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# Exploration of Extracellular Phytase Production by *Amycolatopsis vancoresmycina* S-12 in Submerged Fermentation

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**A B S T R A C T**

In an attempt to explore extracellular phytase producing Actinomycete, the stain S-12 a soil isolate was selected on the basis of clearing zone formation on PSM plate and by assaying of fermented broth. The isolate S-12 identified as *Amycolatopsis vancoresmycina* through morphological, cultural and molecular characterization. As the fermentation process is significantly influenced by various physical and chemical parameters, hence, the isolate was tested in varied fermentation conditions. The maximum production of phytase was observed at 120 hour of incubation at 37°C and at pH 6.5. The most suitable carbon and nitrogen sources were 1.5% glucose and 1% potassium nitrate, supplementary carbon source 0.5% Na-Phytate. As surfactant, Tween 80 (0.5%) showed enhancement of phytase production. Notably, the *A. vancoresmycina* S-12 is able to enhance phytase production close to 8 times by medium optimization only at its wild state.

## Introduction

Phosphorous is an essential component of living organisms, it is found as orthophosphate, pyrophosphate, sugar phosphate, nucleic acids, and derivatives of other form of phosphate. Phytic acid (myo-inositol hexakis phosphate) is an anhydrous storage form of phosphate, which comprises up to 80% of total phosphorous of most plant products like seeds and pollen grains (Omoyeni and Adeyeye, 2009). Phytic acid acts as an antinutritional factor by chelating metals ($Ca^{+2}$, $Mg^{+2}$, $Zn^{+2}$ and $Fe^{+2}$), also forming complex with proteins, making them unavailable to the organism, thereby, affecting digestion of animals by inhibiting enzymes like amylase, trypsin, acid phosphatase and tyrosinase (Sasirekha *et al*. 2012).

Since, the phosphate of phytic acid is not metabolized by monogastric animals (pigs, poultry and fishes) due to lack of phytase, it comes to soil environment with excreta, consequently, contributes to the phosphorous mediated pollution problems (Nasi, 1990). Enhancement of phosphorous

concentration in aquatic body forms the cyanobacterial bloom and hypoxia, causing death of animals therein. Since, chemical and physical methods for hydrolysis of phytate reduce the nutrient value of feeds, attempts were made to search for alternative approah (Khetarpaul and Chauhan, 1990). To look into, enzymatic hydrolysis of phytate was worked out (Christel, 1992).

Phytase is a group of enzymes responsible for phytate hydrolysis. Common bacterial phytase (EC 3.1.3.8, myo-inositol hexakisphosphate 3-phosphorylase) is the member of the histidine acid phosphatase subfamily that catalyzes the hydrolysis of phytic acid to inorganic phosphate and myoinositol phosphate derivatives. Commonly, the phytase is an inducible enzyme that encompasses complex gene regulations for its synthesis (Mullaney and Ullah, 2003).

Although several phytase producers have been isolated (Das *et al*. 2013) and characterized (Gulati *et al*. 2007), or cloned (Moura *et al*. 2001) but there is need to search for new isolates with novel property. Most phytase producers are from the soil (Nasrabadi *et al*. 2012) and the common producers are of bacteria (Khetarpaul and Chauhan, 1990) and fungi (Gulati *et al*. 2007, Vats *et al*. 2009), but producers from Actinomycetes are of rare occurrence (Bajaj and Wani, 2013). Thus, attempts have been made to isolate potent Phytase producers from Actinomycetes. This communication deals with, the medium optimization for phytase production by a new isolate *Amycolatopsis vancoresmycina* S-12, an actinomycete for the first time.

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## Materials and Methods

### Isolation and Screening

Isolation and screening were carried out with phytase screening medium (PSM), having composition ($gL^{-1}$): Glycerol 2.0, L-Arginine 1.0, Na-Phytate 4.0, $CaCl_2$ 0.1, NaCl 1.0, $MgSO_4$, 0.01, $FeSO_4$, 0.001, $CuSO_4$, 0.001, $MnSO_4$, 0.001, $ZnSO_4$ 0.001, pH 6.9, Agar 15.0. Visually well separated colonies were selected and transferred to slants of glucose asparagine (GA) agar medium, ($gL^{-1}$): Glucose 10.0, Asparagine 0.50, $K_2HPO_4$ 0.50, pH 6.8, Agar 15.0 and stored at $4^oC$ in a refrigerator. After confirming their purity, the isolates were streaked to PSM medium for qualitative screening. For final selection, screened isolates were grown in PSM broth and phytase assay was made with cell free culture broth, treated as crude enzyme.

### Characterization and Identification

The selected isolate was characterized on the basis of morphological, cultural, biochemical and molecular (16S rDNA sequencing) basis, following the standard procedures. Phylogenetic analysis was made, consulting through NCBI database. The sequence alignment and phylogenetic tree was made using Mega5 software and neighbor joining analysis.

### Biomass Estimation

After fermentation, the broth was decanted to a fresh preweighed filter paper (initial weight), dried over night at $80^oC$ in an oven and weighed again (final weight). The biomass (dry weight) was calculated by simple deduction method.

### Enzyme Assay

Enzyme activity was assessed according to the method of Engelen *et al*., 1994. The fermented broth was centrifuged at 10,000 rpm for 10 min at $4^oC$, to separate the cell mass and the broth was treated as crude

enzyme. The reaction mixture contained 2 mL of substrate solution with 1 mL crude enzyme and incubated for 65 min at 37$^{o}$C. (Substrate solution: 8.40g Na-phytate in 900 mL acetate buffer (pH 5.5) and diluted to 1 L with water). After incubation, 2 mL Color stop reagent (ammonium heptamolybdate solution, ammonium vanadate solution and 65% nitric acid solution) was added with the enzyme mixture and measured at 415 nm in spectrophotometer. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1 µmole phosphorous per minute under standard assay condition.

## Optimization and Fermentation Condition

Effect of various physical and nutritional parameters on growth and phytase production were studied in batch fermentation, using Erlenmeyer flask (250 mL) containing 100 mL medium, inoculated with 5 mL of spore suspension ($1.5x10^{6}$ $CFU.mL^{-1}$) prepared from 7 days old culture. The fermentation was carried at 32$^{o}$C for 7 days if not stated otherwise. To find out a suitable medium several media were checked, supplemented with 0.1% Na-Phytate replacing the recommended carbon source.

After identifying the suitable basal medium, physical parameters like fermentation period, temperature and pH (using different buffer systems) were optimized. The medium ingredients were also varied separately for optimization. In case of nitrogen variation, nitrogen equivalence was maintained. Similarly, medium supplements (trace salts and surfactants) were also tried.
In each set of experiment, the single parameter was standardized at a time, keeping the other factors constant. The experiments were carried out in triplet and statistical analyses (±SE) were determined by KyPlot software.

## Results and Discussion

Phytase is an enzyme for organic phosphate utilization but it shows a potential demand as feed supplement. Studies on phytase have been carried out mostly with bacteria (Singh and Satyanarayana, 2008) and fungi (Gulati *et al*. 2007) but much less with Actinomycetes.

In order to isolate phytase producing soil Actinomycetes, 42 isolates were screened out of 142 that showed growth and clearing zone formation (24 mm) on PSM medium. From the short listed strains, only 7 isolates showed considerably larger clearing zones and notable growth in PSM (Fig 1).

Among them, the isolate S-12 was selected for further studies for its high phytase production potentiality in PSM broth. The PSM was formulated by the modification of AGS agar medium, formerly recommended for isolation of Actinomycetes (Nasrabadi *et al*. 2012). Actinomycetes do not compete with other soil microorganisms in solid media during growth, but other microorganisms may influence the growth of Actinomycetes. Medium with proper growth nutrients, support the growth of Actinomycetes, probably by reducing the activity of competing microorganisms (Nasrabadi *et al*. 2012, Saad *et al*. 2011).

While characterizing the isolate S-12, the colony appeared circular (16 to 19 mm in dia), concave, chalky white and dusty, became orange at maturity. The organism is filamentous branched substrate and aerial mycelia with conidia (Rectiflexible), conidial mass reddish orange. SEM studies showed conidiophores with conidia (Fig 2A).

The alignment of the nucleotide sequence (1358nt) matched with 16S rDNA gene sequences available with NCBI data base

and compared with sequence of the available reference species. The similarity level appeared between 98.45 to 99.33% closely matched with A. *vancoresmycina*. The phylogenetic tree obtained by applying the neighbour joining method is presented in Fig 2B. Thus, the isolate S-12 is identified as *A. vancoresmycina* (Accession No: HE.966414.1), a member of Actinomycete.

Fermentative production depends on the suitability of medium condition since the production depends on its ingredients and its environment (Saad et al. 2011). To find out a suitable medium condition for growth and phytase production, the fermentation was initially tried in seven media. It appeared (Fig 3A) that the isolate could grow and produced phytase in all the test media but maximum growth (0.04 $gL^{-1}$) and production (22.20 $UL^{-1}$) observed with AGS broth. But, *Mitsuokella jalaludinii* (Lan et al, 2002), *Candida melibiosica* (Georgiev et al. 2013), and also the actinomycete *Nocardia* sp. MB 36 (Bajaj and Wani, 2013) were found to produce maximally in medium containing wheat bran. *Streptomyces hygroscopicus* NRRL B-1476 produced highest amount of extracelluar phytase in Czapek Dox broth (Ali et al. 2007).

Incubation period, temperature and pH are the certain most important parameters for fermentative production of metabolites. To determine the optimum period, fermentation was continued up to 168 h with an interval of 12 h. Where the phytase production was detected since 36 h (1.76 $UL^{-1}$) and it increased progressively with time (Fig 3B). Maximum production was observed at 120 h (24.70 $UL^{-1}$), but the growth was maximum (0.04 $gL^{-1}$) at 156 h and continued at the same rate up to study period. In case of bacteria, the optimum phytase production was observed within 72 h but in Actinomycetes fermentation period was comparatively longer. However, Actinomycetes showed extracellular phytase production in more than 120 h (Nasrabadi et al. 2012), but the *Nocardia* sp. MB 36, a nonfilamentous actinomycete, produced maximally at 40 h (Bajaj and Wani, 2013).

The effect of temperature for phytase production was studied between 25 to 45$^{o}$C with an interval of 2$^{o}$C. Least growth and production observed at 25$^{o}$C but, it showed the maximum biomass (0.04 $gL^{-1}$) and phytase production (25.63 $UL^{-1}$) at 37$^{o}$C (Fig 3C). However, the biomass appeared inversely proportion with the production, with the increase of temperature. Though, the mesophilic organisms grow well between 25$^{o}$C to 40$^{o}$C but there is exception as *Nocardia* sp. MB 36 produces maximum phytase between 40$^{o}$C to 50$^{o}$C (Bajaj and Wani, 2013). In this study, the maximum production was observed at 37$^{o}$C and the production decreased with further increase in temperature. Phytase production was found to vary between 30 to 80$^{o}$C (Hara et al. 1985).

In order to examine the effect of pH on phytase production, experiment was carried out in different pH, using buffer systems. Though the biomass was maximum (0.04 $gL^{-1}$) at pH 7.0 but the production (29.26 $UL^{-1}$) was maximum at pH 6.5 (Fig 3D). However, biomass and phytase production decreased, on either side of the optimum pH. The working isolate *A. vancoresmycina* S-12 preferred to grow at pH 6.5 for optimum phytase production. Whereas, *M. jalaludinii* showed maximum phytase production between pH 6.82 to 7.02 (Lan et al, 2002). But, the *Nocardia* Sp. MB 36 showed maximum phytase production at pH 5.0 (Bajaj and Wani, 2013).

To identify the suitable substrate for phytase production by *A. vancoresmycina* S-12, glucose was found (Fig 3E) as the most suitable (41.48 $UL^{-1}$).

**Table.1** Effect of Vitamins on Growth Kinetics and Phytase Production by A.*Vancoresmycen* S-12

| Vitamins | Concentrations (µg/L) | Biomass (g/L) | Production (U/L) |
|---|---|---|---|
| Riboflavin | 0.1 | 0.17 | 71.41±0.06 |
| | 1 | 0.17 | 71.54±0.24 |
| | 10 | 0.17 | 70.55±0.18 |
| Pyridoxine | 0.1 | 0.18 | 71.66±0.15 |
| | 1 | 0.17 | 71.44±0.17 |
| | 10 | 0.17 | 70.56±0.19 |
| Thiamin | 0.1 | 0.18 | 72.45±0.13 |
| | 1 | 0.19 | 87.67±0.13 |
| | 10 | 0.19 | 71.44±0.10 |
| Biotin | 0.1 | 0.17 | 70.45±0.08 |
| | 1 | 0.17 | 69.34±0.20 |
| | 10 | 0.16 | 68.51±0.12 |
| Control | ---- | 0.18 | 72.53±0.21 |

**Table.2** Effect of Surfactants in Growth Media for Production of Phytase by A.*Vancoresmycena* S-12

| Surfactants | Concentrations (%) | Biomass (g/L) | Production (U/L) |
|---|---|---|---|
| Tween 80 | 0.1 | 0.18 | 131.15±0.03 |
| | 0.2 | 0.17 | 142.23±0.05 |
| | 0.4 | 0.16 | 152.12 ±0.96 |
| | 0.5 | 0.16 | 176.25±0.03 |
| | 1.0 | 0.15 | 114.11±0.05 |
| Teepol | 0.1 | 0.18 | 103.10±0.03 |
| | 0.2 | 0.18 | 107.66±0.04 |
| | 0.4 | 0.17 | 112.26±0.03 |
| | 0.5 | 0.17 | 126.25±0.03 |
| | 1.0 | 0.16 | 141.35±0.03 |
| EDTA | 0.1 | 0.18 | 85.39±0.02 |
| | 0.2 | 0.17 | 81.57±0.03 |
| | 0.4 | 0.17 | 75.32±0.02 |
| | 0.5 | 0.17 | 60.25±0.03 |
| | 1.0 | 0.16 | 56.28±0.03 |
| SDS | 0.1 | 0.17 | 93.48±0.05 |
| | 0.2 | 0.16 | 97.55±0.06 |
| | 0.4 | 0.16 | 99.25±0.03 |
| | 0.5 | 0.15 | 107.45±0.03 |
| | 1.0 | 0.15 | 118.45±0.05 |
| Control | ---- | 0.18 | 87.91±0.02 |

**Figure.1 A** Clearing Zone; (B) Growth in PSM Broth

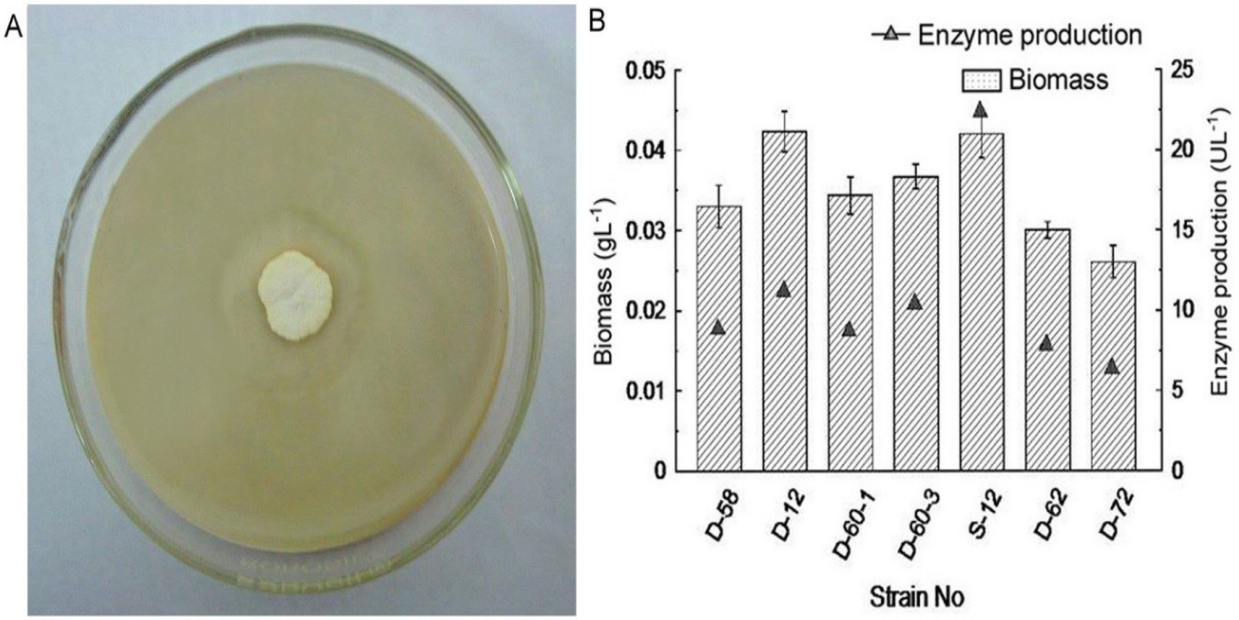



**Figure.2** (A) SEM Photograph; (B) Phylogenetic Tree Construct of Isolate A. *vancoresmycina* S-12 Based on 16S DNA Sequencing

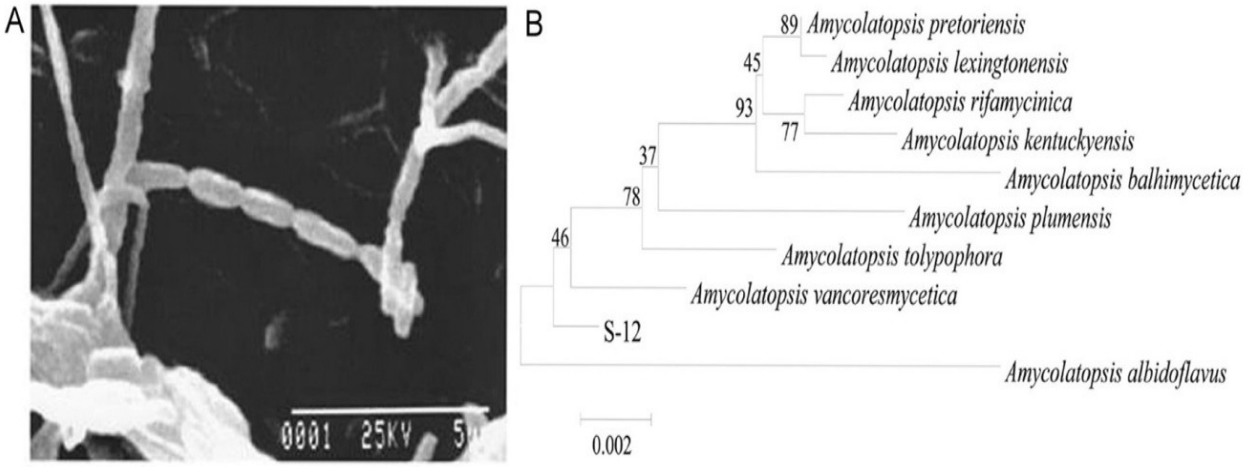
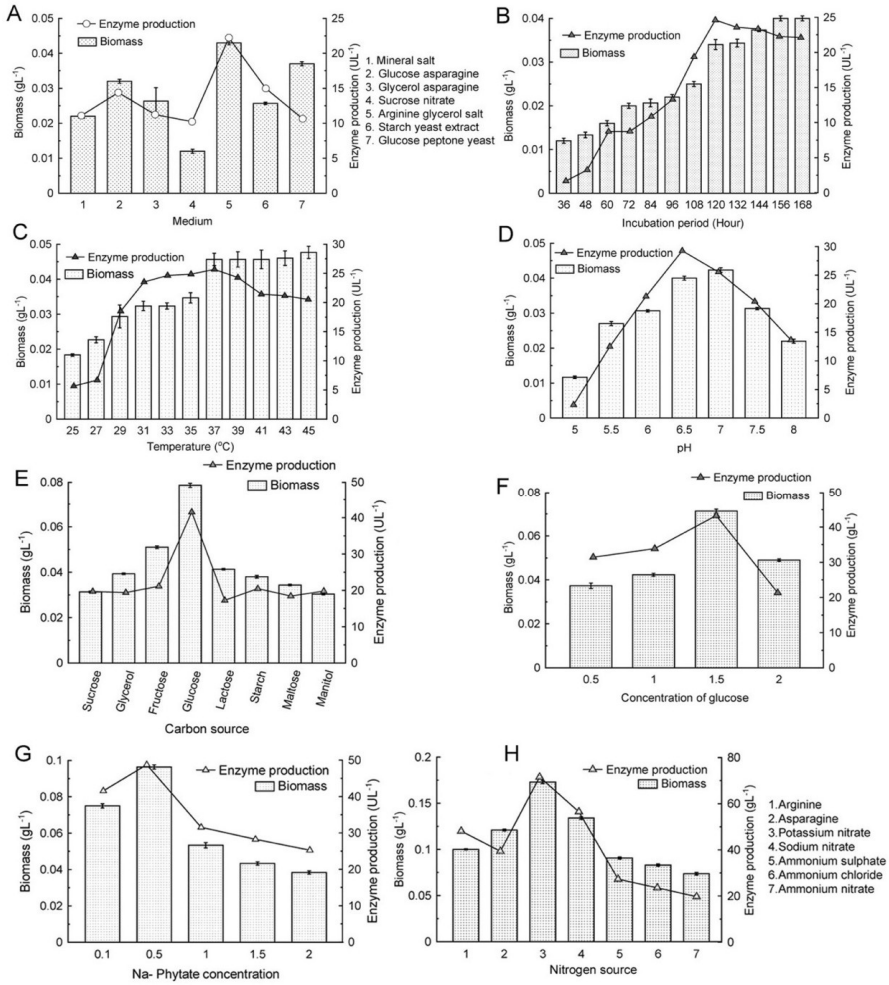


**Figure.3.**Optimization of Fermentation Conditions for Growth and Phytase Production by A. *vancoresmycina* S-12 (A) Selection of Suitable Medium; (B) Effect of Incubation Period; (C) Effect of Temperature; (D) Effect of pH; (E) Effect of Carbon Source; (f) Effect Glucose Concentration; (G) Effect of Na-Phytate Concentration; (H) Effect of Nitrogen Source




But the, lactose showed inhibiting activity (17.13 $UL^{-1}$) and found even lower than the control (glycerol). Further to ascertain the glucose concentration of the medium, the isolate was grown in a range of glucose (0.5 to 2%) and optimum phytase production (44.25 $UL^{-1}$) was observed at 1.5% of glucose (Fig 3F). However, the biomass yield was maximum (0.09 $gL^{-1}$) at 0.5% glucose. In *M. jalaludinii* with the presence of glucose phytae production not only decreased significantly with the presence of glucose (Lan et al, 2002) but also inhibited with higher concentration (Lata et al. 2013). But the present study contradicts the reported ones.

The effect of phytate concentration was tested for phytase fermentation and it appeared that the phytate concentration positively influenced the phytase production. Optimum biomass (0.10$gL^{-1}$) and phytase production (48.88 $UL^{-1}$) observed at 0.5% Na-Phytate (Fig 3G). Phytase production decreased with the increase of sodium phytate concentration

above 2%, but at lower level it stimulates phytase synthesis (Soni and Khire, 2007). *A. vancoresmycina* S-12 showed maximum phytase production at 0.5% Na-phytate but inversely proportional with the increased concentration of sodium phytate, also with the increased amount of inorganic phosphorous.

The effect of nitrogen source on phytase production was studied using various inorganic nitrogen sources (maintaining nitrogen equivalence), in place of the recommended nitrogen of the selected basal medium. Among the nitrogen sources tested, the organism utilized all of them but growth and production varied with the source (Fig 3H). Maximum growth was observed with sodium nitrate (0.18 $gL^{-1}$), while production was optimum (71.65 $UL^{-1}$) with potassium nitrate. The use of ammonium sulphate (0.1%) as nitrogen was found to enhance phytase production by bacterial organisms (Yoon et al, 1996). In case of the isolate S-12 ammonium sulphate did not show significant effect but the production was maximum in potassium nitrate. However, growth was optimum with sodium nitrate but producing insignificantly. Potassium nitrate was also suitably used by *Nocardia* MB 36 for phytase production (Bajaj and Wani, 2013).

Vitamins are the important growth factors for every organism as it activate the enzymes for metabolism. While understanding the effect of vitamins, it was found (Table 1) that Thiamin (1 $\mu gL^{-1}$) played a significant effect on phytase production (87.61 $UL^{-1}$). But no other vitamin showed positive effect either in growth or in production. *Klebsiella pneumonia* SCTb2 shows a positive effect on phytase production, while the medium supplemented with Thiamin (Das *et al*. 2013).

Surfactants were also attempted (Table 2) for enhancement of enzyme production. It is observed that Tween 80 (0.5%) could influence phytase production maximally (176.32 $UL^{-1}$), but not the biomass (0.16 $gL^{-1}$). Also, it is found that SDS and Teepol increased the phytase production to certain extent but EDTA (1%) inhibited the production significantly (56.34 $UL^{-1}$). It is recorded further that the incorporation of surfactants in medium influences the extracellular phytase production (Pederson and Nielsen, 2000). The production of enzyme was maximum in the presence of surfactant in medium (Lealam and Gashe, 1994). However in the present study, production of phytase in presence of surfactant was found optimum at 120 h. Possible alteration of membrane permeability makes the positive influence of phytase production, since, Tween 80, Teepol, SDS, and EDTA are mainly reacting to increase the cell membrane permeability (Haq et al. 2005).

In conclusion, among the isolates, S12 was selected as potent and identified as *A. vancoresmycina*. The isolate S12 could produce extracellular phytase with common nutrients and does not require much energy input. Tween 80 as surfactant enhances enzyme production notably, which is close to 8 times at the initial experiment. It indicates the suitability of the isolate for easy maneuvering. Till date there is no report for phytase production by *Amycolatopsis* sp., thus, it carries enough promise for future exploration.

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