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Full Length Article

Production and Characterization of Phytase from *Streptomyces luteogriseus* R10 Isolated from Decaying Wood Samples

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Abstract

Microorganisms are the richest source of phytase which catalyze hydrolysis of phytate to myo-inositol and phosphate. 50 Actinomycete isolates were isolated from heated soil, sand, wastewater, animal faces and some plant ecological wastes on Starch nitrate agar containing 15 µg / mL of antibiotics (Tetracycline + Amphotericin B, w/w). Out of 50 Actinomycetes isolates, 20 isolates (40%) produced extracellular phytase enzyme on solid medium containing wheat bran as carbon source. In liquid medium, the phytase activity was measured as U/mL and the most active isolate in phytase production was R10. Using morphological, physiological and biochemical studies, it was identified as *Streptomyces* sp. Using 16S rDNA analysis, it was identified as *S. luteogriseus* R10. Growth in medium containing 1% Na-phytate (pH 6.5) at 40°C for 7days increased phytase production. The maximum phytase activity was achieved using wheat bran, straw, rice husk and hay after seven days of incubation at 40°C but low activity was obtained using Sawyer and baggage as a carbon source. The molecular weight of the purified phytase is 65 kDa and it exhibits optimum activity at pH 5 and 45°C. All tested metal ions at 10 mM enhanced phytase activity except Ba^{2+}, Co^{2+}, Cu^{2+}, Ag, Fe^{3+} and Hg^{2+}. Improvement of phytase production was carried out using protoplast fusion between *S. luteogriseus* R10 and *S. niveu*s MM1. Fusant F7 was the best phytase producer (3 time higher) compared to its parents. © 2015 Friends Science Publishers

Keywords: Phytase; *Streptomyces*; Protoplast; Fusant; Phytic acid

Introduction

For humans and animals, legumes, cereals and oilseed crops are the main source of nutrients, which contain phytic acid as storage form of phosphorus (Reddy *et al*., 1989). To satisfy phosphorus requirement, inorganic phosphate is added to monogastric animals diets because they cannot metabolize phytic acid present in their diet. In monogastric animals, phytic acid is chelating various metal ions including calcium, copper and zinc (Graf, 1983). Therefore, phytic acid hydrolysis into less-phosphorylated myo-inositol derivatives using phytase is of great interest. Phytase can be used to improve the nutritional value of feed and/or to decrease the amount of phosphorus excreted by animals. Phytase enzyme produced by bacteria is extracellular which are more appropriate than the intracellular phytase produced by yeast in breaking down phytic acid (Konietzny and Greiner, 2004). The aim of the present study was isolation and identification of thermo-stable phytase producing bacterium and factors affecting production were studied. Phytase was purified and characterized. Enhancement of enzyme production was carried out using protoplast fusion to solve the difficulty of low level of phytase in primitive natural microbes.

Materials and Methods

Bacterial Isolation

Soil, sand, plant materials, wastewater and plant waste products from farms in Huda Al Sham, Saudi Arabia were collecting and transported in sterile plastic bags to the Microbiology lab. Serial dilutions were prepared and about 0.1 mL of each sample was spread on starch nitrate medium (Shirling and Gottlieb, 1966) which composed of g/L: 20 Starch, 1.0 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 3.0 $CaCO_3$, 2.0 KNO_3, 0.5 NaCl and 1 mL trace salt solution) and for solid medium preparation, 20 g/L agar was added. Filter sterilized mixture of Tetracycline+Amphotericin B (w/w) was added at concentration 15 µg/mL to the prepared medium (Aly *et al*., 2011b). Incubation of the plates was at 37°C for 7 days and the purified colonies were streaking on ISP2 medium composed of g/L: 4 yeast extract, 4 glucose, 10 malt extract, 2 $CaCO_3$ and 20 agar and maintained at 4°C until used.

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Bacterial Growth and Screening Conditions

Several Actinomycete isolates were screened on modified LB with wheat bran medium (LBWB medium). LB medium composed of (g / L) 10 g tryptone, 10 g NaCl and 5 g yeast extract in addition to 50 g of wheat bran, pH was adjusted to pH 7.0. Agar (20 g / L) was added, when solid agar medium was needed. In liquid broth medium, the bacterial isolates with phytase activities were cultivated in 50 mL LBWB broth medium in 250 mL Erlenmeyer flasks. Each flask was inoculated with 2mL (4×10^6 CFU⁄mL) of bacterial suspension, previously grown at 37°C for 2 days at 200 rpm in starch nitrate broth medium. The inoculated flasks were maintained for 5 days at 37°C on a rotary shaker (200 rpm). After centrifugation at 10,000 rpm for 15 min, cell-free supernatant was for phytase assay.

Phytase Assay

Cell-free supernatant was used for determination of phytase activity by measuring the amount of phosphorous released from Na-phytate, which develops a color with ammonium molybdate. The color density was quantified spectrophotometrically at 700 nm (Chi *et al*., 1999). Under assay conditions, one phytase unit was the amount of the enzyme, released of 1 nM of inorganic phosphate / min.

Optimization of Phytase Production Process

In liquid medium, the effect of different factors on growth (optical densities at 540 nm) and phytase production was determined (El-Sabbagh *et al*., 2003). LB broth medium with different concentrations of Na-phytate was prepared. The inoculated flasks were incubated at 37ºC and 200 rpm for 5 days. Effect of different incubation temperatures (20-50°C) and medium pH (6.0-8.0) were determined after 5 day of growth in LB medium with 1% Na-phytate. At the end of growth period, growth and phytase were measured. Effect of different incubation periods ranging from 1 to 7 days of growth in modified LB medium (pH 6.5) at 40°C and 200 rpm was determined. All the experiments were carried out in triplicate and averages were reproduced.

Phytase Production Using Various Waste Products

Some agriculture waste products were collected, dried, powdered and sterilized using the autoclave. They were used as carbon source as described by Lanciotti *et al*. (2005) and phytate hydrolysis was determined after 7 days of incubation at 40°C and 200 rpm.

Enzyme Purification and Molecular Weight Determination

Proteins in the culture filtrate were collected after 80% Ammonium sulfate precipitation at 4°C. The collected precipitate at 10,000 rpm was dissolved in phosphate buffers (pH 7), dialyzed against the same buffer for 2 days (El-Sabbagh *et al*., 2003), concentrated under vacuum, applied to a column (30 × 1.5 cm) of diethyl aminoethyl cellulose (DEAE cellulose) and eluted using 1 M NaCl in phosphate buffer (80 mL / h). The eluent was collected in 5 mL fractions. The active fractions with phytase activity were collected and concentrated under vacuum. The concentrate was applied to carboxymethyl-cellulose followed by Sephadex G75 column and elution was carried out using phosphate buffer. The active fraction was collected, lyophilized and was analyzed. Gel electrophoresis was carried with 15% SDS polyacrylamide electrophoresis at room temperature where 20–30 µL (40 µg / well) from the protein standard (Merck) were applied and gel was stained with Coomassie brilliant blue R-250.

Characters of the Pure Phytase Enzyme

Using the standard phytase assay conditions (Chi *et al*., 2008), effect of different pH values, 3–9, on phytase was determined after the pure enzyme suspension in 0.2 M acetate buffer (pH 3–6) or 0.2 M $Na_2B_4O_7.10\ H_2O$ / H_3BO_3 buffer (pH 7–10). Effect of temperature ranged from 20-70ºC on the purified enzymes was determined in the selected buffer. The pre-incubated enzyme at 37°C was used as a reference to calculate activity. Effect of some additives incorporated in the reaction mixture including some metal ions and EDTA on the enzyme assay was studied and the relative activities were compared with the activity obtained for control (without additive) (El-Sabbagh *et al*., 2003).

Characterization of the Selected Actinomycete Isolate (Taxonomical Studies)

The selected phytase producing actinomycete isolate was characterized and identified. It was grown on starch nitrate agar medium for fresh prepared culture. Gram and endospore stains were carried out and bacterial morphology was conducted using light and electron microscopy (XL30-ESEM environment scanning electron microscopy). Physiological and biochemical characters in addition to chemical analysis of the whole cell sugar composition and type of the diaminopimelic acid isomer were determined as described by Aly *et al*. (2011a, 2012) and Hasegawa *et al*. (1983), respectively. Cell fatty acids and phospholipids were extracted and determined using gas chromatography and two-dimensional thin-layer chromatography (Butte, 1983; Hoischen *et al*., 1997).

Phylogenetic Analysis of 16S rDNA Sequence

QIAamp DNA Mini Kit was used for genomic DNA extraction of the selected isolate R10. The forward primer 5' AGTTTGATCATGGTCAG-3' and reverse primer 5' GGTTACCTTGTTACGACT 3' were designed (Weisberg

et al., 1991) and 16S rDNA gene was amplified, sequenced and the DNA sequence was compared to the GeneBank database.

Improving Phytase Production

Improving phytase production using protoplast fusion (Yari *et al*., 2002; Aly *et al*., 2011a, b) between *Streptomyces* R10 and *S. niveus* (MM1) which was highly resistant to NaCl (Aly *et al*., 2003) was carried out. On Mueller Hinton medium, antibiotic pattern using paper disc diffusion assay for the two tested bacteria were as the following: *Streptomyces* R10 was strep.$^{-}$ tet.$^{+}$ and *S. niveus* was strep.$^{+}$ tet^{-}. The two species were grown at 37°C for 24 h in starch nitrate broth medium (Shirling and Gottlieb, 1966). From the previous culture, 1 mL was mixed with 10 mL of a starch nitrate broth with 0.5% (w / v) glycine in100 mL conical flask and the flasks were incubated at 30°C overnight. The growth was collected by centrifugation (5,000 rpm for 15 min) and sonicated for 3min for protoplast formation (Matsushima and Baltz, 1985). The percentages of real protoplasts for each species were calculated (Aly *et al*., 2011b) and the obtained protoplasts were mixed gently in 3 mL of Modified R2 sterile medium containing the appropriate concentration of tetracycline (1µg / mL) and streptomycin (5 µg / mL). After 7 days of incubation at 30°C, the obtained fusants were picked and maintained on the same medium.

Statistical Analysis

Mean of three replicates and standard deviations were recorded. Data were statistically analyzed and difference between mean values was determined using Student's t-test. The differences were significant when $P<0.05$.

Results

This research aimed to isolate many Actinomycete isolates, producing phytase, which can hydrolyze some agricultural wastes. In this connection, 50 bacterial isolates with different colony colors and shapes were obtained from soils, sand and plant materials in addition to ecological wastes on starch nitrate agar-containing antibiotic. All the isolates were grown on LBWB agar medium and 20 isolates out of 50 grow well on the previous medium hydrolyzing the phytic material by phytase, which was detected as pale clear zone around the colony (Fig. 1). Diameter of the clear zone was differed for each organism. Six phytase producing bacterial isolates were selected and grown in liquid broth medium (Table 1).

In liquid medium, the most active isolate in phytase production was isolate R10, which was isolated from decaying wood sample on SN agar with antibiotic. The morphology of the phytase-producing bacterium and Gram reaction were determined after examination with light

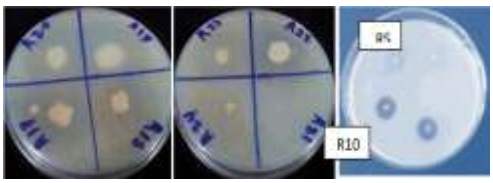

Fig. 1: Screening of some Actinomycete isolates on LBWB medium for phytase production after 5 days of growth

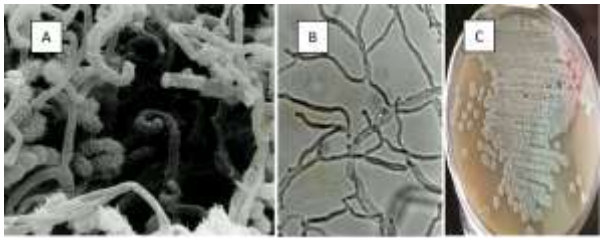

Fig. 2: The selected Actinomycete isolate R10, A: under scanning electron microscope (x 20 000), B: under light microscope x1000, C: after growing on Oat meal agar medium for 10 days at 37°C

microscope using oil emersion lens. It was belonging to filamentous Gram + ve bacteria with gray color (Fig. 2). The isolate R10 was identified according to morphological, physiological and biochemical characters (Table 2, 3, 4 and 5). Cell wall composition and the characteristics sugars, lipids and fatty acids were determined (Table 6). Presence of L-isomer of diaminopimelic acid (L-DAP) and glucose indicated a wall chemotype IV and whole cell sugar pattern as type A, while analysis of phospholipids indicated phospholipids type PII. Saturated fatty acids with no mycolic acids were detected using gas chromatograpy. The phylogenetic tree based on 16S rDNA sequence using neighbor joining tree method was drowning (Fig. 3).

Phytase production or / and bacterial growth varied with phytate concentration, initial pH of the medium, incubation temperature and incubation period. The best phytate recovery was 1.0% (Fig. 4). The effect of temperature and pH on phytase production was showed in Fig. 5 and 6, where incubation at 40°C and initial pH 6.5 recorded maximum phytase production. However, phytase production dropped significantly at pH 9.0 and no production was observed at pH 9.5. The maximum phytase production was recorded after 7 days of growth at 40°C (Fig. 7). Different waste product including hay, straw, and bran (Fig. 8) were used as substrate (1% w / v) for phytase production. Among all the substrates, the maximum phytase activity was observed with bran and straw. The lowest production was observed for sawyer and baggage.

The selected isolate R10 was grown using the best conditions of phytase production. The enzyme was precipitated and purified using different column chromatography and profile of elution was determined.

Table 1: Source, colony color and phytase production by the most active Actinomycetes in phytase production

Bacterial isolate	Source of isolation	Colony color	Phytase production	
			On solid medium	In liquid medium
			Presence of clear zone	Lipase activity (U /mL)
R1	Wastewater	White	+	1.0±0.09
R10	Decaying wood	Gray	+++	1.9±0.05
R24	Contaminated soil	White	++	1.7±0.03
R36	Contaminated soil	Yellow	+	1.7±0.01
R47	Contaminated soil	Pink	+	1.1±0.04
R 49	Animal feces	Yellow	+	1.1±0.05

+: Moderate production, ++: High production, +++: Very high production

Table 2: Cultural characteristics of the Actinomycete isolate R10 grown on different agar medium at 30°C

Agar medium	Growth	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigment
Glycerol-asparagine agar (ISP-5)	Moderate	Dark gray	Yellow	+
Glucose Asparagine agar	Heavy	Dark gray	Yellowish gray	+
In-organic salts-starch iron (ISP-4)	Moderate	Pale gray	Yellowish-white	+
Tyrosine agar (ISP-7)	Scanty	Yellowish gray	Pale yellow	+
Yeast extract-malt extract (ISP-2)	Moderate	Yellowish brown	Yellow	+
Oatmeal agar (ISP-3)	Moderate	White	Pale yellow	+

+: Soluble pigment present

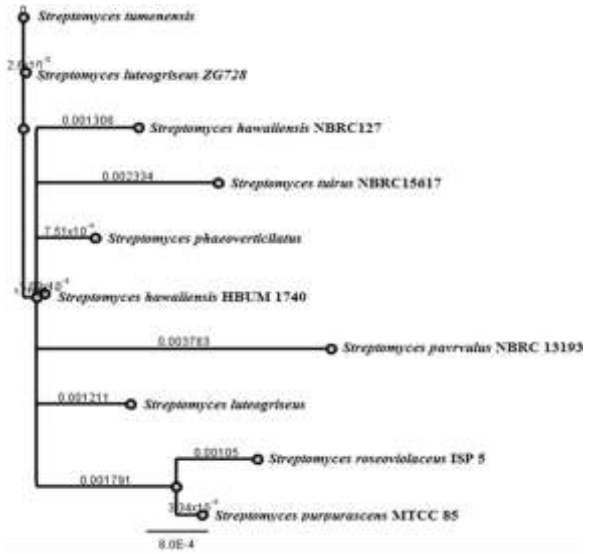

Fig. 3: Phylogenetic tree based on 16S rDNA sequence comparisons of *Streptomyces* R10, using neighbor joining tree method, maximum sequence difference =0.002

The active fractions that showed the maximum phytase activity were collected, lyophilized and used for enzyme characterization and molecular weight determination. Phytase molecular weight was 65 kDa, detected using gel electrophoresis (Fig. 9) and exhibits optimum activity at pH 5.0 and 45°C (Fig. 10). Phytase activity was significantly affected by most of the ions tested and all these ions at 10 mM acted as enhancer for the phytase activity except Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Li^{+} and Hg^{2+} (Table 6).

Protoplast fusion between the identified *S. luteogriseus* R10 and *S. niveus* MM with two different antibiotics resistance profiles (10 µg / mL tetracycline and 400 µg / mL streptomycin), was carried out using PEG 6000.The regenerated protoplast percentage of the two species was 88% and 80%, respectively. Ten recombinant fusants were obtained and fusant F7 showed higher phytase production compared to the two wild types (about 3 fold increases) (Table 7).

Discussion

Different actinobacteria were obtained from the collected samples on agar medium containing antibiotics. Addition of antibiotics to growth medium or samples heating prevent the growth of unwanted bacteria and allow antibiotic resistant actinobacteria to dominate (Velho-Pereira and Kamat, 2011). Similarly, on modified glycerol arginine agar, an initial screening was performed to isolate common Actinomycetes and on modified medium to isolate rare Actinomycetes (Ghorbani-Nasrabadi *et al*., 2012).

In solid LBWB agar medium containing wheat bran as inducer, all Actinomycete isolates were screened for phytase production. As it is well known, the phytase enzyme was inducible, and the presence of phytate, wheat bran or some other inducer in the medium is necessary for enzyme formation (Tambe *et al*., 1994; Konietzny and Greiner, 2004). In bacteria, induction and expression of phytase are regulated and are not controlled uniformly among different bacteria. Phytase may not require during balanced bacterial growth but synthesized under energy and / or nutrient limitation (Konietzny and Greiner, 2004). In this work, 40% of the screened actinobacteria were phytase-producing and this activity was detected as clear zones accompanying the growth in solid agar. Similarly, 46.3% of the Actinomycete isolates had phytate-degrading capacity (Ghorbani-Nasrabadi *et al*., 2012). Out of 21 bacterial isolates from

Table 3: Morphological character of the selected isolate R10

Tested character	Results
Gram stain	Gram positive
Source of isolation	Decaying wood
Motility of spore	Absent
Shape of spore	Cylindrical (5-6 and, 6-9 µm)
Spore chain	Spiral chain
Spore Surface	Hairy
Number of spore / chain	5-20
Aerial and substrate hyphae	Well developed
Zoospore, sporangium, sclerichia, fragmented mycelia	Absent

Table 4: Physiological characteristics of the isolate R10

Character	Reaction	Character	Reaction
Melanin pigment production	+ve	Tolerance to NaCl	5-10%
Enzyme activities:		pH range	6-10
Proteolysis	+ve	Growth temperature:	15-45ºC
Lecithinase	-ve	Resistance to antibiotic	
Lipolysis	+ve	Penicillin	
Chitinase	+ve	Cephalosporine	+
Gelatinase	+ve	Kanamycin	+
Pectinase	+ve	Rifampin	+
H_2S Production	-ve	Tetracycline	+

-ve: negative results, +ve: positive results, ++: Growth, +: Resistance

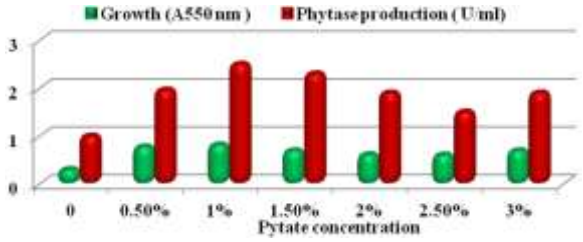

Fig. 4: Effect of different concentration of phytate on growth and phytase production by the selected strain of Actinomycetes isolate R10

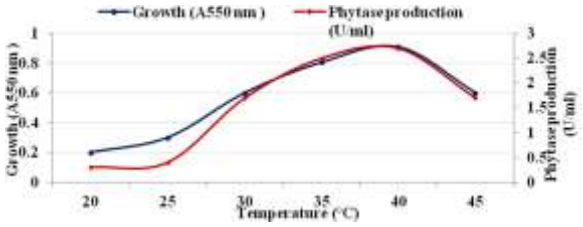

Fig. 5: Effect of different temperature on growth and phytase production by the selected strain of Actinomycetes R10

water habitats, eight showed phytase production by clear zone formation around growth on solid agar medium (Shamna *et al*., 2012). Furthermore, 67 isolates of soil actinomycetes were potentially producing extracellular phytate-degrading activity and two isolates of the genus *Streptomyces* were the most active in phytase production (Ghorbani-Nasrabadi *et al*., 2012).

Phytase detection was obtained using plate-clearing technique and / or measuring the enzyme activity in liquid medium. For bacteria, growth on agar medium is usually easier than in a liquid broth medium (Choi *et al*., 2001), thus, no further assessment was conducted for isolates that could not grow or showed weakly growth on solid agar medium containing wheat bran as source of phytate. The lack or weak phytase activity in some Actinomycete isolates may be due to a loss of trait in the isolate or the used culture conditions were not effective in inducing phytase. Hence, at this point, it is unclear whether phytate hydrolysis is a common trait within the Actinomycete group. Generally, some bacterial enzymes are mostly cell associated, whereas the phytases obtained by fungi are extracellular. Concerning the isolate R10, phytase production was extracellular in the culture filtrate and it was identified using morphological, physiological, biochemical analysis in addition to 16S rDNA analysis. According to morphological description (Pridham and Tresner, 1974), biochemical comparison and physiological analysis of genus *Streptomyces* with other described isolates (Williams *et al*., 1989), the isolate R10 belongs to the genus *Streptomyces*. Identification was made using 16S rDNA, a powerful tool for deducing evolutionary relationships and phylogenetic among eukaryotic organisms, bacteria and archaebacteria (Olmezoglu *et al*., 2012), was used. The 16S rDNA sequence reported that isolate R10 was closely related to *S. luteogriseus* by 93% and can be identified as *S. luteogriseus* R10.

The knowledge on the participation and role of Actinomycetes in hydrolysis of phytate and organic phosphorylated compounds is extremely limited and is strongly dependent on the Actinomycete strain as well as media composition. Furthermore, the importance of Actinomycetes in dephosphorylation of soil organic compounds needs to be elucidated in further studies. The results of Ghorbani-Nasrabadi *et al.* (2012) showed Actinomycetes as a source of phytase. On contrast, it was clear that production of phytases were characterized from some Gram-negative and positive bacteria including *Klebsiella, Enterobacter, Bacillus* and *Pseudomonas* (Yoon *et al*., 1996; Greiner *et al*., 1997; Richardson and Hadobas, 1997; Kerovuo *et al*., 1998; Richardson *et al*., 2001). Although, phytases have found in some animal tissues and in plants, phytases from bacteria have many commercial applications due to substrate specificity, catalytic efficiency and resistance to proteolysis (Konietzny and Greiner, 2004). In liquid medium, maximum enzyme production by *S. luteogriseus* R10 was occurred using LB medium continuing 1% Na-phytate as inducer, incubation of flasks at 40°C, initial pH 6.5 and 200 rpm after 7 days. *Cladosporium* sp. FP-1 showed maximum phytase production in a medium containing 1.0 g phytate (Quan *et al*., 2004). Temperature is one of the most critical parameters to be controlled in any bioprocess and the optimum temperature for production of phytases by many microorganisms was 25-37°C (Vohra and Satyanarayana,

Table 5: Utilization of different carbon and nitrogen sources using the selected isolate R10

Carbon source	Utilization	Nitrogen source	Utilization
Positive control (glucose)	++	$NaNO_3$	++
Negative control	-ve	NH_4NO_3	++
D-mannitol	++	KNO_3	++
Glycerol	++	NH_4OH	++
Raffinose	-ve	NH_4Cl	++
D – galactose	-ve	$NaNO_2$	-ve
Sucrose	++	Phenyl alanine	+
Fractose	++	Valine	+
D-xylose	++	Peptone	+

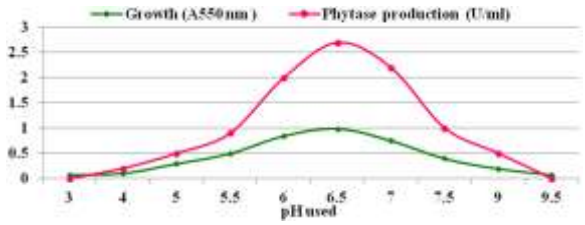

Fig. 6: Effect of different initial pH value on growth and phytase production by the selected strain of Actinomycetes R10

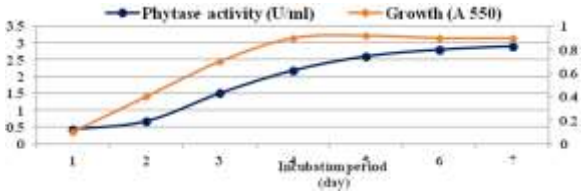

Fig 7: Effect of different incubation periods on growth and phytase production by the selected strain of Actinomycetes R10

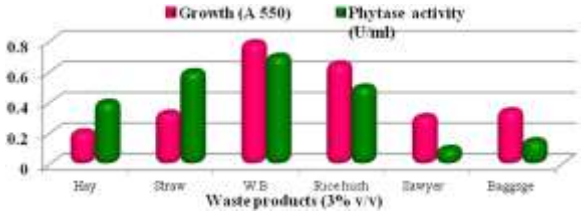

Fig. 8: Growth and phytase production of the selected strain of Actinomycetes isolate R10using different waste products (10 g/L) as carbon source

2003). At pH 6.5, maximum phytase production was recorded and increasing medium pH decreased the activity of the enzyme due to charges on the amino acids within the active site and no formation of the enzyme-substrate complex. Similar results were obtained for phytase of *Pseudomonas* spp, (Sasirekha *et al*., 2012). For *Enterobacter* sp., the optimum phytase production was at pH 5.5 after 3 days of growth at 37°C (Yoon *et al*., 1996). Phytase of *Streptomyces* was very specific for phytate and efficiently hydrolyzed phytate of wheat bran, hay, straw, rice hush sawyer and baggage. Similar results were obtained by Kim *et al*. (1998) where, phytase was very specific for phytate and had little activity on other phosphate esters and efficiently hydrolyzed phytate in oat flour rice and wheat. Using the best conditions for phytase production, the enzyme was collected and purified using different column chromatography. After purification, phytase has molecular weight of 65 kDa and its activity was not significantly affected by most of the ions tested. However, EDTA, Ag^{+}, Cd^{2+}, Hg^{2+}, Cu^{2+} inhibit the phytase activity. Similarly, two species belonging to the genus *Streptomyces* produced phytase with optimum temperature of 55°C and 37°C and optimum pH values of 5 and 7 (Ghorbani-Nasrabadi *et al*., 2012). The purified enzyme of *Bacillus* had maximal phytase activity at pH 7 and 55°C and required calcium for its maximum activity but was readily inhibited by EDTA (Kerovuo *et al*., 1998). The optimum pH of phytases from *E. coli, Klebsiella* or *Aspergillus* were in the range 4.5-5.5 (Greiner *et al*., 1993; Wyss *et al*., 1999, Sajidan *et al*., 2004; Elkhalil *et al*., 2011). In contrast to those phytases, *Bacillus* phytase displayed a narrow pH optimum between 7.0 and 7.5 (Kerovuo *et al.*, 1998; Kim *et al.*, 1998).

Some bacterial phytases with pH optimum of 6.0 to 8.0 benefits in poultry feed additives where their pH optimum was close to the physiological pH of the poultry crop (Kim *et al*., 1998; Choi *et al*., 2001). In contrast, purified phytase from *Bacillus* sp. DS11 had molecular weight of 44 kDa by SDS-polyacrylamide gel electrophoresis and optimum temperature of 70°C in presence of calcium ions but its activity was greatly inhibited by metal ions such as Cd^{2+}, Mn^{2+} and EDTA (Kim *et al*., 1998). Moreover, phytase with lower MW of 32.6 kDa, optimum temperature of 40°C, optimum pH of 3.5, stimulated by dithiothreitol and 2-mercaptoethanol, and inhibited by Ba^{2+} and Pb^{2+} was obtained (Quan *et al*., 2004). Phytases from *Enterobacter* sp. and *Bacillus subtilis* were inhibited by 1 mM EDTA (Yoon *et al*., 1996; Kerovuo *et al*., 1998). On contrast, Greiner (2004) found that phytase was not inhibited by EDTA at concentration of 1 mM whereas phytase from *A. niger* van Teighem was enhanced by EDTA (0.1-2.0 mM) for about 50% (Vats and Banerjee, 2002). The maximal phytase activity of *Enterobacter* sp. 4 was observed at pH 7.0-7.5 and at 50°C but above 60°C, the enzyme activity was gradually lost and was inhibited by each addition of 1 mM Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and EDTA (Yoon *et al*., 1996).

Furthermore, enhancement of phytase production using protoplast fusion was carried out between the identified *Streptomyces* and *Streptomyces niveus*, which showed different antibiotic resistance profiles. Percentage of successfully regenerated protoplasts of the two used *Streptomyces* was 88% and 80%, respectively. Out of 10 recombinant fusants obtained, one (Fusant F7) showed higher phytase production compared to both parents (3

Table 6: The biochemical tests (sugar, amino acid, phospholipids, and fatty acid composition of the cell wall or cell hydrolysate) of the isolate R10

Type of the reaction	Results
Sugar in the cell hydrolysate (glucose)	+
Amino acids in the cell wall (diaminopimelic acid, DAP)	L-form
Phospholipids (phosphatidylethanolamine and phosphatidylinositolmannoside)	+
Fatty Acids	Iso. and Antiso fatty acid

Table 7: Effects of different metal ions and chemicals on phytase activity

Metal	Relative activity (%)
$CaCl_2$	120
$MgCl_2$	120
$MnCl_2$	120
$ZnSO_4$	100
$CuSO_4$	30
$FeSO_4$	90
LiCl	55
$AgNO_3$	40
$HgCl_2$	20
$CoCl_2$	57
$BaSO_4$	90
EDTA	50

Table 8: Phytase production using two tested *Streptomyces* isolates and their fusants

Used bacteria	Phytase activity (U/mL) × 10^2	Used bacteria	Phytase activity (U/mL) × 10^2
Streptomyces niveus	0.40	Fusant F5	0.0
Streptomyces R10 (control)	1.7	Fusant F6	0.08
Fusant F1	0.09	Fusant F7	7.90*
Fusant F2	0.80	Fusant F8	0.80
Fusant F3	0.04	Fusant F9	0.70
Fusant F4	0.05	Fusant F10	5.0*

*: significant difference at $p < 0.05$

fold increase). The previous results were in agreement with those obtained by many authors (Chassy, 1987; Kanatani *et al*., 1990; Ward *et al*., 1993) who used protoplast fusion in genetic manipulation and bacterial improvement. After protoplast fusion between *Streptomyces cyaneus* and *Streptomyces griseoruber*, Teeradakorn *et al*. (1998) isolated new fusants with rearrangement in their genetic materials and produced high levels of xylanase. Lin *et al*. (2007) isolated several fusants with increased lipase activity (317%). Through protoplast fusion, fibrinolytic enzyme production from *Bacillus* was improved 4-5 time compared to wild type (Liang and Guo, 2007). Moreover, Aly *et al*. (2011a, 2012) used the previous technique to enhance chitinase and lipase production by *Streptomyces* and mutate *Bacillus* to enhance chitinase production.

Conclusion

Decaying wood contained many actinomycete isolates with excellent phytase production and *S. luteogriseus* R10 was the most active isolate which can degrade some plant waste products including hay, straw and bran using phytase. Optimization of bacterial growth conditions enhanced phytase production which can be used in bioremediation

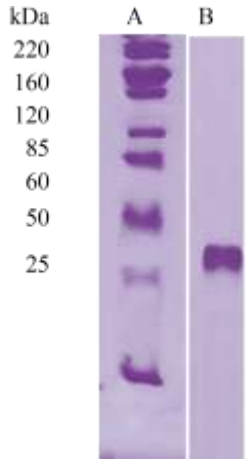

Fig. 9: SDS-PAGE profile of purified phytase, Lane A: standard protein marker, lane B: purified phytase

and many industrial applications. The purified phytase enzyme has 65 kDa and was stable at 45°C and pH5. Genetic improvement using protoplast fusion enhanced phytase production.

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