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# Biosynthesis of Zinc Nanoparticles and Its Effect on Enzymes Production by *Bacillus subtilis* and *Pseudomonus flourescens* Using Different Agricultural Wastes

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**Abstract** Biological synthesis of zinc nanoparticles is an environmental eco-friendly.*Bacillus subtilis* and *Pseudomonas fluorescens* which has been isolated from healthy beans plants and identified in plant pathology department at ( NRC ) were used for production of pectinase and chitinase enzymes in media containing different agriculture wastes and zinc nitrate to initiate nanoparticles synthesis. Pomegranate peel and Colocasia peel were the most promising wastes in zinc nanoparticles synthesis 100, 200 nm as proven by Transmission electron microscopy (TEM) and UV visible spectral analysis. The effectiveness of different agricultural wastes on the linear growth of green bean root rot fungi *in vitro* was investigated. Also, Pomegranate peel and Colocasia peel were the most effective on reducing linear growth of all pathogens. And enzymes activity from two bacterial strains was used different agricultural wastes as substrate and its antagonistic effects on pathogenic fungi.

**Keywords:** *B.subtilis*, *P.fluorescens*, zinc nanoparticles, enzymes, Agricultural wastes

## Introduction

*Bacillus subtilis* and *Pseudomonas fluorescens* were isolated from rhizosphere of healthy bean plants and identified in Plant Pathology department at (NRC). Soil born fungal strains (*Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani, Macrophomina phaseolina* and *Sclerotium rolfsii) which* are the causative agents of bean damping-off and root-rot diseases, were isolated from bean roots that showed the disease symptoms (El-Mohamedy *et al.*, 2015 and 2017).

Agriculture is an important profitof most developing countries. Modern technology can be used for helping agriculture to boost the yield. Nanotechnology is the application ofscience andtechnology to controlmatter at the molecular level, the applications of nano-technology in agriculture also

include fertilizers to improve plant growth and yield, sensors for monitoring soil quality and pesticides for pest and disease management (Khabat *et al*., 2011).

Nanotechnology has the ability to increaseplant protection, food quality, global foodproduction, detection of plant and animal diseases, monitoring of plant growth and reduction of waste for "sustainable amplification" Nanotechnology is the ability for controlling shape, size, at nanometer scale for designing, application of characterization and structure of it (Sastry *et al*., 2003 ; Mansoori, 2005; Sonkaria *et.al*., 2012).

Nanoparticles are the building blocks of nanotechnology which play an important role in agricultural biocontrol, pharmaceutical and biotechnological industrial applications. Nanoparticle was prepared to increase benefit of green synthesis for decreasing cost and safe strategies of environment (Abdallah *et al.*, 2016). Biosynthesis of metal nanoparticles is considered a new technique for nontoxic chemicals, resolve environmental problems, creation of renewable materials from yeast, fungi bacteria, and plant extract (Bhattacharya and Rajinder, 2005 and Gruère *et al*., 2011). Nanoparticles produced has high surface area, structures unusual size and crystal which make an excellent stability or long shelf life with organic antimicrobial agents,Kolodziejczak-Radzimska*et.al.,* 2014.Metal nanoparticles are deeplystudied due to their exclusive optical, electrical and catalytic properties (Rafiea *et al*., 2012).

The aim of this work was to study enzymes activity from two bacterial strains of *Bacillus subtilis and Pseudomonas fluorescens* using different agricultural wastes as substrate and its antagonistic effects against some pathogenic fungi, moreover production of zinc bio nanoparticles was invistagated.

## Materials and methods

*Bacillus subtilis and Pseudomonas fluorescens* were isolated from rhizosphere of healthy bean plants and identified in Plant Pathology department at (NRC). Soil borne fungal strains (*Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani, Macrophomina phaseolina* and *Sclerotium rolfsii) which* are the causative agents of bean damping-off and root-rot diseases, were isolated from bean roots that showed the disease symptoms. The fungi in pure culture were identified after pathogenicity test according to the keys given by Barnett and Hunter (1972) and Nelson *et al*. (1983). Cultures of bacteria were kept on nutrient agar media slants and kept at 4◦C.

### *Substrates*

Different agricultural wastes (Moringashell ,Soybean straw, Corncobs,Olive mill waste, Cottonstack, Wheat bran, wheat straw, rice straw, Pomegranate peel and Colocasia esculenta peel (20 gm/l) were used as sole carbon source and tested for antifungal activity against pathogens and effect on production of chitinase and pectinase enzymes. All wastes were washed, dried at 70 ℃in oven and cut into small pieces before use.

### *In vitro evaluation ofantifungal activity of different agriculture waste*

To determine the antifungal activity of different agriculture wasteagainst fungal phytopathogens species, treatments were PDA plates with different agriculture wasteadded into the medium and autoclaved. Disks from each isolate of pathogens (5 mm. in diameter) were inoculated on the center of PDA medium three replicates were used for each isolate. The inoculated plate with pathogens without wastes were used as control and incubated for 7 days at 28c. The linear growth of pathogens was recorded.

### *Fermentation media*

The ability of the two tested bacterial strains for chitinase and pectinase production was examined in media containing 50 ml of nutrient broth medium in addition of one gram from the different agricultural wastes. Erlenmeyer flasks was inoculated with 1 ml of *B.subtilis, P.fluorescens* separately and incubated at 28-30 ℃ for 48 h. in incubator shaker at 200 r.p.m.

### *Enzymes assay*

#### Pectinase

Pectinase activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; pH 5.5) and suitably diluted crude enzyme, was incubated at 50 ℃ in water bath for 30 min. The reaction was stopped with 1.0 ml dinitrosalycylic acid solution (Miller, 1959) after which the mixture was boiled for 10 min and cooled. The color was read at 540 nm using a spectrophotometer. A standard graph was generated using standard glucose solution. One unit of pectinase activity was defined as the amount of enzyme which liberated 1μm glucose per min.

**Chitinase**

Colloidal chitin was prepared from chitin powder (Sigma Co.). Determination of enzyme activity was carried out according to the method of Monreal and Reese (1969. Take one ml of 1 % colloidal chitin in citrate phosphate buffer (pH 6.6) in test tubes. One ml of culture filtrate was added and mixed by shaking. Tubes were incubated in a water bath at 37 ℃ for 60 minutes, then cooled and centrifuged before assaying. Reducing sugars were determined in 1 ml of the supernatant by 3,5-dinitrosalysilic acid (DNS). Optical density was measured at 540 nm. The colloidal chitin suspension was adjusted to pH 7.0 with 1 N NaOH and re-centrifuged. The pelleted colloidal chitin was stored at 4 ℃ until used.

***Preparation of zinc nitrate nanoparticles and enzymes assay***

The same fermentation media contained Pomegranate peel and *Colocasia esculenta* peel were carried out by replacing sodium nitrate 2.0 g/l by the same weight of zinc nitrate,and incubated by the two tested strained under the same conditions,thenenymes activity was determined.

***Characterization of zinc nanoparticles***

**UV visible spectral analysis**

The bio reduction of zinc in suspension was observed by ultraviolet-visible spectroscopy (UV-Vis) of the solution between 200 and 500nm by using Perkin-Elmer LAMBDA 35 UV-Vis spectrophotometer (USA).

**Transmission electron microscopy (**TEM)

For the confirmation of size and shape, (TEM) measurements were carried out using drop coating method in which a drop of solution containing nanoparticles was placed on the carbon-coated copper grids and kept under vacuum desiccation till dryness. TEM and high-resolution (HR)-TEM micrographs of the sample were taken using the JEM-2100F TEM instrument. The instrument was operated at an accelerating voltage of 200 kV.

***Statistical Analysis***

All data were subjected to statistical analysis according to the procedures reported by Snedecor and Cochran (1982).

## Results

Results in Table 1 indicated that pomegranate peel and rice straw the most effective waste against all pathogenic fungi given 100% reduction in linear growth, also as well as *Colocasia esculenta* reduced linear growth *of F.solani and M.phaseolina 100%, F.*oxysporum 93.8%, and 77.8% in case of *R.solani* and *S. rolfsii* compared with other waste as well as control .

On the other hand cotton stack, moringa, soybean and wheat bran wastes have no effect on the tested pathogens. Meanwhile olive mill waste, wheat straw and saw dust have moderate effect on all pathogens.

**Table 1.** Effect of different agricultural wastes on the linear growth of green bean root rot fungi on PDA in vitro.

| **Agricultural wastes** | ***F.solani*** | | ***F.oxysporum*** | | ***R.solani*** | | ***S.rolfsii*** | | ***M.phaseolina*** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | L.G. | R.% | L.G. | R% | L.G. | R% | L.G. | R% | L.G. | **R%** |
| Colocasia esculenta | 0.0c | 100 | 1.5c | 98.3 | 2.0c | 77.8 | 2.0c | 77.8 | 0.0c | ***100*** |
| Cotton stack | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | ***0.0*** |
| Moringa | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | **0.0** |
| Olive mill waste | 30.0b | 66.7 | 35.0b | 61.1 | 20.0b | 77.8 | 25.0b | 72.2 | 40.0b | **55.6** |
| Pomegranate | 0.0c | 10 | 0.0c | 100 | 0.0c | 100 | 0.0c | 100 | 0.0c | **100** |
| Rice straw | 0.0c | 100 | 0.0c | 100 | 0.0c | 100 | 0.0c | 100 | 0.0c | **100** |
| Sawdust | 3.0c | 66.7 | 3.5c | 61.1 | 40.0b | 55.6 | 40.0b | 55.6 | 50.0b | **44.4** |
| Soybean | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | **0.0** |
| Wheat bran | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | 0.0 | 90.0a | **0.0** |
| Wheat straw | 55b | 38.9 | 90.0a | 0.0 | 90.0a | 0.0 | 80.0a | 11.1 | 65b | **27.8** |
| Control | 90.0a | ---- | 90.0a | ---- | 90.0a | ---- | 90.0a | - | 90.0a | **-----** |

For each column, means followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Results in tables (2, 3) showed that when different agricultural wastes were added to the culture media marked changes in the activity of pectinase and chitinase enzymes were detected, Pomegranate peel and *Colocasia esculenta* greatly affected the enzymes production. Pomegranate peel give the highest activity of pectinase enzyme about42.6 U/ml by *P.fluorescens* and 61.8 U/ml for *Bacillus subtilis,* followed by *Colocasia esculenta* which give pectinase activity 26.8 U/ml by *P.fluorescens* and 41.6 U/ml for *Bacillus subtilis* .In case of chitinase production Pomegranate peel followed by *Colocasia esculenta* also achieved the activity of enzymes by the two tested bacterial strains giving 17.42,17,3 (U/ml) with *P.fluorescence* and 16.31,12.76 (U/ml)with *bacillus subtilis.*

**Table 2.** Effect of supplemented nutrient broth medium with different agricultural wastes on pectinase production by*P.fluorescens* and *B subtilis*

| Agricultural wastes | Pectinase activity (U/ml) | | | |
|---|---|---|---|---|
| | *P.fluorescens* | Change % | *B.subtilis* | Change % |
| **Control** | 10.91a | ---- | 8.71a | ---- |
| **Moringa** | 8.27a | 75.8 | 28.90c | 331.8 |
| **Soybean** | 12.91a | 118.3 | 20.28b | 232.8 |
| **Rice straw** | 11.70a | 107.2 | 21.44b | 246.2 |
| **Saw dust** | 16.36b | 149.95 | 22.87b | 262.6 |
| **Olive mill** | 24.13c | 221.2 | 24.75b | 284.2 |
| **Wheat straw** | 10.42a | 95.5 | 22.11b | 253.8 |
| **Wheat bran** | 22.45c | 205.77 | 12.30a | 141.21 |
| **Cotton stack** | 23.28c | 46.82 | 26.30c | 301.95 |
| **Pomegranate peel** | 42.68d | 391.2 | 61.80d | 709.52 |
| **Colocasia esculenta** | 26.82d | 245.8 | 41.65d | 478.2 |

For each column, means followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

**Table 3.** Effect of supplemented nutrient broth medium withdifferent ricultural wastes on Chitinase production by *P.flourescens*and *B.subtilis*.

| Agricultural wastes | Chitinase activity (U/ml) | | | |
|---|---|---|---|---|
| | *P.flourescens* | Change % | *B.subtilis* | Change % |
| **Control** | 6.39a | ---- | 4.84a | ---- |
| **Moringa** | 8.13a | 0.013 | 8.59a | 1.77 |
| **Soybean** | 12.11b | 1.89 | 5.94a | 1.22 |
| **Rice straw** | 5.33a | 0.83 | 7.64a | 1.58 |
| **Saw dust** | 9.53a | 1.49 | 9.56ab | 1.98 |
| **Olive mill** | 16.76b | 2.6 | 10.69bc | 2.21 |
| **Wheat straw** | 5.33a | 0.83 | 6.47a | 1.34 |
| **Wheat bran** | 12.50b | 1.95 | 10.11bc | 2.09 |
| **Cotton stack** | 11.76b | 0,02 | 9.56ab | 1.98 |
| **Pomegranate peel** | 17.42c | 2.72 | 16.31c | 3.37 |
| **Colocasia esculenta** | 17.30c | 2.70 | 12.76c | 2.64 |

For each column, means followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Comparison between enzymes production in fermentation culture of free and in presence of nano-zinc by *B. subtilis* and *P. flourescens* formulated on pomegranate peel and colocasia esculenta. From the above results colocasia esculenta and pomegranate peel were the most suitable agricultural wastes for production of chitinase and pectinase and reduce the linear growth of soil born fungi of bean plant, so the two wastes were selected to be added in fermentation media containing Zinc nitrate as a source for nanoparticle production and studying the effect of Zinc nanoparticles on chitinase and pectinase production using the two organisms *B.subtilis* and *P.flourescens*.

Results in table 4 showed that , maximum chitinase activity was produced by *P. flourescens* using colocassia esculenta as carbon source produced 114.85 U/ml,while pectinase reached its maximum activity by *B.subtilis*grown in fermentation medium containing pomegranate produce (95.1U/ml). (Mandal *et al.*, 2006) proved that cell-free culture supernatants of five psychrophilic bacteria *Phaeocystis antarctica*, *Pseudomonas proteolytica*, *Pseudomonas meridiana*, *Arthrobacter kerguelensis* and *Arthrobacter gangotriensis* and two mesophilic bacteria *Bacillus indicus* and *Bacillus cecembensis* have been used to biosynthesize silver NPs (~6–13 nm). The microbial cell reduces metal ions by use of specific reducing enzymes likeNADH-dependent reductase or nitrate dependent eductase. The culture supernatants of Enterobacteriaceae (*Klebsiella pneumonia*, *E. coli and Enterobacter cloacae*) also rapidly synthesized silver nanoparticles by reducing Ag+ to Ag0.

**Table 4.** Comparison between enzymes production in fermentation culture of free and in presence of nano-zinc by *B.subtilis* and *P.flourescens* formulated on pomegranate peel and colocasia esculenta.

| **Treatments** | **Chitinase activity(U/ml)** | **Pectinase activity(U/ml)** |
|---|---|---|
| *B. subtilis* + Pomegranate | 16.31 | **15.2** |
| *B. subtilis* + Colocasia | 12.75 | **11.4** |
| *B. subtilis* + Colocasia + zinc-nitrate | 103.27 | **95.1** |
| *B. subtilis* + Pomegranate + zinc-nitrate | 78.76 | **80.69** |
| *B. subtilis* Control( free) | 23.21 | **41.89** |
| *P. flourescens* + Pomegranate | 17.42 | **22.0** |
| *P. flourescens* + Colocasia | 17.30 | **18.4** |
| *P. flourescens* + Colocasia + zinc-nitrate | 114.85 | **66.05** |
| *P. flourescens* + Pomegranate + zinc-nitrate | 74.65 | **81.45** |
| *P. flourescens* Control(free) | 40.85 | **40.15** |

### *Transmission electron microscope*

Transmission electron microscope (TEM) was used for detection of purity and particle size *B. subtilis*and *P. flourescens* were adjusted in fermentation media containing Colocassia or Pomegranate waste as carbon source. Results in figure (1, 2) showed that spherical and granule morphology of zinc nanoparticles were presented specially at the particle size were 100 and 200 nm. Zinc nanoparticles produce spherical and unique distribution particles.

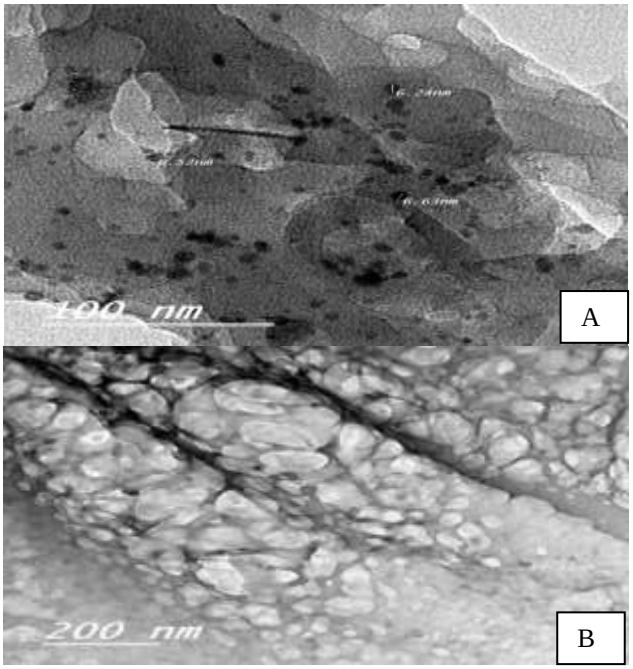


**Figure 1.** TEM of zinc nanoparticles of *P. flourescens* on Pomegranate (A) and Colocasia waste (B).

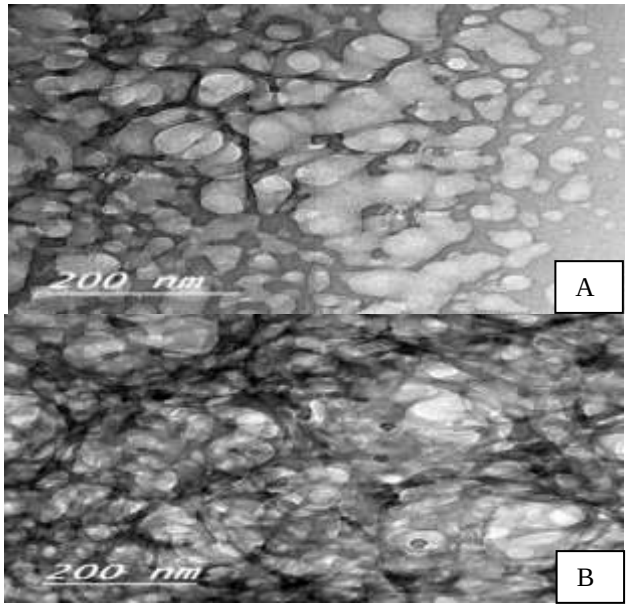


**Figure 2.** TEM of zinc nanoparticles of *B.sutilis* on Pomegranate (A) and Colocasia waste (B).

***UV-Vis spectroscopy***

Biosynthesis of zinc nanoparticles were indicated by visual observation change in culture filtrate of *B. subtilis* and *P. flourescens*, the color of the bacterial cultural filtrates was change from colorless to turbid yellow clearly indicates the formation of zinc nanoparticles depending on the proteins in cultural media represented in pectinase and chitinase which play important role in reduction of zinc ions to zinc nanoparticles takes place extracellularly. The stability and formation of the reduced zinc nanoparticles in bacterial cultural filtrate were monitored by UV-visible spectroscopy. In the current study, the absorption spectra of zinc nanoparticles synthesized by *P. flourescens* and *B. subtilis* with pomegranate peel as agricultural waste showed maximum surface Plasmon absorption band at peak ≈ 380 nm represented in Figure (3 &5), while the UV-Visible spectroscopy of zinc nanoparticles synthesized by *P. flourescens* and *B. subtilis* with colocasi*a* peel agricultural waste showed maximum surface Plasmon absorption band at peak ≈ 270 nm represented in Figure (4 &6).

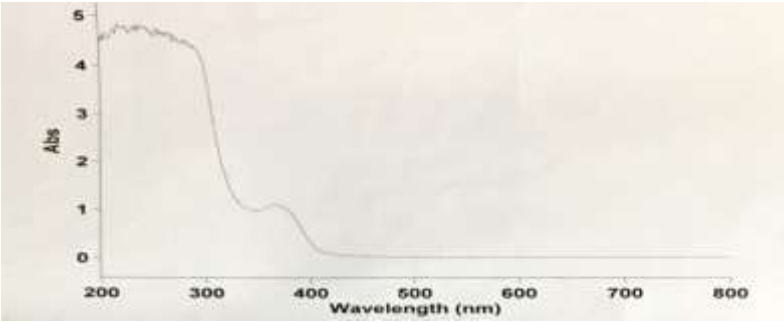


**Figure 3.** UV-Vis spectra of nanozinc produced by *P. flourescens*in prescence of pomegranate

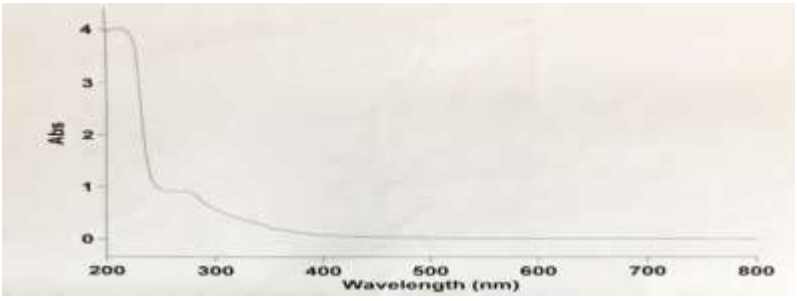


**Figure 4.** UV-Vis spectra of nanozinc produced by *P. flourescens*in prescence ofcolocasia esculenta

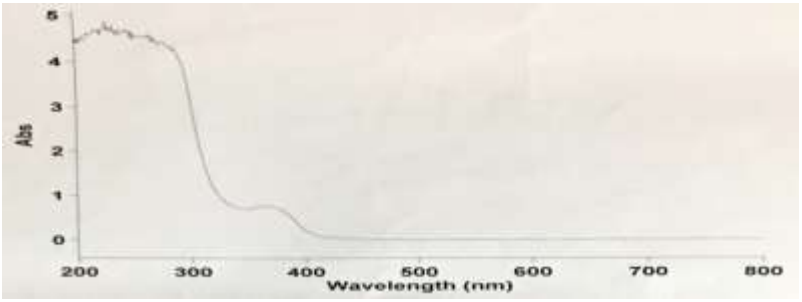


**Figure 5.** UV-Vis spectra of nanozinc produced by *B. subtilis* in prescence of pomegranate

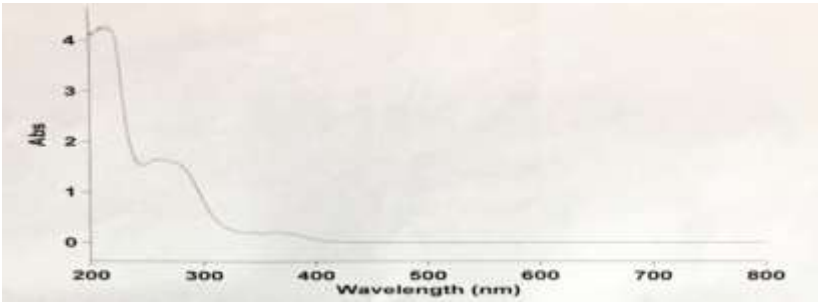


**Figure 6.** UV-Vis spectra of nanozinc produced by *B.subtilis*in prescence ofcolocasia esculenta

## Discussion

El-shami and Atalla (2015) found that maximum invertase activity produced when *Aspergillus terrus* was grown on a culture media contain 40 g/l of pomegranate peel. Nema *et al.* (2015) found that corn husks 0.5% (w/v) was the best waste for production of cellulase in submerged fermentation using *Bacillus* cereus strains isolated from local Syrian soils. Sarika Chaturvedi *et al.* (2015) enhanced xylanase production by adding wheat bran 40g/l to the fermentation media of wood decaying bacteria *Bacillus licheniformis*.

With the addition of piperitone, silver ion reduction was partially inhibited, which showed the involvement of nitroreductase enzymes in the reduction process (Kanan *et al.,* 2010). As a result, the spherical and granule morphology of zinc nanoparticles were presented at the particle sizes of 100 and 200 nm. Zinc nanoparticles produced spherical and unique distribution particles (Aliaa *et al*., 2015). The variations in the peak absorbance of SPR 270 to 380 nm refer to the green synthesis of zinc nanoparticles with poly-dispersed narrow sizes particles in colloidal solution. Other reports found that the

characterization peak of zinc nanoparticles is lied around wavelength of 320-390nm (Vennila *et al.*, 2017; Fouda *et al.*, 2018).

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