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Estimation of Microbial Diversity in Poultry Litter Using Terminal Restriction Fragment Length Polymorphism and Isolation of Phosphate Accumulating Bacteria from Poultry Litter

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ESTIMATION OF MICROBIAL DIVERSITY IN POULTRY LITTER USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM AND ISOLATION OF PHOSPHATE ACCUMULATING BACTERIA FROM POULTRY LITTER

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Estimation Of Microbial Diversity In Poultry Litter Using Terminal Restriction Fragment Length Polymorphism And Isolation Of Phosphate Accumulating Bacteria From Poultry Litter

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES

LIST OF TABLES

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The contamination of fresh water by phosphates in poultry litter results in substantial eutrophication of fresh water causing fish kills and other types of environmental damage. The poultry industry in Kentucky is expanding rapidly. The number of broilers is increasing as more poultry farms are established in the state producing waste that needs disposal. Investigations were made to study the possibility of using microorganisms normally found in poultry litter to sequester phosphate, thereby delaying phosphate runoff after litter is applied to croplands. Little is known, however, about the microflora of poultry litter. Terminal restriction fragment length polymorphism of 16S rDNA from bacteria was used to investigate the bacterial diversity of poultry litter. Poultry litter was collected from a local producer. DNA was isolated using commercial kits and amplified using the polymerase chain reaction with primers specific for bacterial 16S rDNA. The amplified fragments were digested using *HhaI* restriction endonuclease and the DNA fragment lengths were determined. To determine the sensitivity of this method, known quantities of *Escherichia coli* cells were spiked into litter prior to DNA extraction. Successful amplification of the bacterial rDNA was highly variable but could be improved by passing the purified DNA through two purification columns in lieu of only one column. The detection threshold for *E. coli* was 10 cells, however, the results also varied widely.

Bacteria capable of hyper-accumulating intracellular phosphate were isolated from poultry litter as possible tools for phosphate remediation in poultry litter. Five strains of phosphate accumulating bacteria were successfully isolated from poultry litter. Poultry litter was suspended in sterile nanopure water and 100µl was plated on BHI plates containing an addtional 750mM K_2HPO_4. Isolated colonies were screened for intracellular metachromatic granules using the Nile blue stain, a presumptive test for polyphosphate. Positive colonies were cultured in BHI and BHI with supplementation of K_2HPO_4 and free intracellular phosphate concentrations were determined in cell extracts. Total phosphates were measured in cell extracts subjected to hydrolysis by addition of 12N HCl and heating at 100°C for 60 min. Polyphosphate was determined by subtraction of free phosphates from total phosphates. Results showed five isolates of gram-positive bacteria were obtained from poultry litter. All isolates were cocci arranged in chains or clusters and were catalase positive. All isolates showed considerable levels of intracellular phosphate accumulation, which were comparable to *Microlunatus phosphovorus*, a bacterium known to hyper-accumulate phosphate. Biolog analysis indicated four of the five strains isolated were *Staphylococcus sp.* and one strain was unidentified.

INTRODUCTION

The poultry industry in Kentucky is expanding rapidly. The number of poultry broilers in the state has increased from almost none in 1988 to more than 60 million in 1994, and 200 million in 1998 (http://www.nrdc.org/water/pollution/factor/stken.asp). The numbers are expected to continue to increase as more poultry farms move into the state. As poultry production increases in the state there is an expanding problem for farmers to dispose the waste. Chicks are fed with heavy supplements of calcium and phosphorus to facilitate bone strength and faster growth, with most of the elements excreted. Poultry litter is a mixture of broiler excreta and bedding material and contains substantial amounts of phosphorus.

Currently, poultry litter is used as a fertilizer on agricultural fields to provide essential plant nutrients and improve soil quality. However over-application often occurs. Excess phosphate poses a serious environmental threat. Run-off from the litter-treated fields may contain substantial amounts of phosphorus, which seriously diminishes the quality of water by causing eutrophication and growth of algal blooms which results in the killing of fish and other aquatic life (14).

The present study attempts to both characterize the bacterial composition and diversity of poultry litter by employing terminal restriction fragment length polymorphism (T-RFLP) as well as develop a practical approach to remediate phosphates from the poultry waste by isolating phosphate hyperaccumulating bacteria.

The analysis of microbial communities is a difficult task due to the diversity of microorganisms and the complexity of the microbial community. Several approaches for

microbial community analysis have been developed, most of which are culture-independent techniques. One approach, which has gained recent popularity, is the polymerase chain reaction-rDNA-based phylogeny characterization of microbial diversity by determining terminal restriction fragment length polymorphism (9). The technique of T-RFLP was used to determine the microbial diversity of poultry litter in the present study.

Polyphosphates are found in every form of life, including bacteria, cyanobacteria, fungi, protozoa, and algae, but the content differs among organisms and also varies depending on cell size and growth conditions. Polyphosphate occurs in chains where each phosphorus is linked to other surrounding phosphorus atoms via two oxygen atoms. The general formula is expressed as $Mn_{(n+2)}P_nO_{(3n+1)}$ (6). These molecules also are known to form complexes with proteins and nucleic acids (5). Many bacteria produce polyphosphate, which is visible in the form of inclusion granules. In a bacterial cell, polyphosphate granules are seen surrounding the nucleiod, and some also are seen in the periplasmic region and outside the cytoplasmic membrane of the bacterial cell (6).

Microorganisms can be used in industrial settings to remove phosphate from wastewater streams. Enhanced biological phosphate removal (EBPR) has gained lot of prominence in phosphate uptake from sludges and industrial wastes by encouraging the growth of microorganisms, which are capable of hyperaccumulating intracellular phosphate in the form of polyphosphate (5). EBPR is a two-fold process. In the first phase, the wastes containing the orthophosphates are initially maintained in an anaerobic condition. The second phase is an aerobic stage, which sequesters the phosphate from the wastes. During the anaerobic phase, the bacteria export large amounts of phosphate into the media. In the aerobic phase the microorganisms tend to accumulate more phosphate than was excreted during the anaerobic

phase and orthophosphates levels in the media fall drastically. The phosphates are sequestered in the form of intracellular phosphates. The sediments from the aerobic stage are removed from the system however small amounts are used as an inoculum for the next anaerobic phase. A few phosphate hyperaccumulating bacteria have been isolated from these systems and have been identified as *Microlunatus phosphovorus*, the gram-negative bacteria *Lampropedia* and the *Acinitobacteria*.

MATERIALS AND METHODS

POULTRY LITTER COLLECTION

The samples were collected from a local poultry-producing farm. All samples were collected from the surface of the broiler barn floor by transferring into 100ml sterile plastic vials. All samples were stored at 4˚C after transportation to the laboratory.

PCR AMPLIFICATION of 16S rDNAs

DNA was extracted from 0.25 g samples of poultry litter samples using a MoBio Ultraclean Soil DNA Kit (Solano Beach, CA) as described by the manufacturer's protocol. Amplification of the DNA was performed by using a 5'-FAM-labeled primer 27f (5'-AGAGTTTGATCMTGGCTCAG-3'; and unlabeled primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3'; Applied Biosystems, Fermont, CA). The primers are specific for bacterial domain rDNA (7). Reactions were carried out with the following reagents in 50-µl mixtures; approximately 50ng of DNA, 0.5µM each of 27f and 1492r primers, 1.5mM $MgCl_2$, 0.8mM dNTPs, and 1U Taq polymerase (Promega Corp, Madison, WI) in buffer supplied by the manufacturer. Polymerization temperature and thermocycling for the samples were as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 1 min; 55°C for 1 min, and 72°C for 1 min and final extension at 72°C for 10 min. PCR amplification reactions were analyzed using agarose gel electrophoresis. DNA (5 µl; ~100ng) was mixed with 5 µl of loading dye containing: 0.24% bromophenol blue, 0.25% xylene cyanol, 15% ficoll type 400, 120 mM EDTA and subjected to electrophoresis. Electrophoresis was performed using 1% agarose gels in TAE buffer at a voltage of 150 V and were stained using a 10 µg/ml concentration of ethidium bromide.

TERMINAL RESTRICTION FRAGMENT ANALYSIS

The PCR amplification mixture was cleaned by removing excess primers, dNTPs, and buffers. The nucleic acid was concentrated using a MoBio UltraClean PCR Clean-up kit according to the manufacturer's protocol. Fluorescently labeled DNA (~50ng) was cut with the 10U of restriction endonuclease, *HhaI* (New England Biolabs, Beverly, MA), in the manufacturer's recommended reaction buffer for 6 h at 37° C. DNA was ethanol precipitated (1), and the DNA was dissolved in 24.5 µl of formamide and 0.5 µl of Rox 500 size standards (PE Applied Biosystems, Foster City, CA). Prior to analysis, the DNA was denatured at 95° C for 5 min and was snap-cooled at 4°C. Samples were analyzed using a ABI Prism 310 Genetic Analyzer in GenScan mode. The parameters for this procedure were as follows; the temperature was maintained at 60°C, filter set D and Pop 4 polymer was used (PE Applied Biosystems).

TEST FOR SENSITIVITY

To estimate the minimum quantity of bacteria detectable by the T-RFLP method, known quantities of *Escherichia coli* ATCC 25922 cells were spiked into the poultry litter prior to DNA extraction. *E .coli* were grown overnight at 37°C, shaking in LB (tryptone: 12g; yeast extract: 8g; NaCl: 12g; volume was adjusted to 1L) broth. Serial dilutions of *E. coli* in sterile nanopure water (1ml) were spiked into 0.25g of poultry litter samples. To determine the colony forming units of *E. coli* the dilutions were simultaneously plated on LB agar and were incubated at 37°C overnight. Subsequently colonies were counted and the number of *E. coli* in the original sample was calculated by multiplying the number of colonies by the reciprocal of the dilution factor.

The fragments were analyzed using the Ribosomal Database Project (RDP) found in a website called T-RFLP analysis program (TAP) located at (http://www.cme.msu.edu/RDP/trflp/#program) (9). This website allows the user to analyze the data based upon the forward and reverse primers used and the restriction enzyme used to cut the template. The lengths and peak areas of T-RFs were determined with the GeneScan software.

ENRICHMENT FOR PHOSPHATE ACCUMULATING BACTERIA

Samples were prepared by adding 0.5g of poultry litter to 10ml sterile nanopure water and vortexing for 5 min at room temperature. This mixture was then allowed to settle for 10 min. The inocula (100µl) were plated on brain heart infusion (BHI)-agar plates with addition of 100, 500, or 750 mM of K_2HPO_4. All plates were incubated for 3 days at 25°C. Isolated colonies were screened for phosphate inclusion granules using Nile blue staining method (15). The heat-fixed smears of the isolates were prepared with 1% (wt/vol) Nile blue at 55°C for 10 min, followed by a wash with 8% (vol/vol) glacial acetic acid for 1 min. The smears were counter stained with Neisser's methylene blue for 1 min and rinsed with Gram's iodine. Slides were covered and sealed using permount before examination using 1000X objective (Olympus, Melville, NY). The presence of dark granules is indicative of inclusion bodies. The isolates were compared with *Microlunatus phosphovorus,* a known polyphosphate-accumulating strain.

TOTAL PHOSPHATE DETERMINATION

Isolates which stained positive for the metachromatic inclusion granules were streaked for isolation four times. These isolates were grown in 5ml of BHI and in BHI with an additional 750mM of K_2HPO_4 (BHI750) media. These cultures (5ml) were subjected to centrifugation (10,000xg; 15min; 4°C) then washed twice in lysis buffer (1.5M NaCl, 10mM EDTA, 1mM NaF) before being suspended in 0.5ml of lysis buffer. To this solution, 0.2 ml of a 1g/ml of

0.1mm glass beads were added. Cells were lysed using a bead beater (BioSpec Products, Inc., Bartlesville, OK) at 5,000 rpm for 2 min with regular 30-second interval. To avoid excessive heating, samples also were cooled in ice water, between the 30-sec intervals of beating. The samples were transferred to a clean centrifuge tube and centrifuged at 13,000xg for 4 min at 4°C. The supernatant was subjected to acid hydrolysis using 1/6 volume of 12N hydrochloric acid 100°C for 1 hour and neutralized with an equal volume of 12N NaOH, to this 1M Tris was added to adjust the pH to 7.0. Polyphosphate concentration was determined for prehydrolysis and posthydrolysis samples, which are estimates of free intracellular and total intracellular phosphate, respectively. Polyphosphate concentrations were computed by subtraction of free intracellular phosphate, values from total intracellular phosphate values.

PHOSPHATE ASSAY

Phosphate was determined using the modified Oslen and Sommer's phosphate assay (13). The following reagents were used for the assay, reagent A, is ammonium paramolybolate (6g) ($[(NH_4)_6Mo_7O_{24}.4H_2O]$) dissolved in 150 ml water and to this potassium antimony tartarate trihydrate ($C_8H_4K_2O_{12}Sb_{23}H_2O$) already dissolved in 50ml of water was added. These two solutions were mixed with 500ml 5N sulfuric acid and water to make a final volume of 1 liter. Reagent B is prepared fresh daily by dissolving 0.264g of ascorbic acid in 50ml reagent A. Sample (1ml) was mixed in 190µl of reagent B and was incubated at room temperature for 1 hour. Absorbance was determined spectrophotometrically at a wavelength of 840nm using a spectrophotometer (Amersham Pharmacia, Piscataway, NJ). Concentration of phosphate was determined by comparison to a standard curve ranging from 0 to 2µg K_2HPO_4 per ml (Figure 8).

PROTEIN DETERMINATION

Protein concentrations were determined by Lowry's method (8) using a commercially available kit (BioRad Laboratories, Richmond, CA). The assay was performed as described in the manufacturer's instructions. A standard curve was prepared ranging from 0 to 1.5 mg/ml of bovine serum albumin (Figure 7)

GROWTH CONDITIONS OF ISOLATES

Isolates 6, 7, 10, 12, and 13 were grown overnight at 25°C in BHI750. Growth was monitored every 3 hours by measuring the optical density (O.D) at 600nm. To test for facultative oxygen metabolism, all strains were streaked for isolation and grown anaerobically using a GasPak® jar at 25ºC overnight.

PHOSPHATE UPTAKE ANALYSIS OF ISOLATE 7

Isolate 7 was grown for 24 hours in minimal media (1.8g glucose; 0.1g of peptone; 0.1 of yeast extract; 0.44g of KH_2PO_4; 0.1g of $(NH_4)_2SO_4$; and 0.4g of $MgSO_4.7H_2O$; pH was adjusted to 7.0 and volume was adjusted to 1L) (12) and harvested at the late logarithmic phase by monitoring the optical density at a wavelength of 600nm. The cells were centrifuged at 10,000xg for one hour and were washed thrice with sterile nanopure water. Cells were suspended in 6mM KH_2PO_4 and 3mM $MgSO_4.7H_2O$ to yield a concentration of 200mg of phosphorus per liter and were incubated at 25°C shaking. Extracellular phosphate was determined as described above. A sample was removed every 2-hours, centrifuged, and washed thrice in sterile water before being placed in a dried 16x100 mm borosilicate glass test tube. The tubes were dried at a constant temperature (70ºC) until a constant weight was reached and the dry mass of the material was determined. The tubes were analyzed for phosphate concentration

using an Inductively Coupled Plasma Mass Spectrometry (ICP) at the Materials Characterization Center, Western Kentucky University (Bowling Green, KY).

PHYSIOLOGICAL AND BIOCHEMCAL CHARACTERIZATION OF ISOLATES

The genus and species were determined using GP2 microplate (Biolog Hayward, CA). All isolates were shipped to Hoosier Microbiological Laboratory (Munice, IN) for Biolog analysis. This technology is based on the reduction of tetrazolium dye on a 96-well microplate containing a wide variety of carbon sources. Possible species identification was achieved by comparison of the reactions to a Biolog proprietary database. Catalase activity was determined by observing the gas evolution during reaction with hydrogen peroxide.

STATASTICAL ANALYSIS

Statistical analysis was achieved using the Komogorov-Smirnov/Lillifors algorithm in SYSTAT (version 9 for windows, SYSTAT 2002) (10). The data for total phosphates could be corrected for normality, however polyphosphate and percentage of polyphosphate data deviated significantly from normality based on Bonferroni adjusted criteria. As the evidence for non-normality could not be determined, no further transformation was applied. Each character was tested for significant differences between the isolates and between the types of media using ANOVA.

RESULTS

The molecular characterization of the microbial community in poultry litter was determined by T-RFLP, a stepwise technique described in the materials and methods (Figure 1). To determine the optimal amount of poultry litter for DNA extractions, 0.25g, 0.5g, and 0.75g of waste were used. DNA was successfully extracted from these samples and the presence of the DNA was confirmed using agarose gel electrophoresis (Figure 2A). A quantity of 0.5g of poultry litter was determined to be optimum for DNA extraction. PCR reactions did not show any amplification of the 16S rDNAs present in the samples (Figure 2B), which suggested that inhibitors were present in the DNA sample. To test this hypothesis, increasing concentrations of *E. coli* chromosomal DNA were added to the samples of poultry litter DNA. Approximately 40ng of poultry litter DNA was mixed with approximately 40, 50, 60, 70, 80, and 100ng of *E. coli* chromosomal DNA in different microfuge tubes and PCR reaction was performed. This PCR reaction exhibited successful amplification (Figure 3) suggesting the absence of inhibitors.

A known quantity of *E. coli* cells ranging from 6 to $6x10^8$ cells were spiked into the litter and the DNA was then extracted and subjected to PCR amplification. The reactions failed, suggesting that inhibitors were present during the DNA extraction. (Figure 4A and Figure 4B). Membrane filter columns, part of manufacturer's kit, allowed DNA to be washed. Failure of PCR amplifications prompted use of a second membrane filter column to wash the DNA in an effort to remove inhibitors (Figure 5A). This DNA was subjected to PCR and the results showed successful amplifications (Figure 5B).

To investigate the sensitivity and consistency of DNA extraction, PCR amplification and T-RFLP, the above experiment was performed with litter in five separate trials. The results obtained from these experiments were highly variable (Table 1). In all trials, DNA extracted from samples spiked with $6x10^6$ *E. coli* showed a consistent signal from T-RFLP. In two of the five trials it was observed that as few as 60 *E. coli* cells were detected, however, all of the five trials failed to indicate the presence of *E. coli* in all samples.

The T-RFLP profiles shown in figure 6 represents bacterial communities in poultry litter. Terminal fragments were produced from an *HhaI* digestion of 16S rDNAs amplified from total community DNA using fluorescently tagged 27F primer and untagged 1492R reverse primer. The terminal fragments were sized on ABI310 an automated sequencer using internal size standards (ABI ROX500) (Applied Biosystems, Foster City, CA) (9).

Table 2 shows the major fragments detected in poultry litter. The DNA was extracted from samples without spiking any *E. coli* cells. The fragments observed were compared against the ribosomal database project (RDP) (9). Fragment lengths at 56.75 and 452.38 nucleotides did not correspond to any organism in the database. The fragment length at 70.32 matched *Spiroplasma spp* and *Leptotrichia* spp. The fragment length of 94.59 nucleotides corresponds to *Campylobacter spp, Bacillus alcalophilus, Desulfobulbus* s*pp*. and *Chlorobium spp.* The fragment length of 215.55 corresponds to *Xanthomonas spp* and *Enterococcus spp. Xanthomonas* belongs to the gamma subgroup of proteobacteria. The fragment lengths of 234.76 and 236. 46 correspond to *Clostridium spp*. The fragment length of 468.38 corresponds to *Streptomyces spp*.

MORPHOLOGIAL AND CULTURE CHARACERISTICS

The characteristics of polyphosphate accumulating Isolates 6, 7, 10, 12 and 13 were all distinctive and indistinguishable. Samples were prepared by adding 0.5g of poultry litter to 10ml sterile nanopure water and vortexed for 5 min at room temperature this mixture was allowed to settle for 10 min. The inocula (100µl) were plated on BHI-agar plates and BHI-agar plates with additional 100, 500, and 750 mM of K_2HPO_4 (BHI750). All plates were incubated for 3 days at 25°C. Four plates were inoculated per condition. Too many colonies were observed to count on all four plates with BHI-agar. Approximately 34 isolated colonies were observed on each BHI plate with additional 100mM of K_2HPO_4. On BHI-agar plates with additional 500mM of K_2HPO_4 approximately 22 isolated colonies were observed. Ten isolated colonies were observed on BHI750 agar plates. Ten isolated colonies from each plate were selected and screened for phosphate inclusion granule using Nile blue stain. Approximately 160 isolated colonies were screened, five of these isolates were positive for Nile blue stain. These five isolates were Gram positive, coccoid, non-motile bacteria (Figure 9). The cells were found in either clusters or chains, all isolates were positive to Nile blue stain and exhibited a polyphosphate inclusion granule under light microscopy at 1000X magnification. The strains were grown in BHI750 media, all the strains exhibited good growth at 25°C (with shaking at 50 rpm) in liquid media (Figure 10). Colonies were observed one day after incubation at 25°C on BHI-agar plates, all colonies were pale yellowish in color.

All strains also showed good signs of growth under anaerobic conditions at 25°C. The cells were streaked for isolation on BHI-agar plates and placed in a GasPak® jar. Isolated colonies were seen one day after incubation and all strains showed pale yellow color colonies.

BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS

All strains were catalase positive, and all the used a variety of organic acid sources and carbon sources. Glucose, cellobiose, fructose, galactose, maltose, mannitol, mannose and melibiose all served as good carbon sources for the isolates. Hydroxybutyric acid, pyruvic acid and lactic acid served as good sources of organic acids. Alanine, thymidine, uridine, serine, glutamic acid, and adenosine were metabolized. The isolates were not found to utilize glycogen, inulin, asparagin, malic acid, propionate, malate or succinate for their growth (Table 3).

PHOSPHATE ACCUMULATON

To determine the optimal condition for sample clarification and elimination of cell debris following bead-beating, samples were centrifuged at three different conditions (13,000xg for 4 min; 25,000xg for 1 min; and 30,000 xg for 30 min) prior to phosphate determination. The data indicate that 13,000xg for 4 min was efficient for centrifugation, (Table 4) as all samples can be conveniently processed using a bench top microfuge when compared to being processed using an ultracentrifuge. Moreover, the bench top microfuge is highly time efficient as it holds a larger number of samples than the ultracentrifuge.

Intracellular phosphate levels were compared among bacterial strains isolated from poultry litter. As described earlier all bacterial strains isolated from poultry litter were grown on BHI and BHI750, incubated at 25° C for two days with shaking at 120 rpm. Results indicate increased free phosphate, total phosphate and polyphosphate levels per milligram of protein in BHI750 to BHI media (Table 5). Free phosphate levels in Isolate 6 increased from 0.017 μg/mg protein when grown in regular BHI media to 0.150 μg/mg protein when grown on BHI750. Total phosphate levels increased from 0.09μg/mg protein to 0.417μg/mg protein, polyphosphate levels increased from 0.072 μg/mg protein to 0.264 μg/mg protein. In Isolate 7, free phosphate levels

increased from 0.019 μg/mg protein in BHI media to 0.150 μg/mg protein when grown on BHI750. Total phosphate levels increased from 0.09μg/mg to 0.417μg/mg protein BHI750, and polyphosphate levels increased from 0.072 μg/mg protein to 0.264 μg/mg protein. In Isolate 10 free phosphate levels increased from 0.010 μg/mg protein in BHI media to 0.022 μg/mg protein when grown on BHI750. Total phosphate levels increased from 0.075μg/mg to 0.600μg/mg protein BHI750, and polyphosphate levels increased from 0.064 μg/mg protein to 0.385 μg/mg protein. In Isolate 12, free phosphate levels increased from 0.022 μg/mg protein in BHI media to 0.218 μg/mg protein when grown on BHI750. Total phosphate levels increased from 0.440μg/mg to 0.076μg/mg protein BHI750, and polyphosphate levels increased from 0.067 μg/mg protein to 0.223 μg/mg protein. In Isolate 13, free phosphate levels increased from 0.014 μg/mg protein in BHI media to 0.191 μg/mg protein when grown on BHI750. Total phosphate levels increased from 0.076μg/mg to 0.500μg/mg protein BHI750, and polyphosphate levels increased from 0.059 μg/mg protein to 0.306 μg/mg protein.

The concentrations of total phosphate, polyphosphate, and percentage of polyphosphate differed significantly among the types of media (Table 6). The variation in total phosphates between BHI and BHI with additional phosphates was highly significant ($p = <0.001$, $F= 20.130$), in contrast the variation of total phosphates among isolates was not significant ($p = 0.989$, $F= 0.074$). The polyphosphate concentration varied significantly between the types of media ($p = 0.006$, $F = 9.449$). The polyphosphate concentration between isolate 10 and isolate 12 showed some variation, however the differences were not significant and the variation of polyphosphate concentration among all the isolates was not significant ($p = 0.971$, $F = 0.233$). The interaction effect did not show any statistical significance. The variation in the percentage

of polyphosphate concentrations between the types of media was significant (p= 0.008, F = 8.738). The percentage of polyphosphate concentrations between isolate 10 and 12 and between 6 and 7 showed variance, however, the differences were not significant and the variation in polyphosphate concentration among all the isolates was not significant (p = 0.393, F = 1.079). The interaction effect was non-significant.

The phosphate accumulating activity of Isolate 7 was compared the phosphate accumulating activity with *Microlunatus phosphovorus* a bacterium known to hyperaccumulate phosphate (Table 7). Free phosphate levels increased from 0.005µg/mg protein in *Microlunatus phosphovorus* to 0.043µg/mg protein in Isolate 7 in regular BHI medium; 0.03µg/mg protein to 0.18µg/mg protein in BHI750; and 0.08µg/mg protein to 0.06µg/mg protein in media used to grow *Microlunatus phosphovorus.* Total phosphate levels increased from 0.04µg/mg protein in *Microlunatus phosphovorus* to 0.16µg/mg protein in Isolate 7 in regular BHI medium; 0.05µg/mg protein to 0.31µg/mg protein in BHI750; and 0.43µg/mg protein to 0.23µg/mg protein in media used to grow *Microlunatus phosphovorus.* Polyphosphate levels increased from 0.035µg/mg protein in *Microlunatus phosphovorus* to 0.117µg/mg protein in Isolate 7 in regular BHI medium; 0.02µg/mg protein to 0.13µg/mg protein in BHI750; and 0.35µg/mg protein to 0.17µg/mg protein in media used to grow *Microlunatus phosphovorus*.

Isolate 7 accumulated substantial amounts of phosphate. The cells harvested at late logarithmic phase showed phosphate accumulating activity from the minimal media as described previously. This was compared with the *M. phoshovorus* a known phosphate accumulating bacterium. The data suggested that the amount of phosphate accumulation was comparable between Isolate 7 and *M. phosphovorus* as both bacteria accumulated equal amounts of

phosphates from the media. The extracellular phosphate levels in the media decreased to 80% at 8 hour time interval in both *M. phosphovorus* and Isolate 7. At 10 hour time interval the phosphate levels decreased to 64% and remained unchanged until a 30 hour time interval in both *M. phosphovorus* and Isolate 7 (Figure 11 and 12). Phosphorus concentrations were also calculated on a dry weight basis. In *M. phosphovorus* the phosphorus concentrations increased by 50% at an 8 hour time interval, decreased by 5% at a 10 hour time interval, and subsequently increased by 80% at a 30 hour time interval (Figure 11). In Isolate 7 the phosphorus concentrations first decreased by 25 % at a 6 hour time interval, increased by 75% at an 8 hour time interval, and decreased again and subsequently remained unchanged up to the 30 hour time interval (Figure 12).

DISCUSSION

Terminal restriction fragment length polymorphism is a quick and easy method to characterize and analyze microorganisms independent of culturing. This technique has been in use for several years as a method for rapid analysis of rDNAs. The knowledge of the microbiology of poultry litter is limited due to the lack of data regarding the precise phylogeny or taxonomy of bacteria present in the litter. The results of the present study suggest there are inhibitors, which prevent the amplification of DNA isolated from poultry litter. A slight modification in the method of purifying the DNA by passing it through a second purification column improved the preparation. However, the method was highly insensitive and not reproducible in detecting known quantities of *E .coli* cells added to the poultry litter. Terminal restriction fragment lengths analyzed using the ribosomal database project showed a diverse microbial population, however, the data were highly variable and inconsistent.

Bacteria isolated from poultry litter, showed distinct morphological and physiological characteristics. These bacteria are gram positive cocci. The isolates exhibited phosphate accumulation and hence are proposed to be polyphosphate accumulating organisms (PAO). The results demonstrated that the methods used were unintentionally selective for *Staphylococcus spp.* The morphological, physiological, and biochemical characteristics of the bacterial strains suggest they belong to the genus *Staphylococcus* and are tentatively assigned to species Staphylococcus *lentus.* This is also evident from the Biolog data that four of the five isolates were characterized to be *Staphylococcus spp. Staphylococcus lentus*, which is a slow grower, coccoid in shape with approximately 0.7-1.2 μm in diameter (4).

It can be hypothesized that these bacteria can be used to sequester phosphate from poultry litter by enrichment techniques, which support the growth of these organisms and enhance intracellular phosphate hyperaccumulation in the form of polyphosphate.

The data obtained suggest the bacteria present in poultry litter have the capacity to accumulate intracellular and polyphosphate levels in poultry litter. A treatment regimen like in treatment plants might encourage bacteria inhabiting the waste to accumulate phosphate and produce polyphosphate. Enhanced biological phosphate removal is one such well established process which can virtually remove all orthophosphate from waste water (5). This could alleviate, in part, phosphate runoff from the land where poultry litter has been applied.

Currently, research is underway to develop a method to possibly eliminate or control pollution caused by phosphate in poultry litter. Broad spectrum metabolic inhibitors can be used for the ability to increase intracellular phosphates by direct treatment of poultry litter as well as treatment of the bacteria isolated from poultry litter.

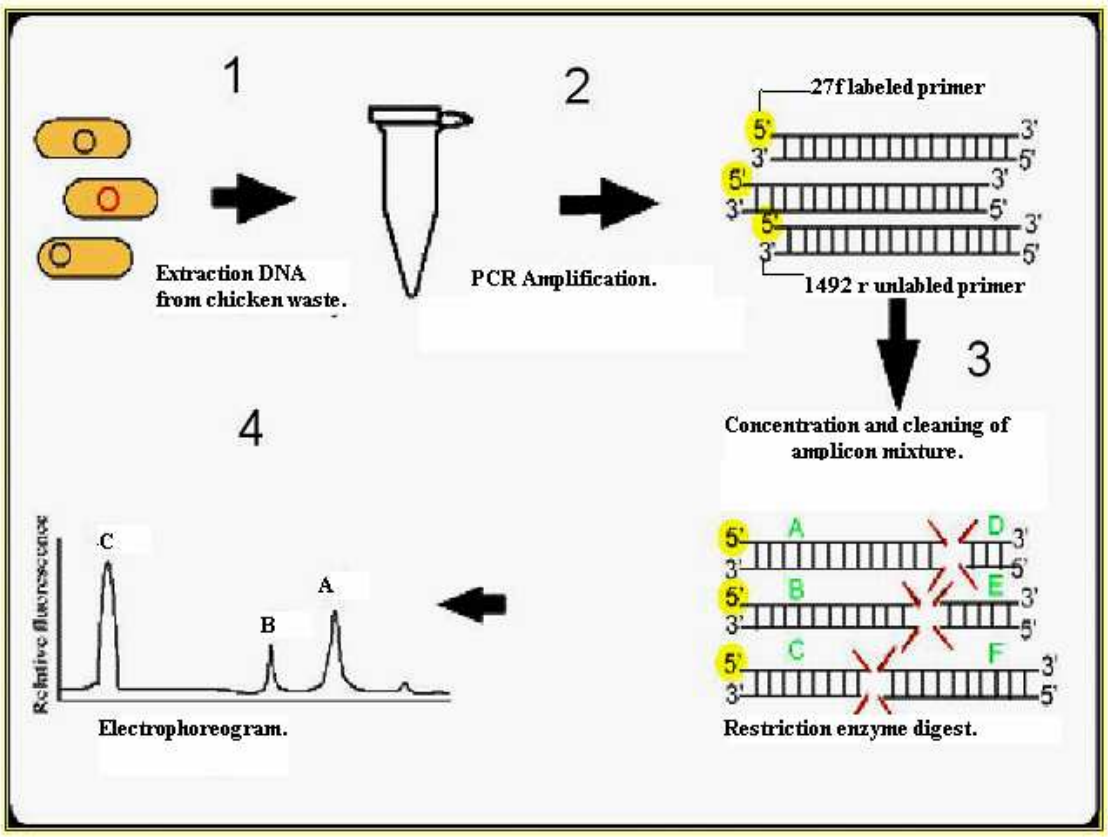
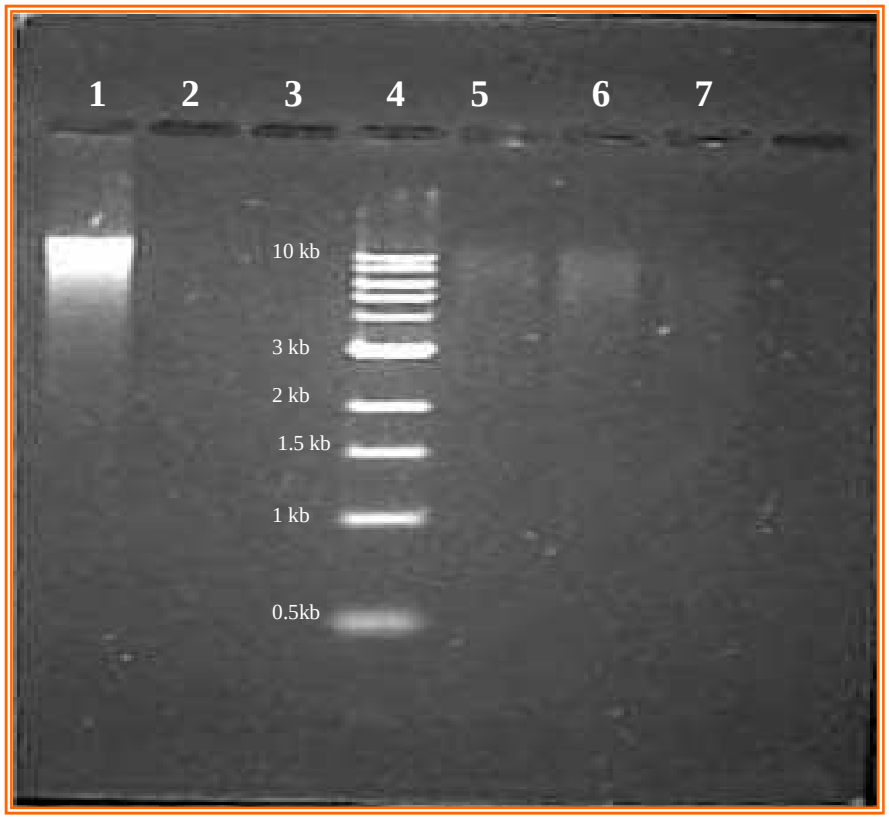


Figure 1. Schematic view of terminal restriction fragment length polymorphism analysis redrawn from (3). Terminal restriction fragment length polymorphism is a stepwise, quick, and easy method to characterize community bacteria. The DNA was extracted from poultry litter. The 16s rDNA was amplified using the polymerase chain reaction (PCR) with 27f, a fluorescently labeled primer, and 1492r reverse primer. The amplified product with the fluorescent label at one end was purified and the amplicon mixture was digested with a restriction enzyme (*HhaI*), which produced fragments of different sizes. These fragments were separated using automated capillary electrophoresis. This automated instrument has a laser reader which detects the labeled fragments and generates a fingerprint for each organism based on fragment lengths (3).

A



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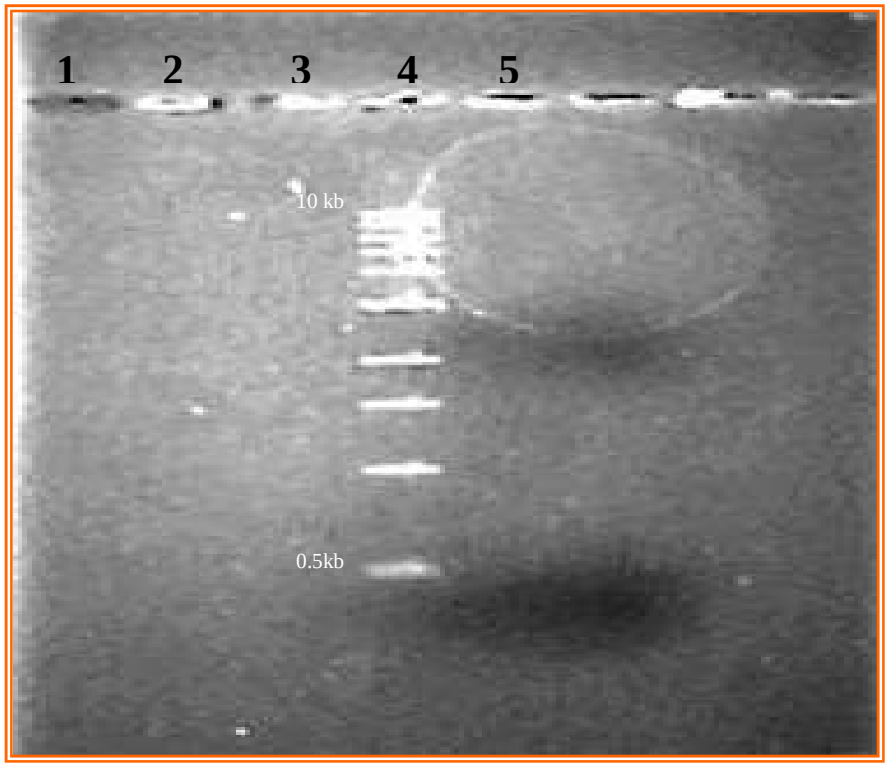


Figure 2. (A) Gel electrophoresis (1% agarose) of DNA extracted from poultry litter. DNA from poultry litter was suspended in a final volume of 50 µl. 5µl of sample was loaded into each well. Lane 1 *E. coli* genomic DNA (50ng); 2,3 Blank; Lane 4, 1kb size standard; Lane 5, DNA from 0.25 g of poultry litter; Lane 6, DNA from 0.5g of poultry litter; Lane 07, DNA from 0.75g of poultry litter. (B). 16S rDNA amplification of DNA extracted from poultry litter. Lanes 1, 2, 3, DNA extracted from 0.25g, 0.5g, and 0.75 g of poultry litter respectively; lane 4, 1kb size standard; lane 5, *E. coli* genomic DNA.

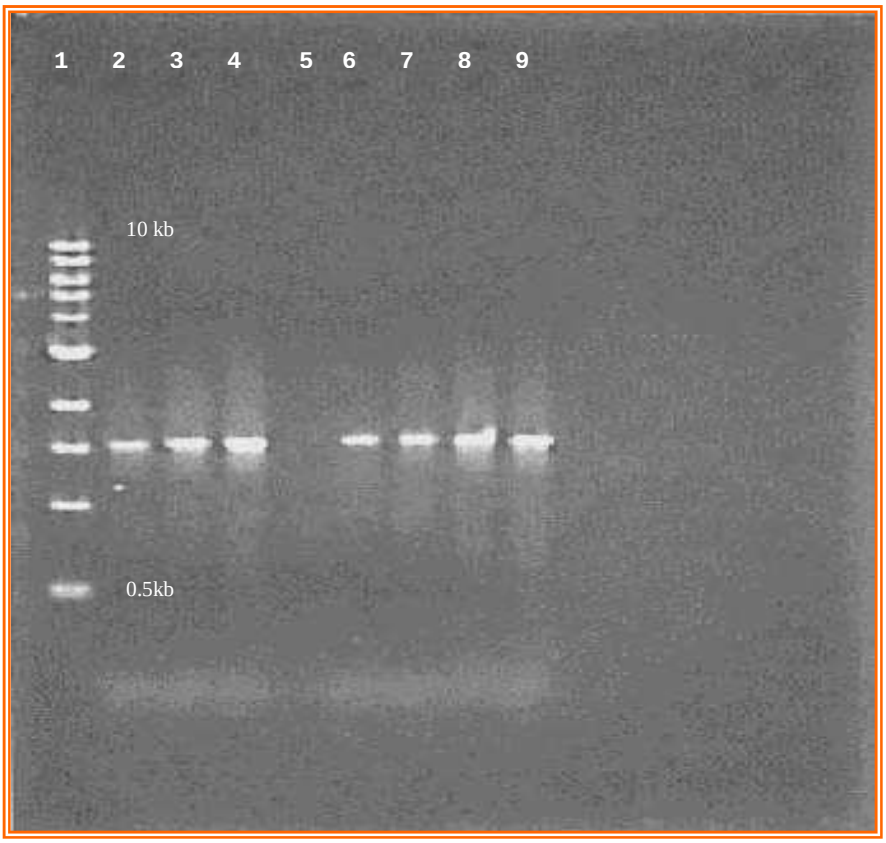
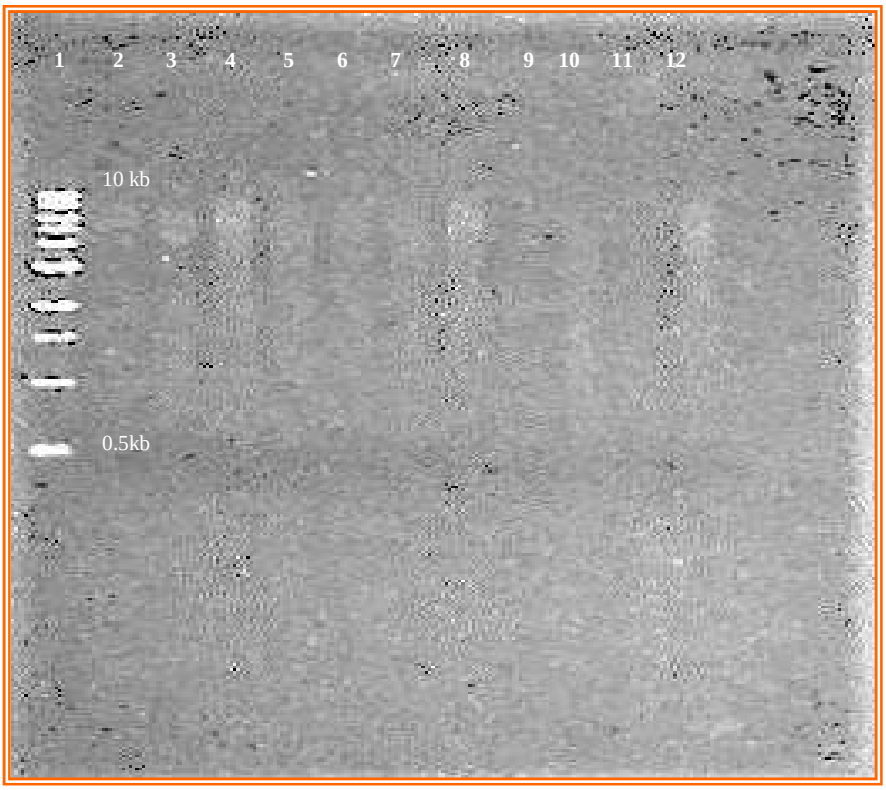


Figure 3. PCR amplification of DNA extracted from poultry litter spiked with *E. coli* chromosomal DNA. Lane 1, 1kb size standard; lane 2, *E. coli* chromosomal DNA; lane 3, 40ng of poultry litter DNA with 40ng of *E. coli* DNA; lane 4, 40ng of poultry litter DNA with 50ng of *E. coli* DNA; lane 6, 40ng of poultry litter with 60ng of *E. coli* DNA; lane 7, 40ng of poultry litter DNA with 70ng of *E. coli* DNA; lane 8, 40ng of poultry litter DNA with 80ng of *E. coli* DNA; lane 9, 40ng of poultry litter DNA with 100 ng of *E. coli* DNA. Polymerization temperature and thermocycling for the samples were as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 1 min; 55 degrees for 1 min, and 72°C for one minute and final extension at 72°C for 10 min.

A



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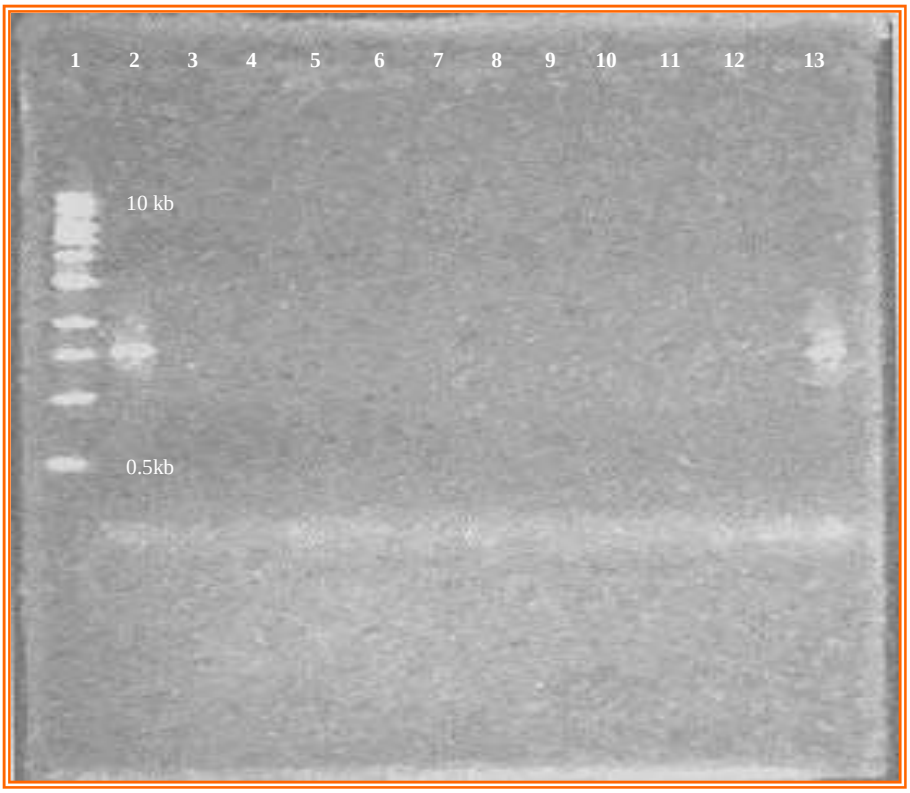
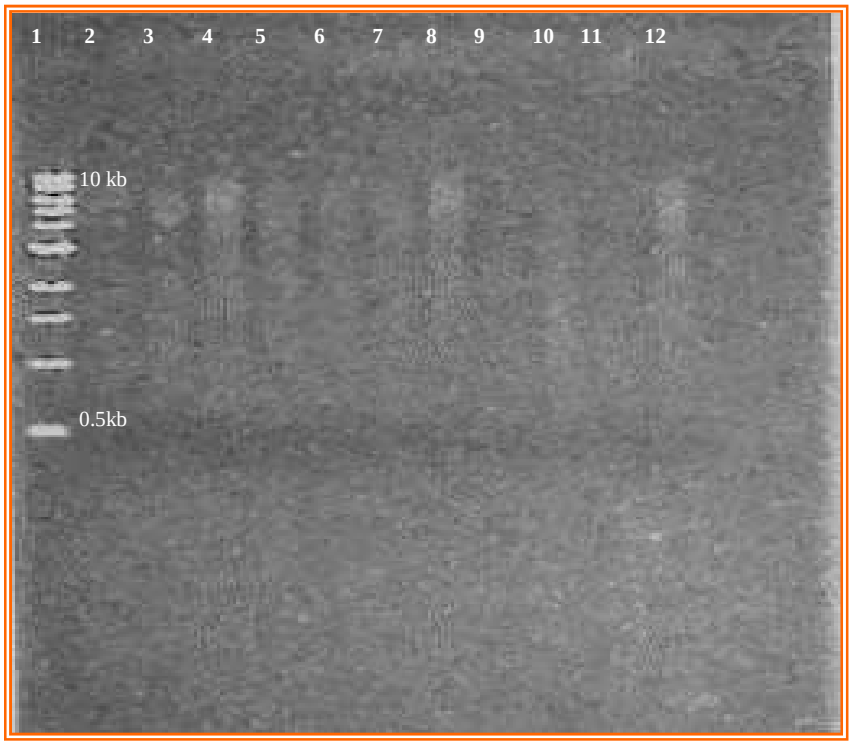


Figure 4. (A) Gel electrophoresis (1% agarose) of DNA extracted from poultry litter with known amount of *E. coli* cells included. Lane 1, 1kb size standard; lane 2, DNA (5µl, ~ 50ng) isolated from poultry litter; Lane 3, *E. coli* genomic DNA (5µl, ~ 50ng) ; Lane 4, DNA (5µl, ~ 50ng) isolated from poultry litter with 6 X 10^8 cells of *E. coli*; lane 5, (5µl, ~ 50ng) DNA isolated from poultry litter with 6 X 10^7 *E. coli* cells; lane 6, (5µl, ~ 50ng) isolated from poultry litter with 6 X 10^6 *E. coli* cells; lane 7, (5µl, ~ 50ng) isolated from poultry litter with 6 X 10^5 *E. coli* cells; lane 8, (5µl, ~ 50ng) isolated from poultry litter with 6X 10^4 *E. coli* cells; lane 9, (5µl, ~ 50ng) isolated from poultry litter with 6 X 10^3 *E. coli* cells; lane 10, (5µl, ~ 50ng) isolated from poultry litter with 600 *E. coli* cells; lane 11, (5µl, ~ 50ng) isolated from poultry litter with 60 *E. coli* cells; lane 12, (5µl, ~ 50ng) isolated from poultry litter with 6 *E. coli* cells. (B). PCR amplification of DNA extracted from poultry litter with known amount of *E. coli* cells included. Lane 1, 1kb size standard; lane 2 *E. coli* genomic DNA 3-12 refer Fig.3A; lane 13 *E. coli* genomic DNA.

A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10 kb
0.5kb

B

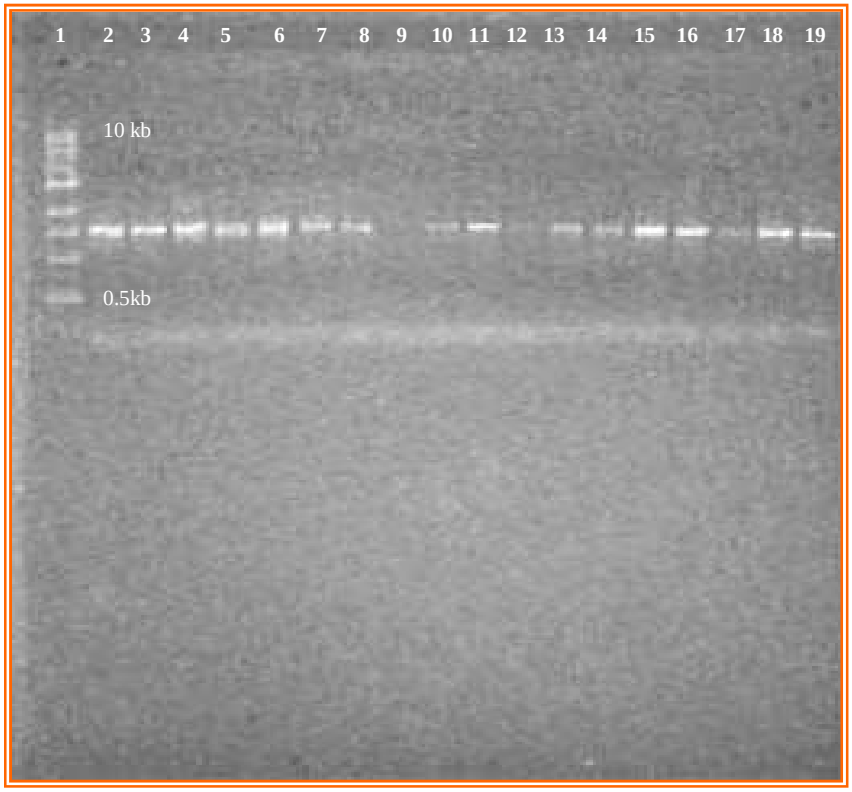
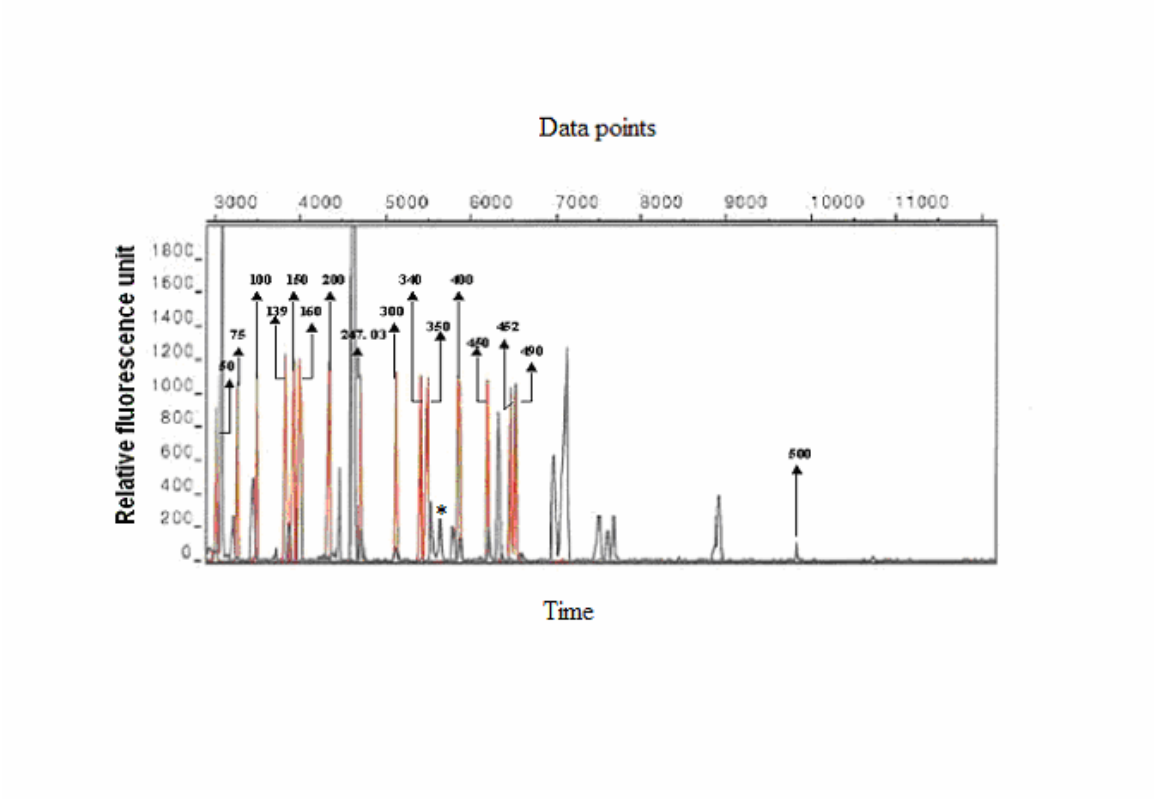
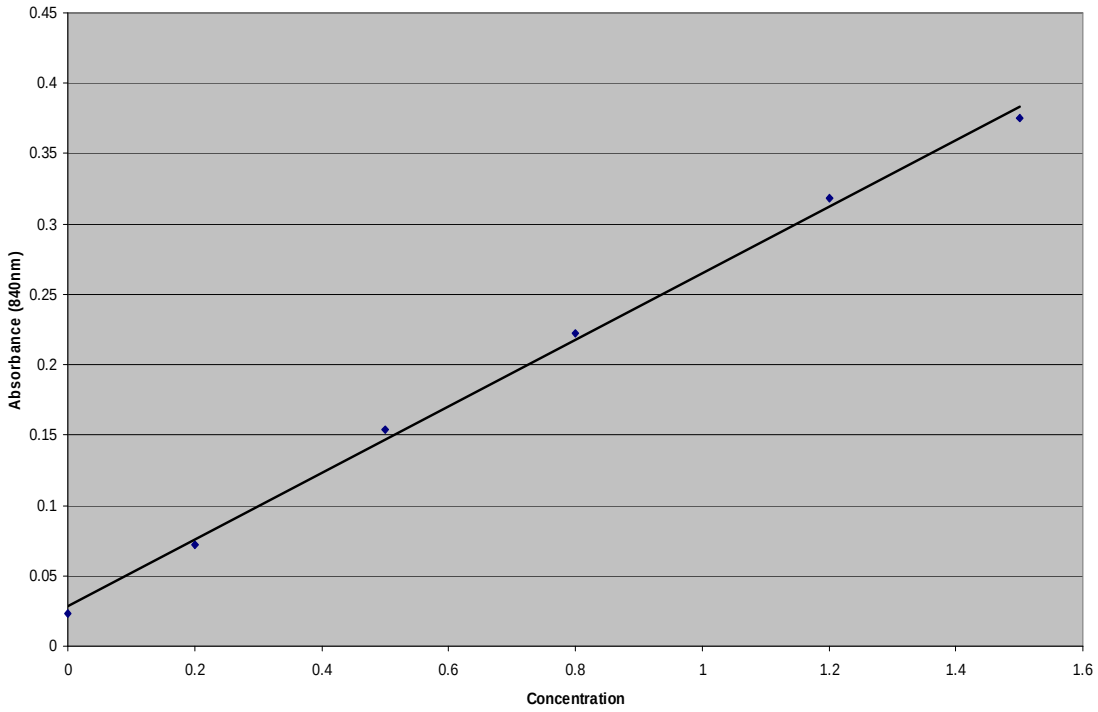

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
10 kb
0.5kb

Figure 5. (A) Gel electrophoresis (1% agarose) of DNA extracted from poultry litter with known amount of *E. coli* cells included, after passing the DNA extracted initially through second purification column. Lanes 1-12 refer Fig. 3A. (B). PCR amplification of DNA extracted from poultry litter with known amount of *E. coli* cells included. Lane 1, 1kb size standard; lane 2 *E. coli* genomic DNA; Lane 3, DNA isolated from poultry litter; lanes 4-12 refer Fig.3A; lane 13-19, 1/10 dilutions of DNA loaded in lanes 8-12. Polymerization temperature and thermocycling for the samples were as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 1 min; 55 degrees for 1 min, and 72°C for one minute and final extension at 72°C for 10 min.

Figure 6. A typical terminal restriction fragment length polymorphism profile from poultry litter. Signal at 370 nucleotides is indicative of 6 X 10^8 *E. coli* cells spiked into the sample.

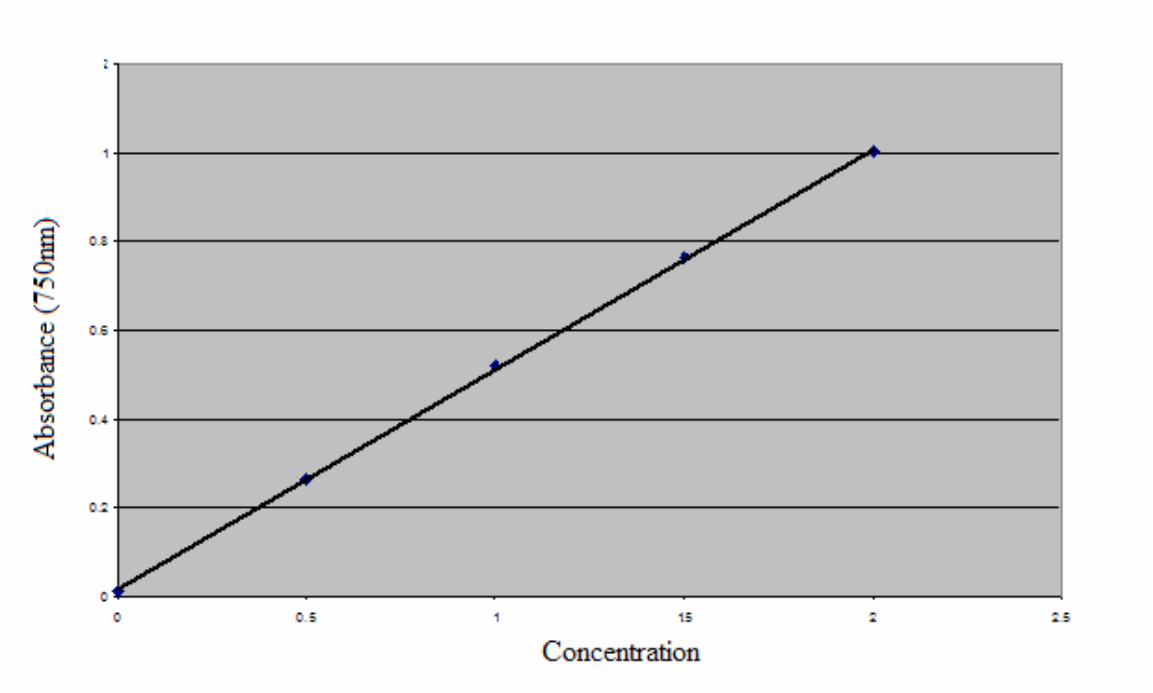


* Indicates *E. coli* signal



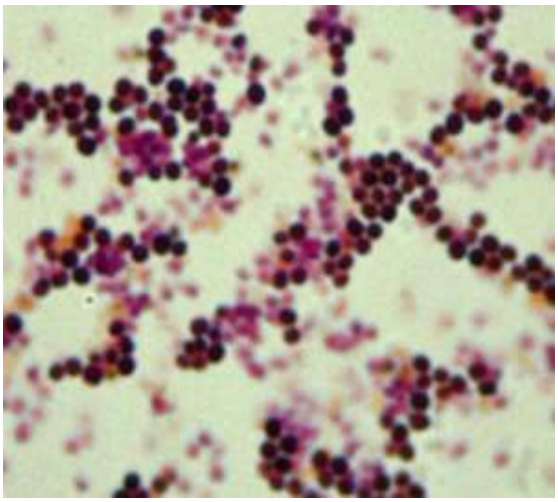
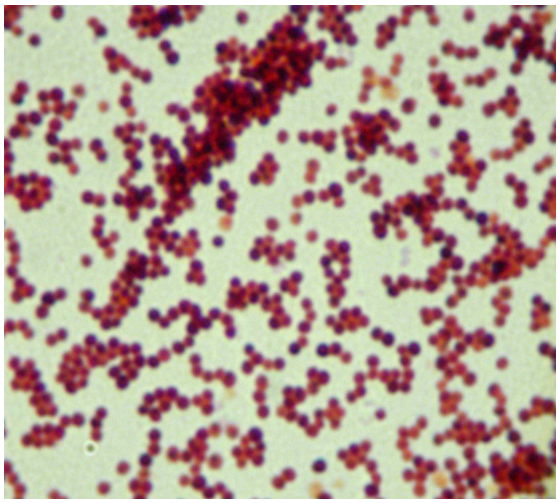
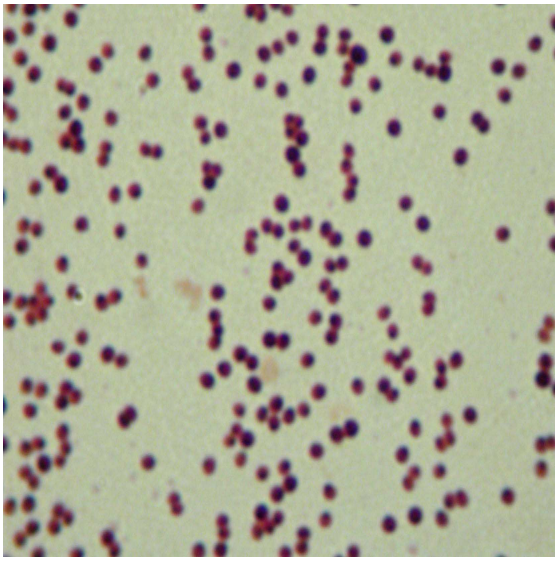
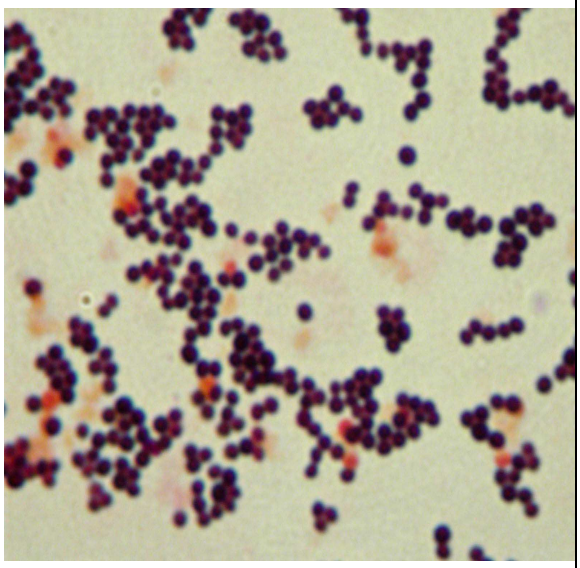
Concentration (K_2HPO_4 mg/ml)

Figure 7. A representative phosphate standard curve used to determine the unknown phosphate levels.



Protein concentration

Figure 8. A representative of protein standard curve used to determine the unknown protein concentrations in the sample.

A 	**B** 
C 	**D** 

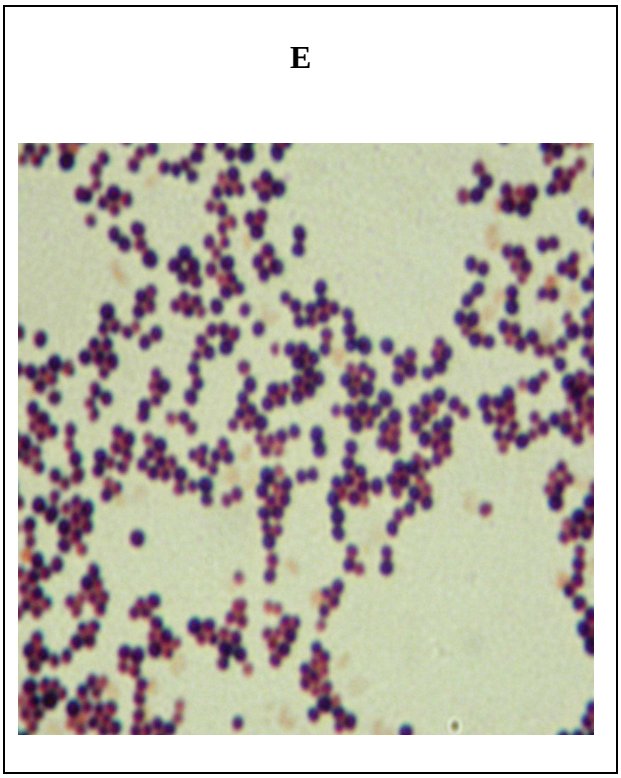
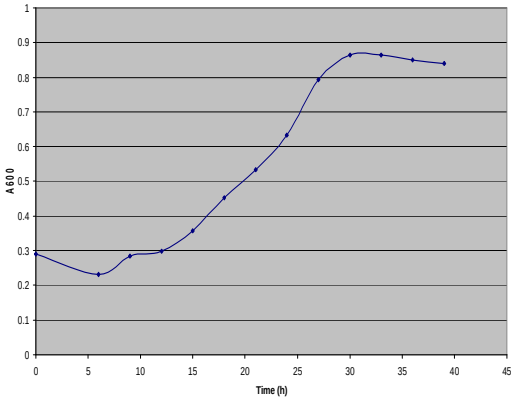


Figure 9. Bright field micrographs of microorganisms isolated from poultry litter on BHI supplemented with 750mM of PO^{-}_{4}. (A) Isolate 6. (B) Isolate 7, (C) Isolate 10, (D) Isolate 12. (E) Isolate 13.

A



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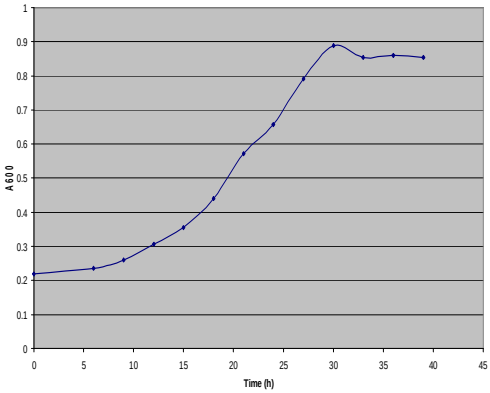

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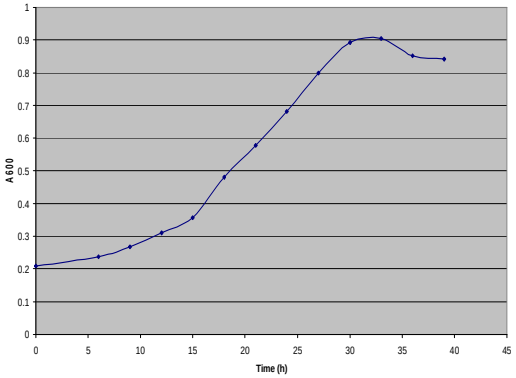

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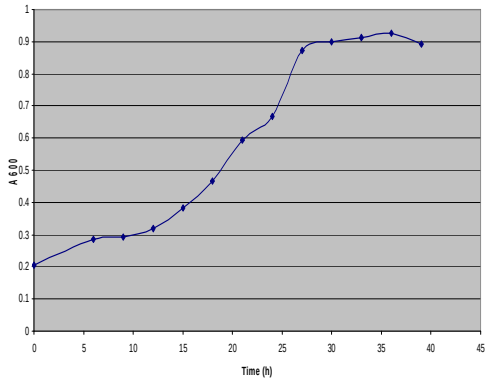

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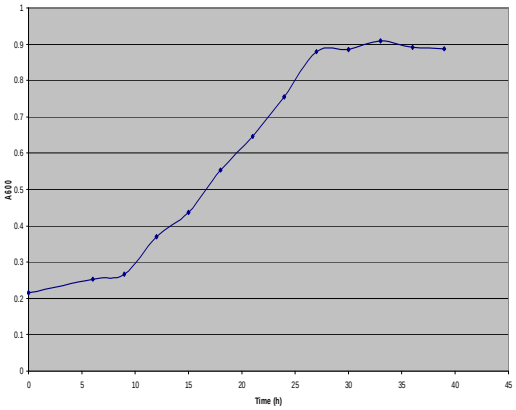

Figure 10. Growth curves of the bacteria isolated from poultry litter. Isolates were grown overnight at 25°C in BHI with 750mM of KH_2PO_4, overnight culture was inoculated in media with growth monitored at 600nm (A) Isolate 6. (B) Isolate 7. (C) Isolate 10. (D) Isolate 12. (E) Isolate 13.

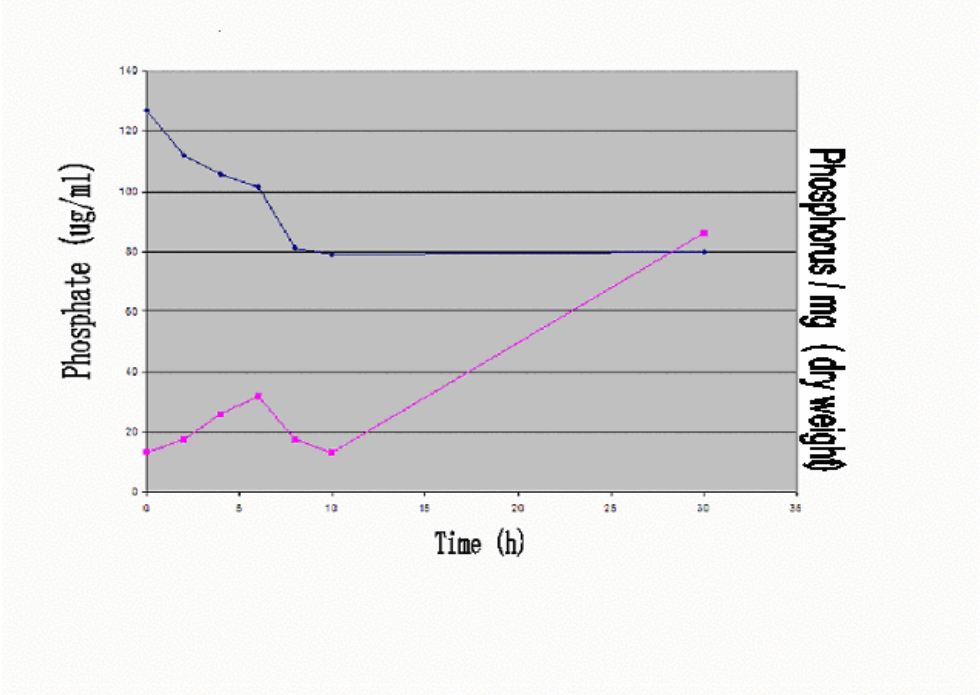

Figure 11. Phosphate uptake by *Microlunatus phosphovorus*. *Microlunatus phosphovorus* was grown in a defined medium and was harvested in the late logarithmic phase, washed and suspended in sterile water with supplementation of 6mM K_2HPO_4 and 3mM $MgSO_4.7H_2O$ under aerobic condition at 25° C. ◊ Extracellular phosphate was determined by ammonium paramolybdated spectophotometric method. □ Dry weight analysis for phosphate was determined by using an ICP.

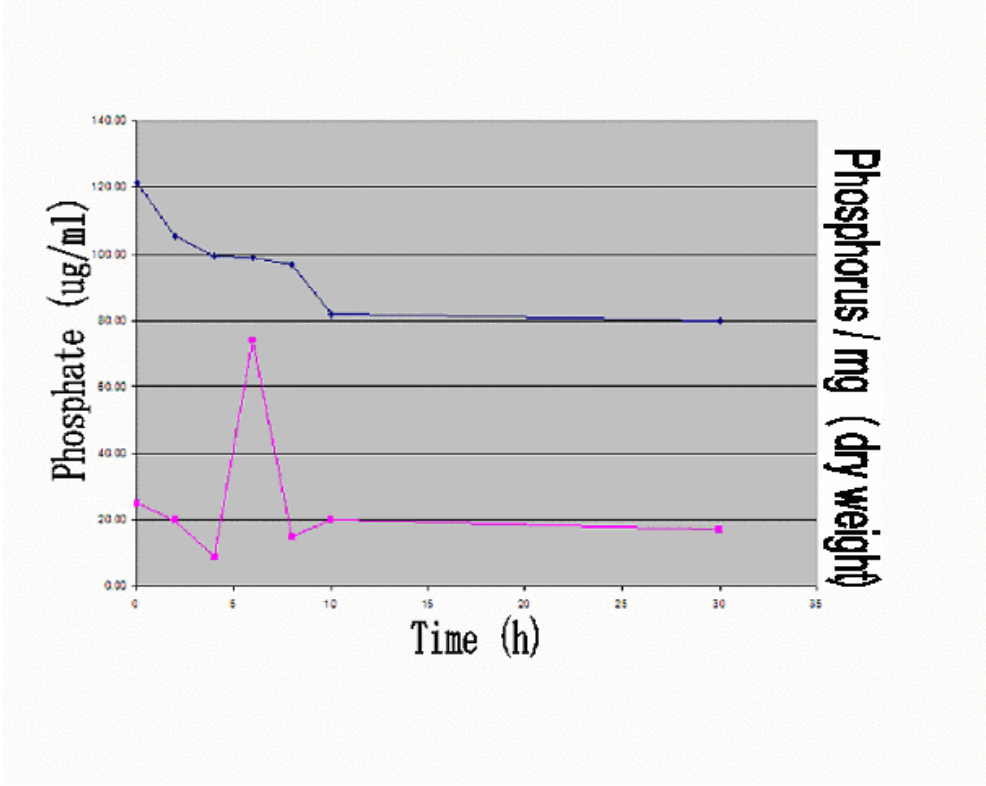

Figure 12. Phosphate uptake by Isolate 7. Isolate 7 was grown in a defined medium and was harvested in the late logarithmic phase, washed and suspended in sterile water with supplementation of 6mM K_2HPO_4 and 3mM $MgSO_4.7H_2O$ under aerobic condition at 25° C. ◊ Extracellular phosphate was determined by ammonium paramolybdated spectophotometric method. □ Dry weight analysis for phosphate was determined by using an ICP.

Table 1. Determination of sensitivity of extraction and detection of *E. coli* in poultry litter using terminal restriction fragment length polymorphism method.

	Quantity of *E. coli* cells spiked into 0.5g poultry litter[a]								
Trial	$6X10^8$	$6X10^7$	$6X10^6$	$6X10^5$	$6X10^4$	$6X10^3$	600	60	6
1	+	-	+	-	-	-	-	-	-
2	+	+	+	+	+	-	-	+	-
2	+	+	+	+	-	+	-	-	-
3	+	+	+	+	-	-	-	-	-
5	+	+	+	-	+	-	+	+	-

[a] + indicates successful amplification of *E. coli* rDNA
- indicates failed amplification of *E. coli* rDNA

Table 2. *HhaI* fragment lengths present in Ribosomal Database for T-RFLP database corresponding to major fragments observed in poultry litter.

HhaI fragments	Organism
56.75	None
70.32	*Spiroplasma spp; Leptotrichia spp*
94.59	*Campylobacter spp; Bacillus alcalophilus spp; Desulfobulbus spp; Chlorobium spp*
215.55	*Xanthomonas spp; Enterococcus spp*
234.76	*Clostridium spp*
236.46	*Clostridium spp; Bacillus sphaericus; Saccharococcus spp*
452.38	None
468.38	*Streptomyces spp*

Table 3. Table showing all substrates observed to be positive in duplicate

Isolate	Sugars	Organic Acids	Aminoacids	Others
Isolate #6	**Glucose, Galactose, Cellobiose, Fructose, Maltose, Mannose, Melibiose, Palationose, psicose, raffinose, ribose, sucrose, trehalose, turanose, xylose**	**N-acetyl-D-glucosamine, N-acetyl mannosamine, gluconica acid, α-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid.**	**Alanine, L-alanine, alanyl-glycine, L-glutamic acid, serine, adenosine, deoxyadenosine, inosine, thymidine, uridine, thymidine-5`-monophosphate, uridine-5`-monophosphate, D-L-α-glycerol phosphate**	**Methyl pyruvate,**
Isolate 7	**Glucose, Galactose, Cellobiose, Fructose, Maltose, Mannose, Melibiose, Palationose, psicose, raffinose, ribose, sucrose, trehalose, turanose, xylose, L-arabionse, L-fucose, D-melezitose**	**N-acetyl-D-glucosamine, N-acetyl mannosamine, gluconic acid, α-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid. D-galacturonic acid, L-pyroglutamic acid**	**Alanine, L-alanine, alanyl-glycine, L-glutamic acid, serine, adenosine, deoxyadenosine, inosine, thymidine, uridine, thymidine-5`-monophosphate, uridine-5`-monophosphate, D-L-α-glycerol phosphate**	**Methyl pyruvate, amygladin, m-inisitol, fructose-6-phosphate, alananinamide,**
Isolate 10	**Glucose, Galactose, Cellobiose, Fructose, Maltose, Mannose, Melibiose, Palationose, psicose, raffinose, ribose, sucrose, trehalose, turanose, xylose**	**N-acetyl-D-glucosamine, N-acetyl mannosamine, gluconica acid, α-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid.**	**Alanine, L-alanine, alanyl-glycine, L-glutamic acid, serine, adenosine, deoxyadenosine, inosine, thymidine, uridine, thymidine-5`-monophosphate, uridine-5`-monophosphate, D-L-α-glycerol phosphate**	**Methyl pyruvate**
Isolate 12	**Glucose, Galactose, Cellobiose, Fructose, Maltose, Mannose, Melibiose, Palationose, psicose, raffinose, ribose, sucrose, trehalose, turanose, xylose**	**N-acetyl-D-glucosamine, N-acetyl mannosamine, gluconica acid, α-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid.**	**Alanine, L-alanine, alanyl-glycine, L-glutamic acid, serine, adenosine, deoxyadenosine, inosine, thymidine, uridine, thymidine-5`-monophosphate, uridine-5`-monophosphate, D-L-α-glycerol phosphate**	**Methyl pyruvate**
Isolate 13	**Glucose, Galactose, Cellobiose, Fructose, Maltose, Mannose, Melibiose, Palationose, psicose, raffinose, ribose, sucrose, trehalose, turanose, xylose**	**N-acetyl-D-glucosamine, N-acetyl mannosamine, gluconica acid, α-hydroxybutyric acid, p-hydroxyphenyl acetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-lactic acid, pyruvic acid.**	**Alanine, L-alanine, alanyl-glycine, L-glutamic acid, serine, adenosine, deoxyadenosine, inosine, thymidine, uridine, thymidine-5`-monophosphate, uridine-5`-monophosphate, D-L-α-glycerol phosphate**	**Methyl pyruvate**

Table 4. Comparison of centrifugation conditions to determine the optimal condition for sample clarification prior to phosphate analysis.

Centrifugal force	Time (min)	Centrifuge	μg PO^-_4 / mg protein		
			Prehydrolysis phosphate	Posthydrolysis phosphate	Polyphosphate
13,000xg	4	Benchtop	0.112	0.591	0.479
25,000xg	1	Ultra centrifuge	0.108	0.642	0.534
30,000xg	30	Ultra centrifuge	0.192	0.556	0.364

[a] Amount of polyphosphate calculated by subtracting total phosphates from free phosphates.

Table 5. Comparison of intracellular phosphate of bacterial strains isolated from poultry litter grown on BHI and BHI with an additional 750mM phosphate.

Strain	Culture condition[a]	μg PO^-_4 / mg protein		
		Prehydrolysis Phosphate[b]	Posthydrolysis phosphate	Polyphosphate
Isolate 6	BHI	0.017 (+/-) 0.004	0.093 (+/-) 0.020	0.072 (+/-) 0.019
	BHI / PO^-_4	0.150 (+/-) 0.040	0.417 (+/-) 0.137	0.264 (+/-) 0.094
Isolate 7	BHI	0.019 (+/-) 0.011	0.083 (+/-) 0.029	0.064 (+/-) 0.019
	BHI / PO^-_4	0.216 (+/-) 0.106	0.492 (+/-) 0.260	0.276 (+/-) 0.018
Isolate 10	BHI	0.010 (+/-) 0.003	0.075 (+/-) 0.018	0.064 (+/-) 0.018
	BHI / PO^-_4	0.215 (+/-) 0.123	0.600 (+/-) 0.350	0.385 (+/-) 0.227
Isolate 12	BHI	0.022 (+/-) 0.014	0.086 (+/-) 0.013	0.067 (+/-) 0.012
	BHI / PO^-_4	0.218 (+/-) 0.051	0.440 (+/-) 0.096	0.223 (+/-) 0.057
Isolate 13	BHI	0.014 (+/-) 0.004	0.076 (+/-) 0.008	0.059 (+/-) 0.005
	BHI / PO^-_4	0.191 (+/-) 0.092	0.500 (+/-) 0.240	0.306 (+/-) 0.157

[a] Isolates were cultured in BHI media (BHI) or BHI supplemented with 750mM K_2HPO_4 (BHI/PO^-_4) incubated at 25° C for two days aerobically shaking at 50rpm.

[b] Cell preparations were lysed by bead beating and subjected to acid hydrolysis prior to PO^-_4 determination. The acid labile portion of total cellular PO^-_4 represents total cellular phosphate.

Table 6. Comparison of phosphate concentrations among bacteria isolated from poultry litter and between two types of media conditions.

Character	Condition	f-ratio	p-value
Total phosphate	Media type	20.130	< 0.001
	Isolates	0.074	0.989
Polyphosphate	Media type	9.449	0.006
	Isolates	0.233	0.971
Percentage of polyphosphate	Media type	8.738	0.008
	Isolates	1.079	0.393

Table 7. Comparison of free phosphate, total phosphate and polyphosphate levels of *Microlunatus phosphovorus* and Isolate 7[a]

Culture condition	µg protein/ml of culture	µg PO^-_4 / mg protein		
		Prehydrolysis phosphate	Posthydrolysis phosphate	Polyphosphate
M.phosphovorus				
BHI	17.639	0.005	0.04	0.035
BHI / PO^-_4	18.850	0.03	0.05	0.02
M.phosphovorus media[a]	2.264	0.08	0.43	0.35
Isolate 7				
BHI	19.395	0.043	0.16	0.117
BHI / PO^-_4	17.276	0.18	0.31	0.13
M.phosphovorus media[a]	2.022	0.06	0.23	0.17

[a] Isolate 7 grown in BHI regular media and BHI supplemented with 750mM of PO_4 at 25° C shaking at 50 rpm.

[b] Bacteria were grown with *M.phosphovorus* media (0.5g glucose, 0.5g of peptone, 0.5g of monosodium glutamate, 0.5g of yeast extract, 0.44g of KH_2PO_4, 0.1g of $(NH_4)SO_4$ and 0.1g of $MgSO_4.7H_2O$, pH was adjusted to 7.0) at 25°, shaking at 50 rpm.

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