doi: 10.1016/j.anifeedsci.2018.03.011

Onyango, E.M., Asem, E.K., Sands, J.S. and Adeola, O. (2004). Dietary phytates increase endogenous losses in ducks and chickens. *Poultry Sci.***83**: 149–150.

Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A. and Soccol, V. T(2001).Production, purification and properties of microbial phytases. *J.Bioresou. Technolo.***77**: 203-214.

Pica, C. U. and Otte, J. (2010). Poultry food Security and poverty in India. Looking beyond the Farm- gate.World's.*J .Poultry Sci.* 66: 309-320.

Ponnuvel, P., Narayankutty, K., Jalaludden, A.and Anitha, P. (2015). Effect ofphytase supplementation in low energy-protein diet on the production performance of layer chicken. *J. Sci. Biotechnolo.***3**: 25–27.

Powar V.K. and Jagannathan, V. (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis.J. Bacteriolo.***151:** 1102-1108.

Promdonkoy, P., Tang, K., Sornlake, W., Harnpicharnchai, P., Kobayashi, R. S. Ruanglek,V., pathanpreecha, T., Vesaratchavest, M., Eurwilaichitr, L. abd Tanapongpipat, S. (2009).Expression and Characterization of *Aspergillus* Thermostable Phytases in Pichia Pastoris. FEMS Microbiolo.**290:** 18 −24.

Reddy,C. S., Achary, V. M. M., Manna, M., Singh, J., Kaul, T. and Reddy, M. K. (2015). Isolation and molecular characterization of thermostable phytase from *Bacillus subtilis* (BSPhyARRMK33). *J. Appl. Biochemi. Biotechnolo.***175:** 3058-3067.

Reta, D. (2009). Understanding the role of indigenous chickens during the long walk to food security in Ethiopia. Livest Res Rural Dev **21**: 116.

Roofchaei, A., Rezaeipour, V., Vatandour, S.and Zaefarian, F. (2017). Influence of dietary carbohydrases, individually or in combination with phytase or an acidifier, on performance, gut morphology and microbial population in broiler chickens fed a wheat-based diet. *Anim Nutr.* doi: 10.1016/j.aninu.2017.12.001

Sa, K., Hr, C.andYs, B. (2013). The effect of phytase enzyme on the performance of broiler flock (A-Review). Poultry Sci. **1**: 117-125.doi: 10.22069/PSJ.2013.1478

Sajidan, A., Farouk, A., Greiner, R., Jungblut, P., Müller, E.-C.and Borriss, R.(2004), Molecular and physiological characterization of a 3-Phytase from the Rhizobacterium *Klebsiella pneumoniae* ASR1. *Appl. Microbiol. Biotechnol.* 25-53.

Sandberg, A.S and Andlid, T. (2002). Phytogenic and microbial phytase in human nutrition. *J. int. Food. Sci. Technolo.,* **37:** 823-834.

Sasirekha, B., Bedashree, T., and Champa, K. (2012). Optimization and partial purification of extracellular phytase from Pseudomonas aeruginosa p6. *European, J.Experi. Biolo,***2**: 95–104.

Scholey, D.V., Morgan, N.K., Riemensperger, A., Hardy, R., Burton, E.J (2018). Effect of supp lementation of phytase to diets low in inorganic phosphorus on growthperformance and mineralization of broilers. *Poult Sci,* **97**: 2435-2440. doi: 10.3382/ps/pey088

Selle, P.H. and Ravindran, V., (2007). Microbial phytase in poultry nutrition. *Anim. Feed Sci. Technol.* **135**: 1–41.

Selvamohan, T., V. Ramadas and M. Rejibeula. (2012). Optimization of phytase production by *Pseudomonas* Sp. Isolated from poultry faces. *Int. J. Mod Engin. Res.***2**: 1326-1330

Seo, M. J. Kim, J. N., Cho, E. A., Park, H., Choi, H. J and Pyun, Y. R. (2005). Purificationand Characterization of a Novel Extracellular Alkaline Phytasefrom Aeromonas Sp. *J. Microbiolo and Biotechnolo.* **15:** 745–748.

Shah, P., Bhavsar, K., Soni, S.K. and Khire, J.M. (2009). Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. *J Ind Microbiol Biotechnol***36:** 373-380.

Shim, J. H. and Oh, B. C. (2012).Characterization and application of calcium-dependent beta-propeller phytase from *Bacillus amyloliquefaciens* DS11. *J. Agri. Food Chem*. **40:** 9669-76.

Shivange, A. V., Schwaneberg, U. and Roccatano, D. (2010). Conformational dynamics of active site loop in escherichia coli phytase. *J. Biopoly.***93:** 994-1002.

Singh, N. K.; Joshi, D. K.; and Gupta, R. K (2003) Isolation of Phytase Producing Bacteria Andoptimization of Phytase Production Parameters. *J. Microbiol*. **6**: 6419.

Singh1, C. S., Singh1, s. Sharma, P.K. and Sharma D.C. (2017).Isolation, Identification and Molecular Characterization of Phytase Producing Bacteria, Pseudomonas Sp. *Aazad. J.pure and applied microbiolo***. 11:** 1845-1850.

Smitt, J.P., Rinzema, J., Tramper, H., Van, M. and Knol, W. 1996. Solid state fermentation of wheat bran by Trichoderma reesei QMQ414. *J. Appl. Microbial. Biotechnol.***46**: 489-496.

Soni, S.K. (2009). Phytase from *Aspergillus Niger* NCIM 563: Isolation, Purification, Characterization and its Applications. M.SC.Thesis, Pune University, India. pp. 1-180.

Spier, M. R., Greiner, R., Rodriguez-León, J. A., Woiciechowski, A. L., Pandey, A., Soccol, V. T., and Soccol, C. R. (2008). Phytase production using citric pulp and other residues of the agroindustry in SSF by fungal isolates. *J. Food Technolo. Biotechnolo.***46**: 178-182. doi:http://dx.doi.org/10.1080/00986445.2010.493115

Sreedevi, S. and Reddy, B.N. (2013). Screening for efficient phytase producing bacterial strains from different soils. *Int. J. Biosci.***3:** 76-85.

Stephen, J. and Jisha, M.S. (2008). Phosphate Solubilization efficiency. *J. Agricu. Environ. Sci.***4:** 110-112.

Sukumar D. and Jalaudeen A. (2003). Effect of supplemental phytase in diet on certain economic traits in layer chicken. *In. J. Anim. Sci.,***73**: 1357–1359.

Tadelle, D., Kijora, C., and Peters, K (2003) Indigenous chicken ecotypes in Ethiopia: growth and feed utilization potentials. *Int J Poult Sci.***2**: 144-152.

Tiwari, K. L, Jadhav, S.K, and Fatima, A. (2007). Culture condition for the production of thermostable amylaseby Penicillium rugulosum. *J. Biotechnol. Biochem.***2**: 21-24.

Tom-Sinoy, E.S., Bhausaheband, C.P. and Rajendra, P. P. (2011). Isolation and identification of feather degradable microorganism. *J.VSRD-Technical and Non-technical.***2:** 128-136.

Touati, E., Dassa, E., Dassa, J. and Boquet, P.L. Acid phosphatase (pH2.5) of Escherichia coli: regulatory characteristics. In: Phosphate metabolism and cellular regulation in microorganisms.American Society for Microbiology, Washington D.C., 1987, p.31-40.

Tumer, B.L., Paphazy, M.J., Hoygarth, P.M, and Mckelvie, L.D. (2002). Inositol phosphates in the environment. *J. Philos. T. Roy. Soc.***357:** 449-469.

Tungala, A, narayanan, k.A. and muthuraman, m. s. (2013). Isolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production. *Inter. J. Pharmacy and Pharmaceut. Sci.***5:** 975-1491.

Turner, B.L. and Leytem, A.B. (2004). Phosphorus Compounds in Sequential Extracts of Animal Manures: Chemical Speciation and a Novel Fractionation Procedure. *J. Environmen. Sci. Technolo.* **38:** 6101-6108.

Vats, P. and Banerjee, U. C. (2002).Studies on the production of phytase by a newly isolated strain of *Aspergillus nigervar teigham* obtained from rotten wood-logs, Process.*J. Biochem.***38**: 211-217.

Vishwanatha, T, Spoorthi, N.J., Reena, V., Divyashree, B.C., Siddalingeshwara, K.G., Karthic, J and Sudipta K.M. (2010). Screening of substrates for protease production from *bacillus licheniformis. Int. J. Engine. Sci. and Technolo.* **2**: 6550-6554.

Viveros, A., Brenes, A., Arija, I., Centeno, C. (2002). Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult Sci, **81**: 11721183. doi: 10.1093/ps/81.8.1172

Vohra, A., Satyanarayana, T. (2003) Phytases: Microbial Sources, Production, Purification and Potential Biotechnological Applications. Critical *Reviews in Biotechnolo.***23**: 29–60.

Wang, X., Upatham, S., Panbangred,W., Isarangkul, D., Summpunn, P., Wiyakrutta, S., and Meevootisom, V. (2004). Purification, characterization, gene cloning and sequence analysis of a phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5. *J. Sci.*

Yanke, L. J., Bae, H.D., Selinger, L.B. and Cheng, K.-J. (1998). Phytase activityof anaerobic ruminal bacteria. *J. Microbiol.***144**: 1565-1573.

Yenesew, A, Agraw A, Yihenew G, Dessalegn M. (2015). Poultry production manual. Bahir Dar university capacity building for scaling up of evidence best practices in agricultural production in Ethiopia (BDUCASCAPE) working paper p 17.

Zamudio, M., González, A. and Bastarrachea, F. (2002). Regulation of Raoultellaterrigena comb.nov. Phytase expression. *J. Microbiol.***48**: 71-81.

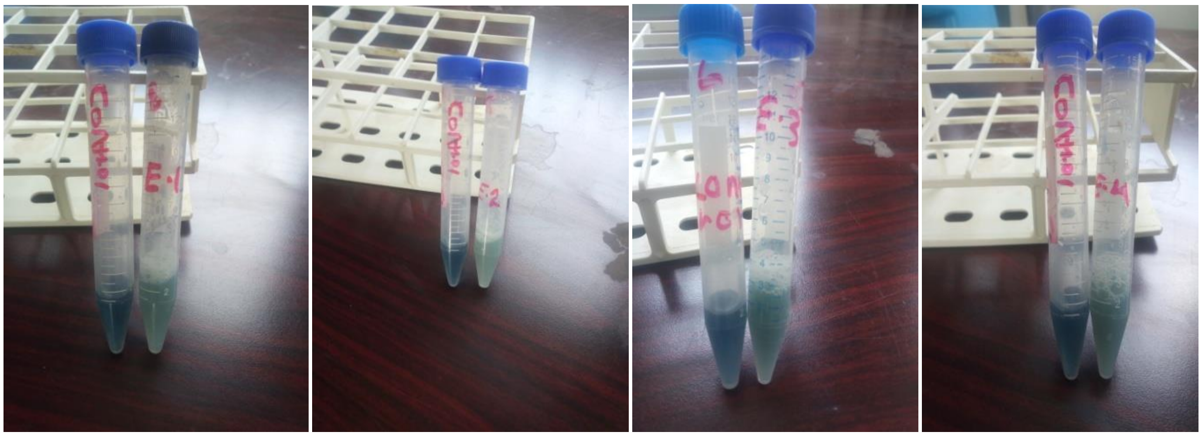
Zuo, R., Chang, J., Yin, Q., Chen, L., Chen, Q., Yang, X., Zheng, Q., Ren, G. and Feng, H. (2010).Phytase Gene Expression in *Lactobacillus* and Analysis of Its Biochemical Characteristics. *J. Microbiolo. Resea.* **165:** 329-335.Powar V.K.andJagannathan, V. (1982). Purificationand properties of phytate-specific phosphatase from *Bacillus subtilis*. *J. Bacteriolo.***151:** 1102-1108.

Appendixes

Appendix A. Results of Process optimization

Appendix 1 Activity of purified phytase

Sample sources	OD at 700nm	Conc. (µl/ml)	Act. (IU/ml)
RS1	0.59	32.777	0.029
RS8	0.543	30.166	0.027
RS10	0.388	21.555	0.019
RS15	1.164	64.666	0.058



Enzyme one Enzyme two Enzyme three Enzyme four

Appendix figure 1 Phytase and its reaction with color reagent

Appendix 2. Crude phytase enzyme activity

Sample sources	OD at 700nm	Conc. (µl/ml)	Act. (IU/ml)
RS1	1.233	68.5	0.061
RS8	1.09	60.555	0.054
RS10	0.999	53.166	0.049
RS15	1.424	79.111	0.071

Appendix 3 the effect of carbon sources on phytase production

	RS1			RS8			RS10			RS15		
Carbon sources	OD	Conc. (µl/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)
Glucose	0.392	21.777	0.019	0.452	25.111	0.022	0.428	23.777	0.021	0.434	24.111	0.021
Sucrose	0.443	24.611	0.022	0.635	35.277	0.031	0.307	17.055	0.015	0.360	20	0.018
Starch	0.524	29.111	0.026	0.602	33.444	0.030	0.405	22.5	0.020	0.355	19.722	0.017
Galactose	0.531	29.5	0.027	0.855	47.5	0.042	0.477	26.5	0.023	0.51	28.333	0.026

Appendix 4. The effect of Nitrogen sources on phytase production

	RS1			RS8			RS10			RS15		
Nitrogen sources	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)
Peptone	0.554	30.777	0.028	0.852	47.333	0.043	0.702	39	0.035	0.655	36.388	0.032
Ammonium Nitrate	0.819	45.5	0.04	0.723	40.166	0.036	0.603	33.5	0.03	0.66	36.666	0.033
Sodium Nitrate	0.487	27.055	0.24	0.568	31.555	0.028	0.61	33.888	0.03	0.495	27.5	0.025
Urea	0.645	35.833	0.032	0.765	42.5	0.038	0.833	46.277	0.04	0.850	47.222	0.043
Ammonium sulphate	0.917	50.944	0.046	1.12	62.222	0.056	0.69	38.333	0.035	0.716	39.777	0.036

Appendix 5. The effect of Time on phytase production

	RS1			RS8			RS10			RS15		
Time	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)	OD at 700nm	Conc. (µL/ml)	Act. (IU/ml)
24h	0.439	24.388	0.022	0.754	41.888	0.037	0.654	36.333	0.033	0.605	33.611	0.030
48h	0.653	36.277	0.033	0.757	42.055	0.038	0.777	43.166	0.038	0.670	37.777	0.033
72h	0.814	45.222	0.04	0.783	43.5	0.039	0.789	43.833	0.039	0.818	45.444	0.04
96h	0.684	33	0.034	0.418	23.222	0.021	0.597	33.166	0.029	0.590	32.777	0.029

Appendix 6. The effect of pH on phytase production

	RS1			RS8			RS10			RS15		
Initial pH of media	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)
pH 3	0.610	33.888	0.03	0.749	41.611	0.037	0.702	39	0.035	0.673	37.388	0.034
pH 4	0.827	45.611	0.04	0..772	42.888	0.038	0.922	51.222	0.046	0.733	40..722	0.037
pH 5	0.832	46.222	0.042	0.749	41.611	0.04	0.986	54.777	0.047	0.802	44.555	0.04
pH 6	0.922	51.222	0.046	0.993	55.166	0.049	0.993	55.166	0.049	0.949	52.722	0.047
pH 7	0.710	39.444	0.036	0.922	51.222	0.046	0.922	51.222	0.046	0.702	39	0.035
pH 8	0.651	36.166	0.033	0.749	41.611	0.04	0.723	40.166	0.036	0.622	34.555	0.03
pH 9	0.592	32.888	0.029	0.789	43.833	0.039	0.682	37.888	0.034	0.579	32.166	0.029

Appendix 7 the effect of Inoculums size on phytase production

	RS1			RS8			RS10			RS15		
Percent Of Inoculums size	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)	OD at 700nm	Conc. (µg/ml)	Act. (U/ml)
100 µl	0.524	29.111	0.026	0.723	40.166	0.036	0.704	39.111	0.035	0.723	40.166	0.036
2 00 µl	0.646	35.888	0.032	0.763	42.388	0.038	0.752	41.777	0.037	0.733	40.722	0.037
400 µl	0.704	39.111	0.035	0.868	48.222	0.043	0.763	42.388	0.038	0.782	43.444	0.039
600 µl	0.751	41.722	0.037	0.889	49.388	0.044	0.880	48.888	0.044	0.815	45.277	0.04
800 µl	0.920	51.111	0.046	0.963	53.5	0.048	0.810	45	0.04	0.799	44.388	0.039
1ml	0.751	41.72	0.037	0.889	49.388	0.044	0.880	48.888	0.044	0.815	45.277	0.04

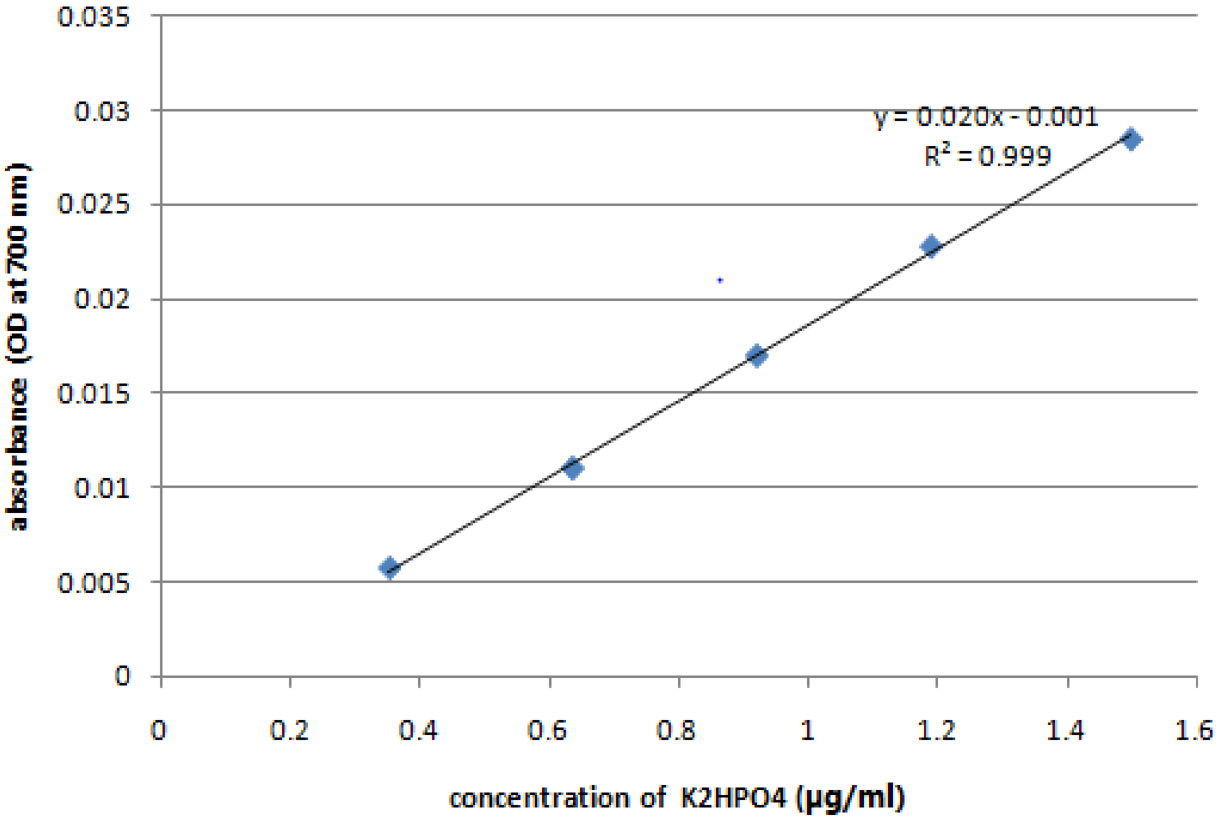
Appendix 8. The effect of Temperature on phytase production

	RS1			RS8			RS10			RS15		
Temperature	OD at 700nm	Conc. (μg/ml)	Act. (U/ml)	OD at 700nm	Conc. (μg/ml)	Act. (U/ml)	OD at 700nm	Conc. (μg/ml)	Act. (U/ml)	OD at 700nm	Conc. (μg/ml)	Act. (U/ml)
30 ^{o}C	0.644	35.777	0.032	0.562	29.611	0.027	0.694	25.444	0.023	0.696	38.666	0.035
37 ^{o}C	0.686	38.111	0.034	0.562	29.611	0.027	0.625	34.722	0.031	0.604	33.555	0.03
40 ^{o}C	0.724	40.222	0.036	0.711	39.5	0.036	0.651	36.166	0.033	0.729	40.5	0.036
50 ^{o}C	0.74	41.111	0.037	0.757	42.055	0.037	0.705	39.166	0.035	0.755	41.944	0.038
55 ^{o}C	0.602	33.444	0.03	0.601	33.388	0.03	0.681	37.833	0.034	0.625	34.722	0.031

Appendixes B. Phytase standard curve (calibration curve)

Appendix 9.Serial dilution of the potassium di phosphates stock and OD readings at 700 nm

NO	4Desired Conc. OfK$_2$HPO$_4$(μg/ml	Dilution needed	Volume stock (ml)	Sodium acetic acid buffer (ml)	Total volume (ml)	Concentrations Of K$_2$HPO$_4$(g/ml)	OD Reading at700 nm
1	300	3.00/7.00	0.500	0.500	1.00	0.0057	0.352
2	350	3.50/6.50	0.450	0.550	1.00	0.011	0.635
3	400	4.00/6.00	0.400	0.600	1.00	0.017	0.92
4	450	4.50/5.50	0.350	0.650	1.00	0.0228	1.19
5	500	5/5	0.300	0.700	1.00	0.0285	1.499



Appendix figure 2. Phytase standard curve (calibration curve)