



# Enzyme Assisted Extraction of Phenolic from Pineapple waste by Bacterial isolates PS6 and VC14

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## Abstract

In the present study, from 8 soil samples, 23 isolates showed xylanase activity. Of the 23 isolates, PS6 showed maximum xylanase activity of 0.16 IU. Of which, PS6 were compatible with VC14 (cellulase producer). Co- culturing of PS6 and *Pseudomonas* VC14 showed maximum cellulase and xylanase activity production of 6<sup>th</sup> day. Different concentration of 1, 2 and 3% of pineapple waste in MSM broth was supplemented with xylanase, cellulase and both in consortia. It showed maximum antioxidant activity in consortia containing 3% waste. Therefore, it shows that phenolic content increases with increase in substrate concentration and the total phenolic content was high in broth containing both the strains.

## Keywords

Xylanase, Pineapple waste, antioxidants

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## INTRODUCTION

Secondary metabolites produced by microorganisms have a tremendous impact on society and are exploited for their antibiotics and pharmaceutical activities such as anticancer, antitumor, immunostimulatory and antioxidants [1]. The secondary metabolite that is commonly found in plants, mushrooms and fungi are Polyphenolics, it's been reported they possess multiple biological effects such as anti-inflammatory, anti-arteriosclerotic, antitumor, anti-mutagenic, anti-carcinogenic and cardio-protective actions including antioxidant activity [2].

It has been shown that free radicals are implicated in the pathogenesis of various human diseases such as arteriosclerosis, cancer, liver injury, skin damage, coronary heart diseases and arthritis [3]. Antioxidant acts as the defense factor against free radicals in the body. Some of the synthetic antioxidants such as butylated hydroxyanisole (BHA) and tert-butyl

hydroquinone (TBHQ) are usually used as food additives by the food industries for prevention for lipid peroxidation. However, their application has been limited because of possible toxic and carcinogenic components formed during their degradation, because of these health concerns it is necessary to find safer, more effective and economic natural antioxidants [4].

In spite of the abundant literature about the presence of phenolic antioxidants in fruits and vegetables, there is not enough information about the influence of variables on the extraction process. Many conventional extraction systems could diminish the activity of the phenolic antioxidants, which are very sensitive to specific solvents and high temperatures. This has impelled worldwide research for developing process to obtain natural antioxidant extracts under mild conditions. Application of low-cost commercial enzymes, used in the food industry, to degrade main components of vegetable cell walls

might ameliorate the phenolic compounds extraction without using denaturing conditions and thus preserving their antioxidant activity.

Recently the interest in xylanases has markedly increased due to the potential application in pulping and bleaching processes using cellulase free preparations, in food and feed industry, textile processes, enzymatic Saccharification of lignocellulosic materials and organic waste treatment [5]. Therefore, the present study aims at isolation of maximum xylanase activity showing microorganism from soil and determination of phenolic content of pineapple waste by enzyme-assisted extraction.

## MATERIALS AND METHODS

### Isolation of Xylanase Producing Microorganism

A total of 8 soil samples were collected from different places of Bangalore. Each soil sample is crushed thoroughly and filtered. One gram of soil samples were suspended in 100ml distilled water then mixed thoroughly. Mixtures were allowed to settle down then serially diluted up to  $10^{-2}$ . From  $10^{-2}$ , 100 $\mu$ l was taken and spread evenly over the surface of minimal salt agar supplemented (g/l)  $MgSO_4 \cdot 7H_2O$  1.5;  $K_2HPO_4$  1.5;  $NH_4NO_3$  1g,  $FeCl_3$  0.025g,  $CaCl_2$  0.02g/l, wheat bran 2g, agar 20g) with 1% wheat bran and incubated aerobically at 37°C for 24- 48 hours. On the basis of colony showing different morphology, colonies were selected and sub-cultured on nutrient agar plates and stored at 4°C [6].

### Screening for Xylanase

#### Congo red staining

The cultures isolated were inoculated into MSM containing 1% xylan. Incubated plates were flooded with 0.1% for 15 to 20 minutes, the stain was poured off and then flooded with 1N NaOH so as to destain for another 15 to 20 minutes and finally 1 N NaOH was also discarded. Colonies showing clear zones around the bacterial colonies indicates xylanase hydrolysis [7].

#### Gram's iodine staining

The plates were flooded with 1 % Grams iodine solution (0.133 g KI and 0.067 g Iodine dissolved in 20 ml distilled water) for 2-3 min to observe the xylanolytic activity. The formation of a clear zone of hydrolysis was observed around the colonies indicated xylanase degradation.

### Xylanase production

Erlenmeyer flasks containing 50 ml of minimal salt media containing 1% xylan was inoculated with 100  $\mu$ l of bacterial isolate. The flask was incubated at 30°C for 24h and centrifuged at 10,000 rpm for 10 min [8]. The resulting supernatant was called as crude

enzyme preparation. Uninoculated broth served as control.

### Estimation of xylanase activity

The xylanase activity of selected bacterial cultures were measured using 3, 5-dinitrosalicylic acid (DNS) method for determination of the amount of reducing sugars released [9]. Crude enzyme was added to 0.5 ml of 0.1 % xylan in 0.1 M phosphate buffer and incubated at 50°C for 10 min. After incubation, reaction was stopped by the addition of 1.5 ml of DNS reagent and boiled at 100°C in water bath for 15 min. Sugars liberated were determined by measuring absorbance at 575 nm. Xylanase production was estimated by using xylose standard curve (0-50 mg). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 $\mu$ mol of xylose per minute under standard assay conditions. Bacterial isolate with the highest activity was selected for optimization of xylanase production (PS6).

### Cross-streak test between VC14 and xylanase producer

The isolates showed maximum xylanase activity were tested for compatibility against VC14 (cellulase producer, isolated in our laboratory). Each of the co-inoculated strains was grown in nutrient agar streaked perpendicular to each other at 30°C for at least 3 days [10].

### Estimation of xylanase and cellulase enzyme activity

Erlenmeyer flasks containing 100 ml of minimal salt media containing 1% xylan and 1% CMC (carboxymethylcellulose) was inoculated with 100  $\mu$ l of suspension of PS6 and VC14 bacterial isolates. The flask was incubated at 37°C and at every 24 hrs of incubation. 5ml of incubated broth is withdrawn and centrifuged at 10,000 rpm for 10 min, [8] the resulting supernatant was called as crude enzyme extract. Uninoculated broth served as control. The xylanase activity of PS6 and cellulase activity of VC14 was measured using 3, 5-dinitrosalicylic acid (DNS) method for determination of the amount of reducing sugars released [9].

### Determination of antioxidant activity by total phenolic content

Fresh Pineapple waste was dried, powdered and then added to 100ml of minimal media and autoclaved at 121°C for 15min. the total phenolic content of the extract was determined by the Follin-Ciocalteu method [11]. Briefly, 200 $\mu$ L of crude extract (1mg/ml) was made up to 3ml with distilled water, mixed thoroughly and 0.5ml of Follin-Ciocalteu reagent was added after 3min, followed by the addition of 2ml of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 90

minutes, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of Gallic acid equivalent/ gram dry weight. Gallic acid was used as standard (0-500mg).the content of total phenolic in the extract was expressed as mg of Gallic acid equivalent/g of dry weight of sample.

#### Statistical analysis

All experiments were performed in triplicates and the data were expressed as mean  $\pm$  standard error.

## RESULTS AND DISCUSSION

### Isolation and Screening for Xylanase Producer

From 8 soil samples 30 bacterial isolates showed zone of clearance on Minimal Salt Media (MSM) containing 1% wheat bran. Of the 30 isolates, 23 isolates (77%) were positive by congo red staining and were positive for xylanase production (Figure 1) these results similar in the context of xylanolytic activity with that of the work of Avtar Singh *et al.* (2015). Shivaji *et al.* (2005) isolated *Bacillus arseniciselenatis* DSM 15340 and *Bacillus arsenicus* from a bore well located in the chakdah region of West Bengal, India. Ghoshal *et al.* (2015) isolated the xylan degrading bacteria from Punjab university campus by soil enrichment technique; they incubated the isolate in MSM, nutrient agar and czapek media. The production of the xylanase by the organism was checked and 43 bacterial, actinomycetes and fungal strains were obtained. One fungal isolate *Penicillium sp.* showed high xylanase activity of 21.65 U/ ml. Thus indigenous microbes could be a potential source of xylanase which can be explored for use in many applications. Wheat bran

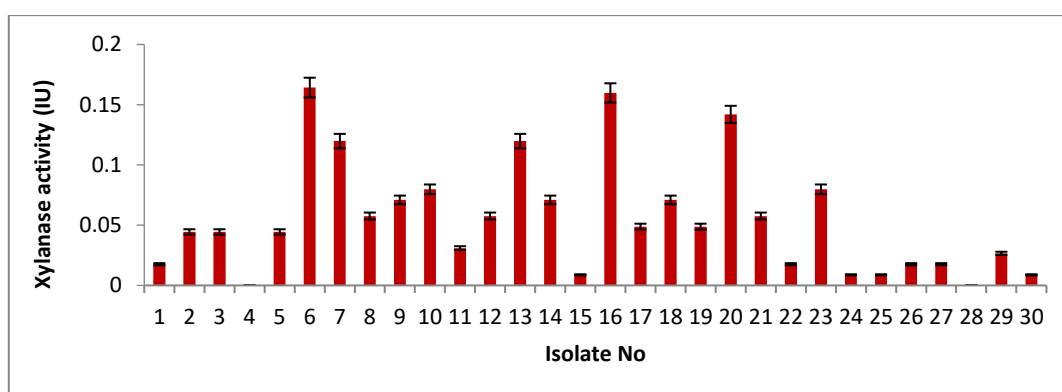
contains 54 % carbohydrates (pentoses and hexoses), 14 % protein, minerals, amino acids, and vitamins, which supports the growth of the bacterium and hence xylanase production.



**Fig. 1:** PS6 showing zone of clearance on addition of Congo red in xylan plates

### Xylanase activity

Microorganisms with positive results in the qualitative analyses were selected for determination of xylanase activity. Among the 30 isolates, 23 (77%) which showed positive result by Congo red staining, PS6 exhibited the highest extracellular xylanase activity (0.16 U/ml) (Figure 2) compared to other isolates. Avtar Singh *et al.* (2015) reported that a maximum xylanase activity of 368 nkat/mL in AVS13. Wang *et al.* (2012) in their study reported that after 4 days of inoculation, the extracellular xylanase activity was 717.7 U/ml. Mohammed Inuwa Jaafaru *et al.* [15] reported Isolates FD26 (1.35mg/ml) and FD18 (1.30mg/ml) produced the highest amount of reducing sugar when grown on oat-spelt xylan.

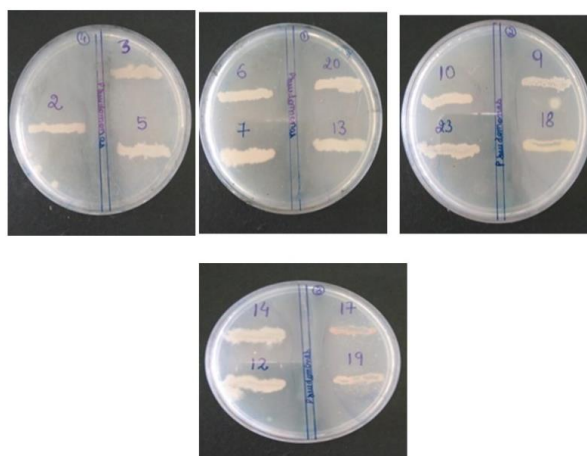


**Fig. 2:** Xylanase activity of the 30 bacterial isolates. Bars indicate mean of triplicates  $\pm$  SE

### Cross-streak test between VC14 and xylanase producer for compatible and incompatible combinations

Co-culturing of VC14 and 15 xylanase producer demonstrating maximum xylanase activity on

nutrient agar plates showed no growth inhibition at the center where the two strains crossed each other indicating that 15 xylanase producing strains are compatible to each other (Figure 3).

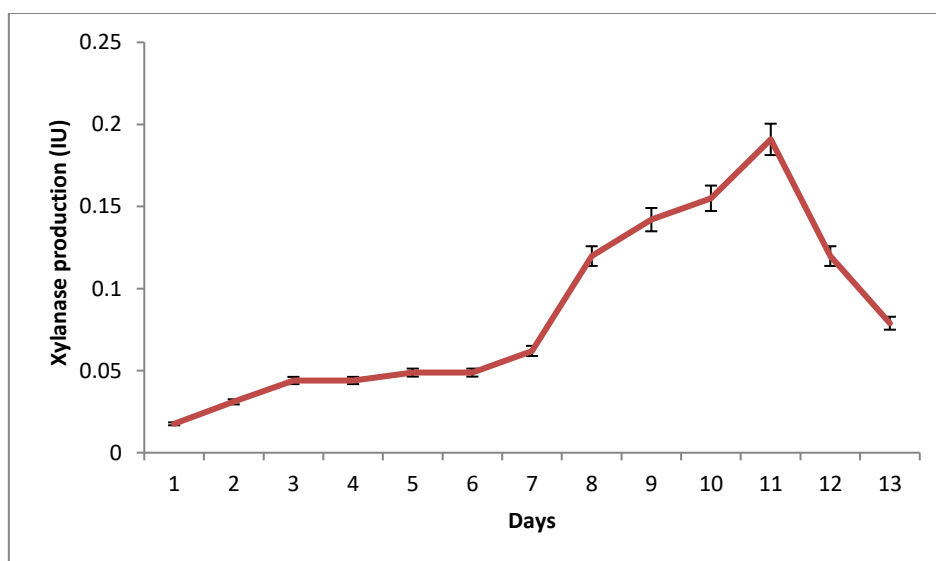


**Figure 3.** Cross-streak tests between co-inoculated strains. No inhibition zone was observed at the intersection between strains.

### Xylanase Activity in PS6

Among the 15 strains, PS6 showing highest xylanase activity (0.16 IU) and was found compatible with VC14 isolate was selected. The enzyme produced was monitored for 13 days at a time interval of 24hrs. Xylanase production started from 1<sup>st</sup> day onwards and reached its maximum on the 11<sup>th</sup> day (0.19IU).

From the 12<sup>th</sup> day onwards, the xylanase production decreased gradually (Figure 4). The production of xylanase is highly influenced by the availability of the nutrients in the substrates. The activity of xylanase increases when there is optimum nutrient present in the basal media as the availability of nutrient decreases activity of xylanase also decreases.

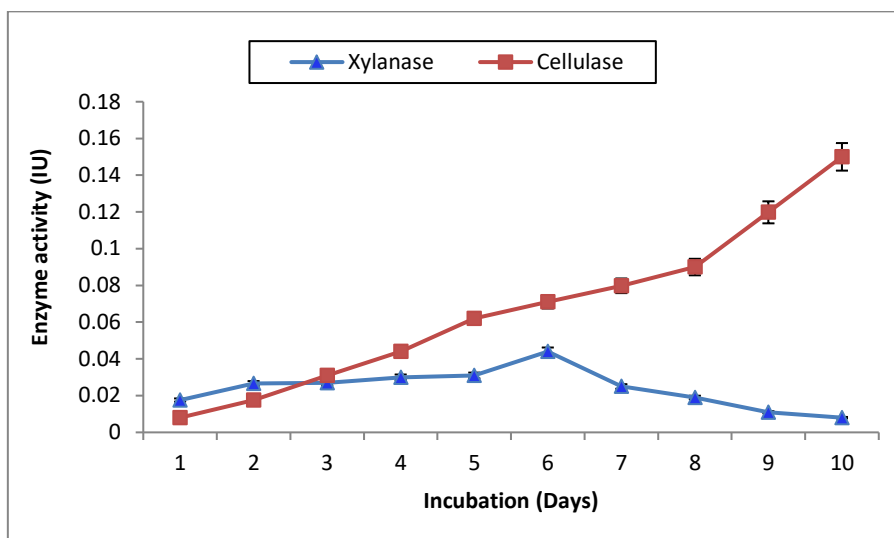


**Figure 4:** -Xylanase activity of PS 6. Bars indicate mean of triplicates  $\pm$  SE

### Xylanase and Cellulase Enzyme Activity

PS6 strain was earlier found to be compatible with VC14 was grown in consortia. It was reported that the production increases from the first 24hrs of incubation of both strains, on 6<sup>th</sup> day PS6 showed the

maximum growth of 0.044IU. PS6 growth gradually decreases after 6<sup>th</sup> day whereas VC14 was stable till 7<sup>th</sup> day and was reported highest on 10<sup>th</sup> day 0.15IU (Figure 5).



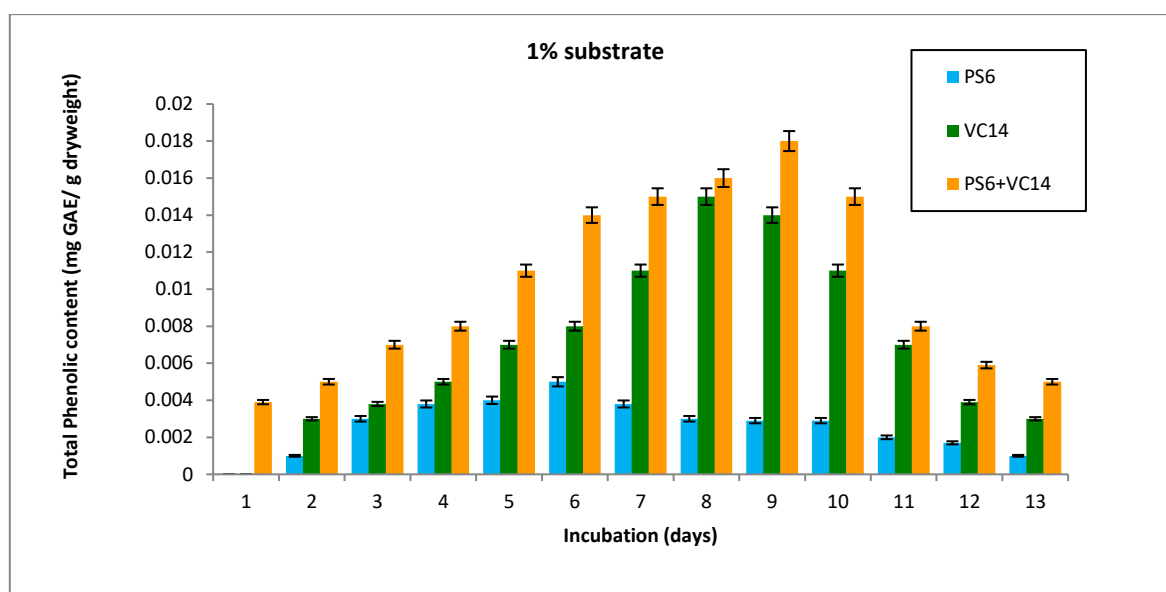
**Figure 5:** - Optimum incubation period for enzyme production by VC14 and PS6 in MSM medium supplemented with xylan and CMC. Bars indicate mean of triplicates  $\pm$  SE.

### Estimation of Phenolic Content

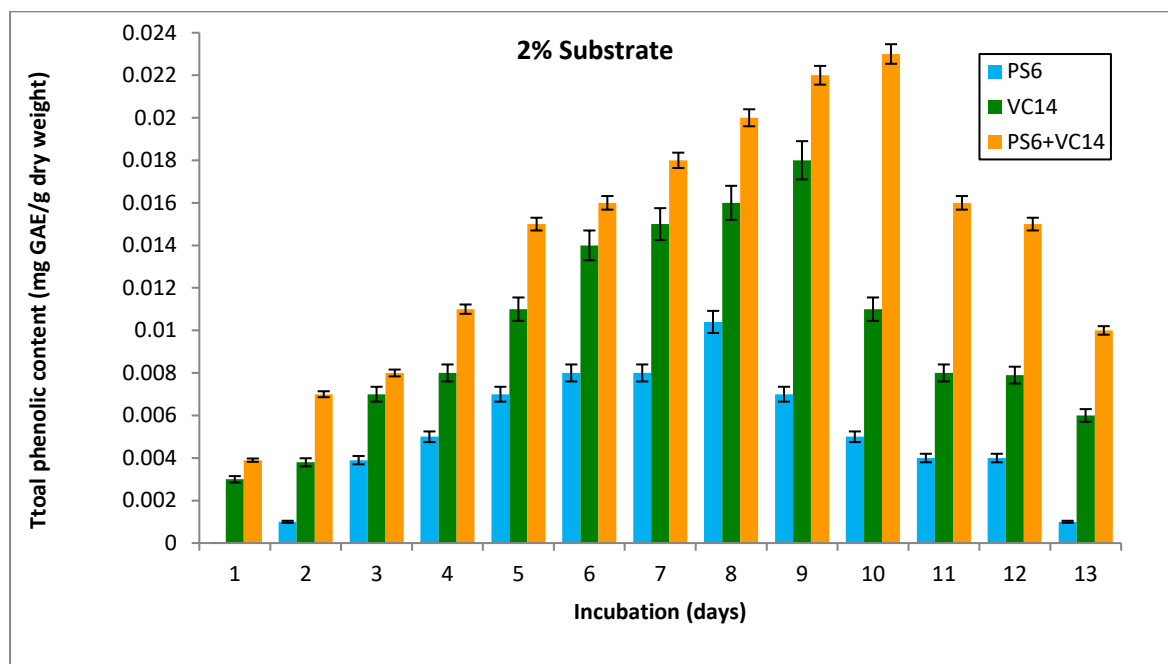
Polyphenols are very important fruit constituent due to their antioxidant activity, their property of chelation of redox-active metal ions, inactivation of lipid free radical chains and prevention of hydroperoxide into oxyradicals [16]. Phenolic content can be used as indicator of antioxidant activity and preliminary screening for antioxidant activity in any natural source [17].

In the present study total phenolic content as a measure of antioxidant potential using different concentration of pineapple waste as substrate was measured. Pine apple waste (3%) treated with PS6

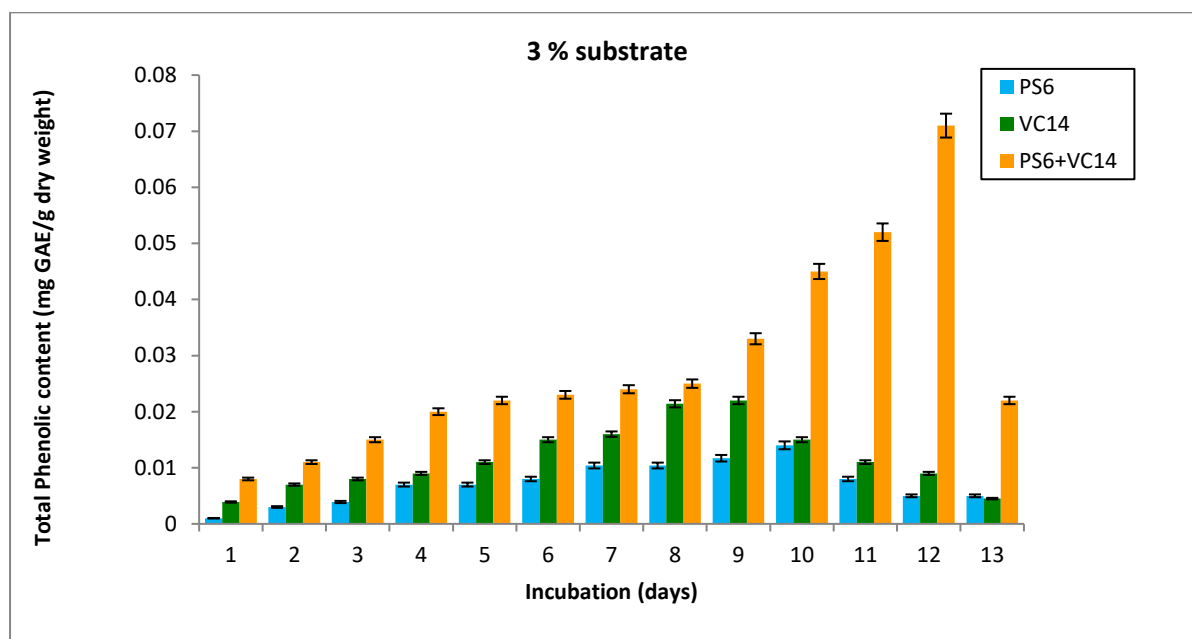
and VC14 exhibited highest phenolic content 0.071mg GAE/ g dry weight, whereas waste treated with VC14 showed phenolic content of 0.022mg GAE/g dry weight and waste treated with PS6 showed phenolic content of 0.014 mg GAE/g dry weight, low in comparison to PS6-VC14 consortia (Figure 6,7,8). Rashad *et al.* (2016) reported the phenolic content of the methanolic extract with various concentrations, the highest phenolic content exhibited was at 8mg/ml of 120 mg GAE /100 g dry weight. Kim *et al.* (2016) have reported the total phenolic content of 6.4-7.8 mg GAE/g in the five rice cultivators grown in china.



**Figure 6:** Total Phenolic content in 1% concentration of Pineapple waste. Bars indicate mean of triplicates  $\pm$  SE



**Figure 7:** Total Phenolic content in 2% concentration of Pineapple waste. Bars indicate mean of triplicates  $\pm$  SE.



**Figure 8:** Total Phenolic content in 3% concentration of Pineapple waste. Bars indicate mean of triplicates  $\pm$  SE.

These values are in broad ranges reported in the literature, the concentration and type of the phenolic content in the fruits and fruit co-products depends on several factors; differences in varieties, ripeness and season, environmental factors such as soil type and climate, genetic factors and processing and extraction methods [18]. The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent

used for the extraction process. Furthermore, solvent polarity plays a key role in increasing phenolic solubility [19].

## CONCLUSION

In this research study, main focus was on isolation of maximum xylanolytic activity showing microorganism in soil which will further help in conversion of xylose present in agricultural/



municipal (organic) waste into other useful products. Disposal of these wastes is expensive due to high cost of transportation and a limited availability of landfills they are unscrupulously disposed causing concern as environmental problems. These microorganisms play an important role in the biosphere by reducing complex polymer xylose into various economically important products. Enzyme assisted extraction as an alternative method can be considered for extraction of useful products from waste.

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