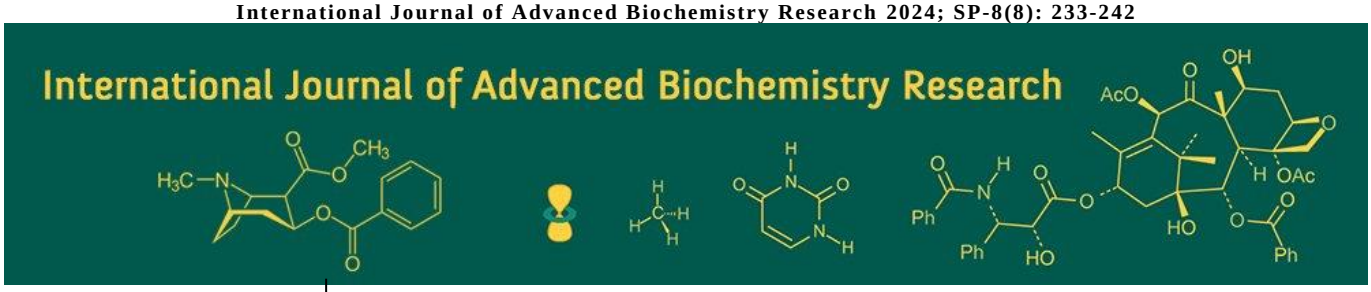


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# Exploring agricultural soils of the Saurashtra region for entomopathogenic bacteria and fungi: Isolation, characterization and phylogenetic analysis

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**Abstract**
The purpose of this research is isolation of entomopathogenic fungi and bacteria that are natural to the soil. Microorganisms' morphological and biochemical characteristics. qualitative examination of possible isolates to determine whether they produce any enzymes. Using the 16S and 18S RNA techniques, powerful isolated bacteria were identified molecularly. Testing and analysis of possible isolated bacteria and fungus *in vitro* against pests, bacteria, and fungi. Study was conducted at Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India during 2018-19. Iidentify and characterize the isolates using cultural and biochemical tools, and study their qualitative and quantitative production of biotechnologically important enzymes, plant growth promoting characteristics, to confirm entomopathogenic isolates and PCR based molecular identification to confirm the species of isolates. Seventy two bacterial and thirty five fungal colonies were isolated from thirty five soil samples collected from agricultural soils of different research station. Based on pesticidal activity, different enzyme activity and plant growth promoting characteristics, fifteen isolates give positive results. ive out of twelve bacterial isolates belongs to genus *Bacillus* while four isolates belongs to genus *Pseudomonas* and remaining three isolate belongs to genus *Enterobacter* and one out of three fungal isolates belongs to genus *Verticillium* while another one isolates belongs to genus *Metarhizium* and remaining one isolate belongs to genus *Beauveria*. The fifteen selected entomopathogenic bacterial and fungal isolates studied were identified by phylogenetic analysis of sequences obtained from partial 16S and 18S rRNA gene sequencing.

**Keywords:** Isolation, characterization, entomopathogenic bacteria, soil, molecular identification, biochemical characteristics

## Introduction
Entomopathogenic microbes are natural enemies of insects and arachnids and contribute to the regulation of their host populations. In agriculture, the microbes have been observed to cause mortality in pest populations and several microbial species have been investigated for their potential as biological control agents (Eilenberg *et al*., 2000) [3]. Therefore knowledge of the community of natural enemies in the agroecosystem as well as the effect of the agronomical practices on these organisms is essential to use a conservation biological control strategy. Thus sampling of host individuals can reveal information about host range and prevalence of fungal species as pathogens in natural host population. However, several entomopathogenic microbes only occur as infections in living hosts for a relatively short period of time during their life cycle. The remainder of the life cycle these species presumably lurk as dormant structures in the soil in the vicinity of the dead host cadaver. Further dispersal from cadavers as focal points presumably occur due to weather (rain and wind), soil manipulation and also insect activity (Meyling *et al*., 2006) [9]. Entomopathogenic fungi that commonly used are *Beauveria bassiana*, *Verticillium lecanii* and *Metarhizium anisopliae*. *M. anisopliae* can infect several insects of the Order Coleoptera, Lepidoptera, Hemiptera and Isoptera. There still numerous number of potential local entomopathogenic which is come from soil that can infect to insect. The aim is to conduct a research about isolation, identification and pathogenicity test entomopathogenic microbes.

Entomopathogenic bacteria are unicellular prokaryotic organisms having size ranging from less than 1 µm to several µm in length. Bacteria with rigid cell walls are cocci, rod-shaped and spiral while bacteria without cell walls are pleomorphic. The majority of bacterial pathogens of insect-pests occur in bacterial families Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae, and Micrococcaceae. These families of bacteria usually represent epiphytes or weak pathogens; however, some of them are highly virulent to their respective hosts.

Entomogenous fungi are potentially the most versatile biological control agents, due to their wide host range that often results in natural epizootics. An attractive feature of these fungi is that infectivity is by contact and the action is through penetration (Nadeau *et al*., 1996) [10]. These fungi comprise a heterogenous group of over 100 genera with approximately 750 species, reported from different insects. Many of these offer a great potential in pest management. The most important fungal pathogens are *Metarhizium spp.*, *Beauveria spp.*, *Nomuraea rileyi*, *Verticillium lecanii* and *Hirsutella spp. V. lecanii* (Zimm.) popularly called the "white holo" is known to cause mycosis in a number of insects belonging to the insect orders Homoptera, Coleoptera and Lepidoptera.

## Materials and Methods

An agricultural soil sampler was used to collect the soil samples from agricultural fields from a depth of 2 to 5 cm around the crop rhizospheres. The representative soil samples (approximately 200 g each) were collected from agricultural soils of different villages. A total of 50 soil samples were collected in sterile HDPE bags and brought to the laboratory. The samples were kept at 4 ℃ till processing.

### Morphological characterization

For morphological characterization, all cultures were plated on Nutrient agar/Potato Dextrose Agar plates and the plates were incubated at 35 ± 2 ℃ for 24 hrs and 28 ± 2 ℃ for 72 hrs in BOD incubator. Observations were taken from the growth on NA/PDA agar plates for morphological, cultural and microscopic characterization as described below.

### Microscopic characterization by gram's staining for bacterial isolates

Thick smear of all cultures were prepared on clean glass slides individually and heat dried for few seconds by passing over a burner. Smears were then stained with 1-3 drops of crystal violet stain and incubated at room temperature for 1 min. The crystal violet stains were then removed by gently washing with tap water. The smears were then covered with 1-3 drops of gram's iodine and incubated at room temperature for 30 to 60 sec. Smears were then decolorized with gram's decolorizer and counter stained with 1-3 drops of safranin and incubated at room temperature for 30 to 60 sec and washed gently with tap water. The slides were then dried in room temperature overnight and observed under light microscope.

### Biochemical characterization

Biochemical tests readymade kits available from HiMedia (HiPure Bacterial Identification Kit) were used. Individual bacterial suspension (50 µl @ $10^7$ CFU/ml) was inoculated in each well of test strips and allowed to incubate for 24 h at 35 ± 2 ℃. After incubation observations were recorded by applying appropriate reagents provided with the kit.

On the base of Morphological, Physiological, Biochemical Characters 15 microbes with highest entomopathogenic characters were selected and its enzyme assay, plant growth promoting characteristics and PCR based molecular identification were performed as per given procedure.

### Extracellular enzyme production test

#### Qualitative chitinase enzyme test

The qualitative chitinase enzyme production test was performed by modifying the standard method followed by Hsu and Lockwood, 1975 [5].

### Quantitative chitinase enzyme assay

For the measurement of chitinase activity, colloidal chitin was used as the substrate. The chitinase activity was then measured by Ruth *et al*., 2016 [15].

### Qualitative cellulase enzyme test

The qualitative cellulase enzyme production test was performed by modifying the standard method followed by Lay Mg Mg *et al.*, 2015 [8].

### Quantitative cellulase enzyme assay

The activity of cellulase was assayed using DNS method by Lay Mg Mg *et al.*, 2015 [8].

### Qualitative protease enzyme test

The qualitative protease enzyme production test was performed in a culture medium by Sadia *et al*., 2009 [16].

### Quantitative protease enzyme assay

The proteolytic activity was determined by caseinolytic method of (Kunitz, 1947) [7] with some modifications, using azocasein as a substrate.

### Phosphate solubilization capacity

#### Qualitative phosphate solubilization test in solid medium

Qualitative phosphate solubilization test in solid medium was carried out in Sperber's medium as per standard protocol followed by Nosrati *et al.*, 2014 [13]. To examine Pi solubilization capabilities, 10 µl of the bacterial and fungal suspensions (~$10^4$ CFU/ml) was spotted with the help of sterile inoculating needle onto the center of Sperber medium plate containing insoluble Pi. The Solubilization Index (SI) was determined by measuring the ratio of halo (clear zone) diameter (mm) and the colony diameter as per the formula followed by Nautiyal *et al*., (1999) [11]. And the Solubilization Efficiency (SE) was calculated by the formula followed by Nguyen *et al.*, (1992) [12].

### Quantitative phosphate solubilization test in liquid medium

Quantitative phosphate solubilization test in liquid medium was carried out in phosphate solubilization medium as per standard protocol followed by Krishnaraj and Dahale (2014) [6]. The amount of Pi (Inorganic phosphorus) released in the broth was estimated from three flasks each after incubation (DAI) in comparison with a set of uninoculated control. The graph of OD versus concentration of phosphate in µg was plotted for the standard and samples were compared to calculate P concentration.

**Nitrogen fixing capacity**

Moderate efficiency of $N_2$ fixing was observed in fifteen isolates obtained form the phyllosphere of different soil samples as evident from their growth in Jensen's medium. The readymade Jensen's agar media was obtained from HiMedia. Jensen's agar medium plates were then prepared by adding 1.5% agar to it to allow proper solidification. The plates were then inoculated by streaking the isolate culture onto the plates and incubated at 35 ± 2 °C for five days. The quantification of the nitrogen fixed was determined by the Microkjeldahl digestion and distillation method.

**Molecular identification**

**Preparation of stock solutions for reagents and buffers for DNA isolation**

The reagents and buffers used for DNA isolation were obtained along with the Qiagen's ready to use DNA extraction DNeasy® Blood and Tissue kit (250).

**16S rRNA amplification**

Bacterial isolate were identified using 16S rRNA sequences at Department of Biotechnology, Junagadh Agricultural University, Junagadh. Bacterial isolates were grown in MRS broth for 36 hours. The genomic DNA was extracted from the bacterial isolate culture (Himedia Bacterial DNA isolation kit). A fragment of the 16S rRNA gene was amplified using 16s universal primers. The 16s rRNA region of bacteria, including 16s Universal primer 005F (5′- TGG AGA GTT TGA TCC TGG CTC AG-3′) and 907r (5′-CCG TCA ATT CMT TTR AGT TT-3′) were amplified. The amplification was performed in 30 µl reaction volume with 0.1 mM of each dNTP and 100pmol of both forward and reverse primer. Veriti PCR (Thermo fisher) was programmed for initial denaturation at 94 °C for 4 min, and 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The amplification was completed with a final extension at 72 °C for 5 min. Further it was sequenced by ABI 3130 capillary sequencing. Partial 16S rRNA gene sequence of studied bacteria was analyzed with nucleotide BLAST search in Gene Bank of NCBI.

**18S rRNA amplification**

Fungal isolate were identified using 18S rRNA sequences at Department of Biotechnology, Junagadh Agricultural University, Junagadh. Bacterial isolates were grown in MRS broth for 36 hours. The genomic DNA was extracted from the bacterial isolate culture (Himedia Bacterial DNA isolation kit). A fragment of the 18S rRNA gene was amplified using 18s universal primers. The 18s rRNA region of fungi, including 18s Universal primer ITS-2F (5′-CGATGAAGAACGCAGCGAAATGCGAT-3′) and ITS-2R (5′- TCCTCCGCTTAGTATATGCTTAA -3′) were amplified. The amplification was performed in 30 µl reaction volume with 0.1 mM of each dNTP and 100pmol of both forward and reverse primer. Veriti PCR (Thermo fisher) was programmed for initial denaturation at 94 °C for 4 min, and 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The amplification was completed with a final extension at 72 °C for 5 min. Further it was sequenced by ABI 3130 capillary sequencing. Partial 18S rRNA gene sequence of studied bacteria was analyzed with nucleotide BLAST search in Gene Bank of NCBI.

**Results and Discussion**

A total of 35 soil samples were collected as the source of entomopathogenic microbial isolation from the rhizosphere of different crops and the details of their sampling varities and locations have been presented in the Table 1.

**Morphological characterization**

*In vitro* multiplications of bacterial isolates were carried out on Nutrient agar plates and the colonial characteristics were recorded in terms of size, shape, elevation, margin, texture, opacity and pigment. All seventy two isolates showed typical entomopathogenic bacterial like colonies on the medium after 24 hr of incubation.

*In vitro* multiplications of fungal isolates were carried out on Potato Dextrose agar plates and the colonial and microscopic characteristics were recorded in terms of Growth pattern, color, texture, No. of days for sporulation and Hyphae. All thirty five isolates showed typical entomopathogenic fungal like colonies on the medium after 4-6 day of incubation. The colony morphologies of the isolates ranged from dense to disperse, majority with small to medium in size with plane growth pattern. The colony color of the isolates included olive green and yellow, white, violet, yellow to light brown. Texture of the isolates like cottony and powdery, smooth cottony and flat powdery consistence with septate hyphae.

**Microscopic characterization**

Microscopic characterization of the isolates were carried out by gram's staining, spore staining and scanning electron microscopy for cellular morphological characterization. However, the scanning electron microscopic characterization was done only for the fifteen main isolates selected for biochemical and further different bioactive compound production parameters while the characterization by gram's staining was carried out for all the seventy two pre-selected isolates. Different isolates displayed different cell sizes and morphologies when viewed under the microscope. The cells ranged from coccus to short or thin long rod shape in single or pairs to bunchy type in organization.

**Biochemical characterization**

**Qualitative chitinase, cellulase and protease enzyme test**

The results of production of chitinase, cellulase and protease enzyme by the isolates. It is obvious that all fifteen isolates were capable of producing chitinase, cellulase and protease enzyme qualitatively tested in the respective enzyme production media as described in methods. These results shows that the isolates were capable of degrading the colloidal chitin, carboxymethyl cellulose and proteose peptone as substrates provided in the respective enzyme production tests.

**Quantitative chitinase assay**

The data pertaining to the results of quantitative chitinase enzyme assay as performed as described in methods. The standard curve obtained by plotting the varying concentration of N-acetylglucosamine against their corresponding absorbance at 540 nm. The determination of standard curve of GlcNAc for estimating the chitinase and specific activity. Chitinase enzyme activity shown by isolates ranged from 0.303 to 0.827 U $ml^{-1}$. The highest chitinase activity was observed in isolate B-8a while lowest in isolate B-4b (Table 2) (Fig. 1). The data of the results pertaining to chitinase enzyme and specific activity are in lines with the report of Chrisnasari *et al.* (2016) [2]. The ability of the isolates to produce chitinase enzyme suggest their ability to degrade chitin compounds as energy source. In most cases, chitinases from bacterial source are reported to act as a fungicidal compound.

**Table 1:** Details of soil sampling varieties and locations

| Sample No. | Crop rhizosphere | Varieties | Locations |
|---|---|---|---|
| 1. | Cotton | GJHV-522 | Cotton Research Station |
| 2. | Cotton | GJHV-544 | Cotton Research Station |
| 3. | Cotton | G. Cot 20 | Cotton Research Station |
| 4. | Cotton | GJHV-523 | Cotton Research Station |
| 5. | Soybean | Gujarat Soybean-1 | Oilseed Research Station |
| 6. | Cotton | GJHV-511 | Cotton Research Station |
| 7. | Soybean | Gujarat Soybean-3 | Oilseed Research Station |
| 8. | Sesamum | Gujarat Sesamum-2 | Oilseed Research Station |
| 9. | Sesamum | Gujarat Sesamum-3 | Oilseed Research Station |
| 10. | Sesamum | Gujarat Sesamum-4 | Oilseed Research Station |
| 11. | Sesamum | Gujarat Sesamum-10 | Oilseed Research Station |
| 12. | Castor | GCH-6 | Oilseed Research Station |
| 13. | Ground nut | TG-73 | Oilseed Research Station |
| 14. | Ground nut | GJG-31 | Oilseed Research Station |
| 15. | Ground nut | TG-37A | Oilseed Research Station |
| 16. | Soybean | Gujarat Soybean-1 | Sagadividi Farm |
| 17. | Soybean | Gujarat Soybean-3 | Sagadividi Farm |
| 18. | Pigeon pea | GJP-1 | Sagadividi Farm |
| 19. | Pigeon pea | GJP-1 | Sagadividi Farm |
| 20. | Ground nut | GJG-22 | Sagadividi Farm |
| 21. | Green gram | - | Pulse Research Station |
| 22. | Chickpea | Gujarat gram-1 | Pulse Research Station |
| 23. | Black gram | Gujarat-1 | Pulse Research Station |
| 24. | Black gram | Gujarat-1 | Pulse Research Station |
| 25. | Chickpea | GG 5 | Pulse Research Station |
| 26. | Chickpea | GJG 3 | Pulse Research Station |
| 27. | Green gram | Gujarat Green gram-4 | Pulse Research Station |
| 28. | Cowpea | Cowpea Gujarat-4 | Pulse Research Station |
| 29. | Kidney bean | Kidny bean Gujarat-1 | Pulse Research Station |
| 30. | Pearl millet | GHB-538 | Near Krishigadh |
| 31. | Ground nut | GJG-22 | Near Krishigadh |
| 32. | Wheat | LOK-1 | Wheat Research Station |
| 33. | Lady's finger | GJO-3 | Vegetable Research Station |
| 34. | Sponge gourd | GSG-1 | Vegetable Research Station |
| 35. | Ridge gourd | Pusa nasadar | Vegetable Research Station |

**Table 2:** Determination of chitinase and specific activity of isolates

| Sr. No. | Culture code | NAG released (mg/ml) | Enzyme activity (U/ml) | Specific Activity (U/mg) |
|---|---|---|---|---|
| 1. | B-4b | 0.124 | 0.303 | 15.922 |
| 2. | B-7a | 0.128 | 0.315 | 15.906 |
| 3. | B-8a | 0.345 | 0.827 | 15.984 |
| 4. | B-8b | 0.213 | 0.514 | 15.839 |
| 5. | B-9c | 0.170 | 0.413 | 15.849 |
| 6. | B-15c | 0.305 | 0.732 | 15.685 |
| 7. | B-17b | 0.322 | 0.771 | 15.845 |
| 8. | B-30a | 0.125 | 0.305 | 15.589 |
| 9. | B-32a | 0.128 | 0.313 | 15.666 |
| 10. | B-33a | 0.137 | 0.335 | 15.752 |
| 11. | B-33c | 0.140 | 0.341 | 15.586 |
| 12. | B-35b | 0.149 | 0.363 | 15.920 |
| 13. | F-7 | 0.141 | 0.344 | 15.867 |
| 14. | F-19 | 0.125 | 0.307 | 15.858 |
| 15. | F-28 | 0.149 | 0.363 | 15.841 |
| S.Em.± | | 0.003 | 0.0069 | 0.0857 |
| C.D. at 5% | | 0.0087 | 0.0199 | 0.2475 |
| C.V. % | | 2.92 | 2.72 | 0.94 |

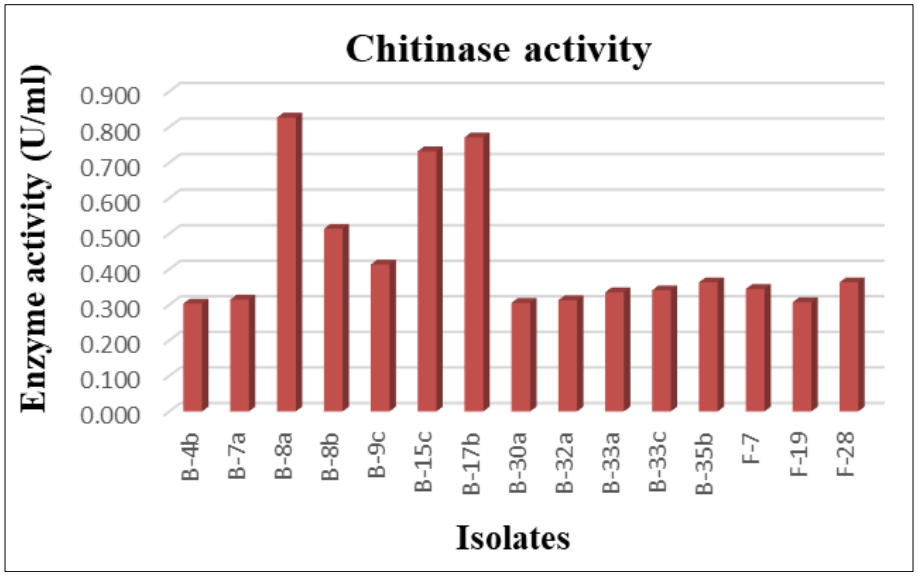


**Fig 1:** Chitinase activity of entomopathogenic isolates (U/ml)

**Quantitative cellulase assay**

The results of quantitative cellulase enzyme assay as performed as described in methods. The standard curve obtained by plotting the varying concentration of anhydrous glucose against their corresponding absorbance at 540 nm. The determination of standard curve of glucose for estimating the cellulase and specific activity. Cellulase enzyme activity shown by isolates ranged from 0.778 to 2.714 U $ml^{-1}$. The highest cellulase activity was observed in isolate B-32a while lowest in isolate F-28. On the other hand, the glucose content in isolates ranged from 2.550 to 7.336 mg $ml^{-1}$ (Table 3) (Fig. 2). The finding of these results pertaining to cellulase enzyme and specific activity are in agreement with the report of Lay Mg Mg *et al.* (2015) [8]. The ability of the isolates to produce cellulase enzyme suggest their ability to degrade cellulose, the most abundant organic compounds as energy source.

**Table 3**: Determination of cellulase enzyme activity of isolates

| Sr. No. | Culture code | Total glucose conc. (mg/ml) | Enzyme activity (U/ml) | Filter paper method (U/ml) |
|---|---|---|---|---|
| 1. | B-4b | 3.219 | 1.191 | 0.596 |
| 2. | B-7a | 2.129 | 0.788 | 0.394 |
| 3. | B-8a | 4.709 | 1.742 | 0.871 |
| 4. | B-8b | 3.984 | 1.474 | 0.737 |
| 5. | B-9c | 6.218 | 2.301 | 1.150 |
| 6. | B-15c | 4.794 | 1.774 | 0.887 |
| 7. | B-17b | 2.704 | 1.000 | 0.500 |
| 8. | B-30a | 4.918 | 1.820 | 0.910 |
| 9. | B-32a | 7.336 | 2.714 | 1.357 |
| 10. | B-33a | 6.024 | 2.229 | 1.115 |
| 11. | B-33c | 5.337 | 1.975 | 0.987 |
| 12. | B-35b | 5.590 | 2.068 | 1.034 |
| 13. | F-7 | 2.550 | 0.944 | 0.472 |
| 14. | F-19 | 3.057 | 1.131 | 0.565 |
| 15. | F-28 | 3.672 | 1.359 | 0.679 |
| S.Em.± | | 0.0632 | 0.0258 | 0.0169 |
| C.D. at 5% | | 0.1825 | 0.0745 | 0.0488 |
| C.V. % | | 2.48 | 2.74 | 3.57 |

S.Em: Standard Error of mean, C.D.: Critical Difference and C.V.: Coefficient of variation.

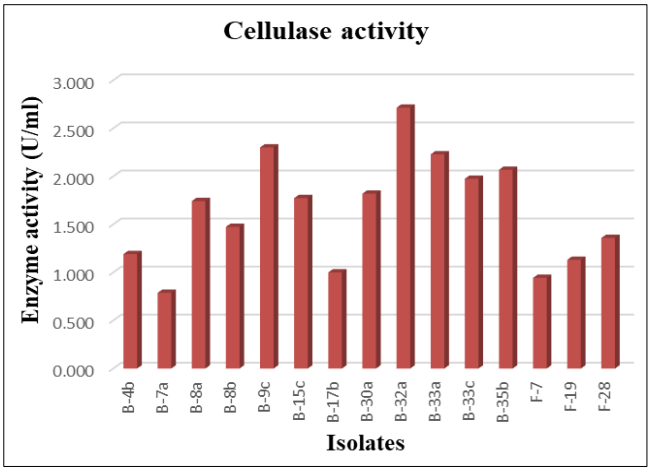


**Fig 2:** Cellulase activity of entomopathogenic isolates (U/ml)

**Quantitative protease assay**

The data pertaining to results of quantitative protease enzyme assay as performed as described in methods. The standard curve obtained by plotting the varying concentration of tyrosine against their corresponding absorbance at 540 nm. The determination of standard curve of glucose for estimating the protease and corresponding specific activity, and BSA for estimating protein content. protease enzyme activity shown by isolates ranged from 0.550 to 0.990 U $ml^{-1}$(Table 4) (Fig. 3). The highest protease activity was observed in isolate B-15c while lowest in isolate F-28. Park *et al.* (2014) [14] also reported the production of protease enzyme in proteolytic bacteria ranging above 100 U $ml^{-1}$ $min^{-1}$. These results thus suggest that the isolates under study presents a suitable candidates for production of protease enzymes for various biotechnological and industrial applications.

**Table 4:** Determination of protein content, protease enzyme and specific activity

| Sr. No. | Culture code | Protein content (µmol/ml) | Enzyme activity (U/ml) | Specific Activity (U/mg) |
|---|---|---|---|---|
| 1. | B-4b | 1.018 | 0.560 | 30.273 |
| 2. | B-7a | 1.385 | 0.762 | 29.913 |
| 3. | B-8a | 1.295 | 0.712 | 30.223 |
| 4. | B-8b | 1.490 | 0.820 | 30.174 |
| 5. | B-9c | 1.268 | 0.697 | 30.036 |
| 6. | B-15c | 1.801 | 0.990 | 30.240 |
| 7. | B-17b | 1.571 | 0.864 | 30.184 |
| 8. | B-30a | 1.254 | 0.689 | 30.243 |
| 9. | B-32a | 1.235 | 0.679 | 29.796 |
| 10. | B-33a | 1.379 | 0.758 | 30.234 |
| 11. | B-33c | 1.680 | 0.924 | 29.896 |
| 12. | B-35b | 1.034 | 0.568 | 29.575 |
| 13. | F-7 | 1.113 | 0.612 | 30.309 |
| 14. | F-19 | 1.163 | 0.640 | 30.091 |
| 15. | F-28 | 1.000 | 0.550 | 30.218 |
| S.Em.± | | 0.0152 | 0.0149 | 0.1468 |
| C.D. at 5% | | 0.0439 | 0.043 | 0.4239 |
| C.V. % | | 2.01 | 3.58 | 0.85 |

S.Em: Standard Error of mean, C.D.: Critical Difference and C.V.: Coefficient of variation.

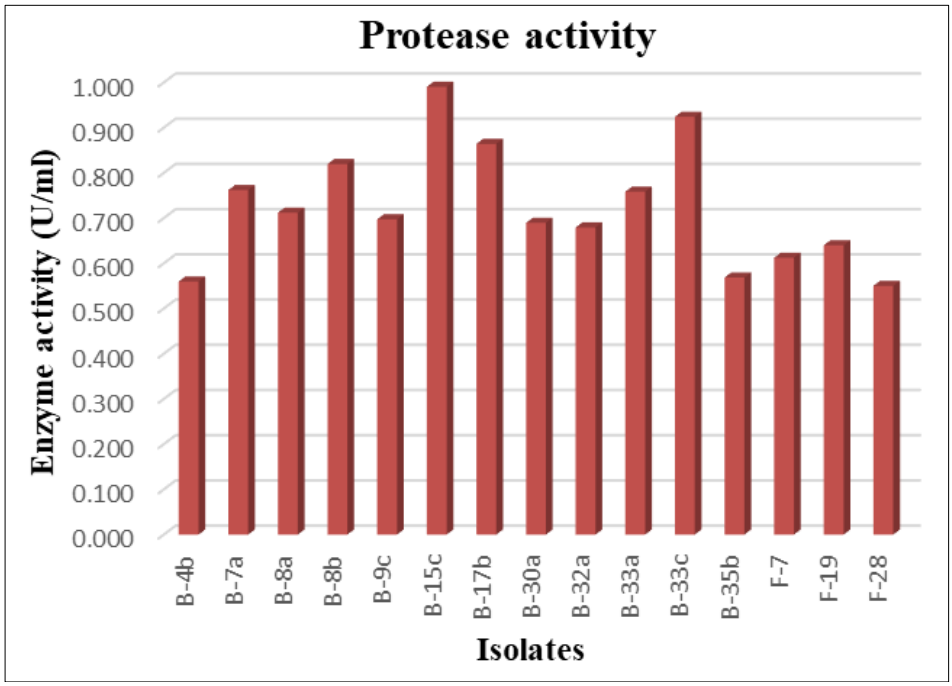


**Fig 3:** Protease activity of entomopathogenic isolates (U/ml)

**Phosphate solubilization capacity**

**Qualitative phosphate solubilization test in solid medium**

All the isolates were tested on TCP containing Sperber's agar media for qualitative phosphate solubilization test. All fifteen isolates showed positive test and zone of solubilization on the Sperber's agar media. The diameter of dissolution halos or zone of solubilization (ZOS) formed by the isolates were measured on 1, 3, 5, 7, 9 and 11 days after inoculation and their corresponding solubilization index and efficiency were calculated according to the formulas described by Nosrati *et al.*, 2014 [13]. The ZOS by isolates were found to increase with the increase in the number of days after inoculation till 9 DAI after which the increment slowed down with little or no increment till 11 DAI. The highest ZOS and thereby solubilization efficiency was shown by isolate B-33a with ZOS of 3, 6, 9, 14 and 16 mm, Phosphate solubilization Efficiency (PSE) of 33.33, 66.67, 100.00, 140.00 and 160.00% on 3, 5, 7, 9 and 11 DAI respectively. The lowest, on the other hand, were shown by B-8a with ZOS of 3, 5, 6, 8 and 9 mm, PSE of 37.50, 55.56, 60.00, 72.73 and 81.82% on 3, 5, 7, 9 and 11 DAI respectively.

**Capacity of native potential isolates for phosphate solubilization quantitatively**

Phosphate solubilizing activity of any bio fertilizers culture has great significance for agricultural use. It enables the microorganisms to solubilize fixed form of soil phosphate, which can thus increase phosphate availability to the crop as well as for microbe itself (Hariprasad and Niranjana, 2009) [4]. The initial pH of the phosphate solubilization broth was adjusted to 7.0 and change in pH of the broth was recorded at 3, 5, 7, 9 and 11 DAI. Decreased pH of the broth increased with advance of incubation time in case of all the isolates. The control tube which was kept without inoculation showed decreased pH up to 6.5 at 11 DAI with very less soluble Pi released. So, there was a negative correlation between pH and Pi release in TCP broth. Decreased pH of the broth increased with advance of incubation time in case of all the isolates and the maximum drop in pH (4.1) was recorded in broth of isolate B-17bat 11 DAI followed by isolates B-32a and B-30a with decreased pH of 4.2 and 4.5 etc. respectively. So, therewas a negative correlation between pH and Pi release in TCP broth.(Table 5) (Fig. 4)

**Table 5**: pH drop and Pi released from TCP broth by isolates on 3, 5 and 7 DAI

| Sr. No. | Culture code | 3 DAI | | 5 DAI | | 7 DAI | |
|---|---|---|---|---|---|---|---|
| | | pH | Pi (µg/ml) | pH | Pi (µg/ml) | pH | Pi (µg/ml) |
| Control | - | 6.8 | 37.17 | 6.6 | 37.88 | 6.6 | 38.60 |
| 1. | B-4b | 5.7 | 270.74 | 5.5 | 374.79 | 5.2 | 400.98 |
| 2. | B-7a | 5.6 | 472.88 | 5.2 | 486.21 | 5.1 | 496.21 |
| 3. | B-8a | 5.8 | 521.21 | 5.6 | 570.74 | 5.4 | 578.83 |
| 4. | B-8b | 6.0 | 411.93 | 4.8 | 449.55 | 4.5 | 483.60 |
| 5. | B-9c | 4.9 | 421.21 | 4.6 | 423.12 | 4.4 | 456.21 |
| 6. | B-15c | 5.8 | 563.83 | 5.6 | 599.31 | 5.3 | 620.74 |
| 7. | B-17b | 4.7 | 685.02 | 4.4 | 707.40 | 4.2 | 723.12 |
| 8. | B-30a | 5.1 | 423.36 | 5.0 | 463.60 | 4.8 | 531.93 |
| 9. | B-32a | 5.0 | 239.07 | 4.7 | 286.21 | 4.5 | 422.17 |
| 10. | B-33a | 6.4 | 285.02 | 5.7 | 322.17 | 5.4 | 381.45 |
| 11. | B-33c | 5.7 | 215.02 | 5.3 | 296.19 | 5.1 | 391.93 |
| 12. | B-35b | 5.4 | 357.40 | 5.2 | 385.02 | 5.0 | 414.79 |
| 13. | F-7 | 5.8 | 243.36 | 5.4 | 256.69 | 5.2 | 396.45 |
| 14. | F-19 | 5.6 | 251.69 | 5.4 | 281.45 | 5.3 | 388.60 |
| 15. | F-28 | 5.9 | 265.98 | 5.6 | 338.12 | 5.4 | 408.60 |
| S.Em.± | | 0.0477 | 6.9742 | 0.0507 | 7.7975 | 0.0467 | 8.65 |
| C.D. at 5% | | 0.1375 | 20.1403 | 0.1461 | 22.5178 | 0.1346 | 24.9797 |
| C.V. % | | 1.47 | 3.22 | 1.66 | 3.25 | 1.59 | 3.17 |

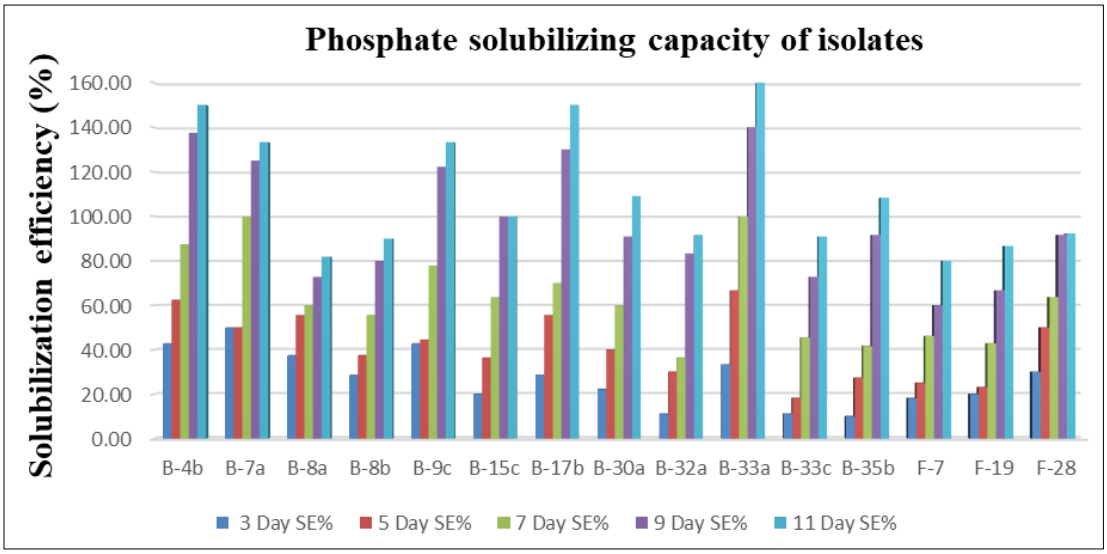


**Fig 4:** Phosphate solubilization efficiency (%) of isolates on TCP media on 3, 5, 7, 9 and 11 DAI

**Nitrogen fixing capacity**

Nitrogen is the most significant yield-limiting element factor in many agricultural production systems. It is known that in legumes, BNF by symbiotic bacteria provides a substantial amount of nitrogen required by the plant. When NF bacterium co-exists as an endophyte within non legumes, the plant's total nitrogen content rises uniformly. Nitrogen accumulation in inoculated non-legumes may be the result of BNF (Boddey *et al*., 1995) [1] or the increase in nitrogen uptake from the soil. Among the 15 isolates tested, all isolates were able to fix nitrogen(Table 6) (Fig. 5). The range of nitrogen fixing ability was from 1.515 to 3.846 mg 'N'/g. Among them, the maximum nitrogen fixing ability (3.846 mg 'N'/g) was recorded from B-35b and minimum nitrogen fixing ability (1.515 mg 'N'/g) was recorded in B-32a.

**Table 6:** Nitrogen fixing capacity of isolates

| Sr. No. | Culture code | Nitrogen (mg 'N'/g) |
|---|---|---|
| 1. | Control | 1.546 |
| 2. | B-4b | 2.594 |
| 3. | B-7a | 1.852 |
| 4. | B-8a | 2.696 |
| 5. | B-8b | 1.964 |
| 6. | B-9c | 2.354 |
| 7. | B-15c | 1.842 |
| 8. | B-17b | 2.058 |
| 9. | B-30a | 1.754 |
| 10. | B-32a | 1.515 |
| 11. | B-33a | 3.670 |
| 12. | B-33c | 1.893 |
| 13. | B-35b | 3.846 |
| 14. | F-7 | 1.846 |
| 15. | F-19 | 1.910 |
| 16. | F-28 | 1.764 |
| S.Em.± | | 0.04 |
| C.D. at 5% | | 0.1155 |
| C.V. % | | 3.09 |

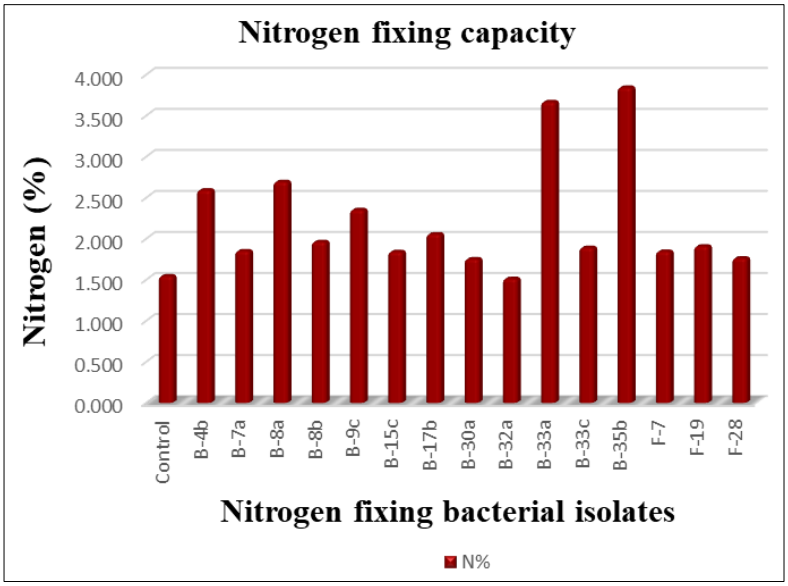


**Fig 5:** Nitrogen fixation capacity of isolates

**Molecular identification by 16s rRNA gene amplification**

The entomopathogenic bacterial isolates under study were identified using 16S rRNA gene sequencing at Department of Biotechnology, Junagadh Agricultural University, Junagadh. The genomic DNA was extracted from the bacterial isolate culture as described in methods. A fragment of the 16S rRNA gene was amplified using 16S universal primers. The sequencing was carried out on capillary sequencer (3130x capillary). Partial 16S rRNA gene sequence of studied bacteria were analyzed with nucleotide BLAST search in GenBank of NCBI.

The BLAST search of Genbank for all isolates provided the percentage similarity between the microorganism tested and those detected in Genbank as shown in Table. Thus, the results showed that the twelve isolates are confirmed as entomopathogenic bacteria.

The 16S rRNA gene sequences of twelve bacterial isolates obtained were compared against the sequences available from NCBI BLASTn Search. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides ). Comparative sequence analysis of BLASTn parameter on NCBI revealed that 5 out of twelve isolates belongs to genus *Bacillus* while four isolates belongs to genus *Pseudomonas* and remaining three isolate belongs to genus *Enterobacter*.

**Table 7:** Identification of entomopathogenic isolates by 16S rRNA gene sequencing

| Culture code | Strain designated | Significant alignments with most closely related organisms | | | |
|---|---|---|---|---|---|
| | | Accession ID | Description | Query coverage % | Identity % |
| B-4b | *P. putida* strain JAU_ B-4b | MK415028.1 | *Pseudomonas putida* strain IAE8 16S ribosomal RNA gene, partial sequence | 96% | 90.28% |
| B-7a | *B. subtilis* strain JAU_ B-7a | KR967391.1 | *Bacillus subtilis* strain AER314-2 16S ribosomal RNA gene, partial sequence | 96% | 99.55% |
| B-8a | *B. subtilis* strain JAU_ B-8a | MK557938.1 | *Bacillus subtilis* strain FC3474 16S ribosomal RNA gene, partial sequence | 99% | 99.66% |
| B-8b | *E. asburiae* Strain JAU_ B-8b | MK467572.1 | *Enterobacter asburiae* strain SA66 16S ribosomal RNA gene, partial sequence | 71% | 95.71% |
| B-9c | *B. thuringiensis* strain JAU_ B-9c | KY003094.1 | *Bacillus thuringiensis* strain VITSJ-16 16S ribosomal RNA gene, partial sequence | 100% | 98.76% |
| B-15c | *B. subtilis* strain JAU_ B-15c | MH141058.1 | *Bacillus subtilis* strain C16 16S ribosomal RNA gene, partial sequence | 100% | 99.54% |
| B-17b | *B. clausii* strain JAU_ B-17b | AB251924.1 | *Bacillus clausii* gene for 16S rRNA, partial sequence, strain: PB-92 | 99% | 98.32% |
| B-30a | *E. cloacae* strain JAU_ B-30a | KU161284.1 | *Enterobacter cloacae* strain BIA072 16S ribosomal RNA gene, partial sequence | 98% | 88.94% |
| B-32a | *P. monteilii* strain JAU_ B-32a | KT881478.1 | *Pseudomonas monteilii* strain JV 16S ribosomal RNA gene, partial sequence | 94% | 98.06% |
| B-33a | *P. knackmussii* strain JAU_ B-33a | KY324901.1 | *Pseudomonas knackmussii* B13 16S ribosomal RNA gene, partial sequence | 99% | 98.34% |
| B-33c | *E. cloacae* strain JAU_ B-33c | JX514409.1 | *Enterobacter cloacae* strain ACC6 16S ribosomal RNA gene, partial sequence | 99% | 100.00% |
| B-35b | *P. fulva* strain JAU_ B-35b | KC293832.1 | *Pseudomonas fulva* strain GGRJ12 16S ribosomal RNA gene, partial sequence | 93% | 98.72% |

**Molecular identification by 18s rRNA gene amplification**

A fragment of the 18S rRNA gene was amplified using 18S universal primers as described in Materials and methods. The sequencing was carried out on capillary sequencer (3130x capillary). Partial 18S rRNA gene sequence of studied fungi were analyzed with nucleotide BLAST search in GenBank of NCBI.

**Table 8:** Identification of entomopathogenic isolates by 18S rRNA gene sequencing

| Culture code | Strain designated | Significant alignments with most closely related organisms | | | |
|---|---|---|---|---|---|
| | | Accession ID | Description | Query coverage % | Identity % |
| F-7 | *Verticillium lecanii* strain JAU_ F-7 | AJ292383.1 | *Verticillium lecanii* partial 18S rRNA gene, internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, internal transcribed spacer 2 (ITS2) and partial 28S rRNA gene, strain IMI 304817 | 100% | 100.00% |
| F-19 | *Metarhizium anisopliae* strain JAU_ F-19 | KJ573520.1 | *Metarhizium anisopliae* isolate Sbz 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence | 100% | 100.00% |
| F-28 | *Beauveria bassiana* strain JAU_ F-28 | KC753382.1 | *Beauveria bassiana* isolate EABb04_01tip 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 100% | 100.00% |

## Conclusion

In conclusion, this study focuses on the vital role of entomopathogenic fungi, including *Beauveria bassiana*, *Verticillium lecanii*, and *Metarhizium anisopliae*, in regulating insect and arachnid populations as biological control agents in agriculture. The research isolates, identifies, and characterizes these microbes from soils, highlighting their capabilities and benefits like enzyme production, phosphate solubilization, and nitrogen fixation. Recognizing the community dynamics of these natural enemies and their interaction with agronomical practices is crucial for effective conservation biological control strategies. The findings support future research to improve efficacy and application of native microbial isolates for sustainable, eco-friendly pest management.

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## References

1. Boddey RM, Oliveira OCD, Urquiaya S, Peis VM, Olivares FLD, Baldani VLD, Dobereiner J. Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant and Soil. 1995;174:195-209.

2. Chrisnasari R, Yasaputera S, Christianto P, Santoso VI, Pantjajani T. Production and characterization of chitinases from thermophilic bacteria isolated from Prataan hot spring, East Java. Journal of Mathematical and Fundamental Sciences. 2016;48(2):149-163.
3. Eilenberg J. Entomophthorales on Diptera. In: Papp L, Darvas B, editors. Manual of Palaearctic Diptera 13. Budapest: Science Herald; 2000. p. 521-533.
4. Hariprasad P, Niranjana SR. Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. Plant and Soil. 2009;316(1):13-24.
5. Hsu SC, Lockwood JL. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Applied Microbiology. 1975;29(3):422-426.
6. Krishnaraj PU, Dahale S. Mineral phosphate solubilisation: Concepts and prospects in sustainable agriculture. Proceedings of the Indian National Science Academy; c2014.
doi: 10.16943/ptinsa/2014/v80i2/55116.
7. Kunitz M. Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor. Journal of General Physiology. 1947;30(4):311-320.
8. Lay Mg Mg Z, Than WM, Myint M. Study on the cellulase enzyme producing activity of bacteria isolated from manure waste and degrading soil. International Journal of Technical Research and Applications. 2015;3(6):165-169.
9. Meyling NV, Pell JK, Eilenberg J. Dispersal of *Beauveria bassiana* by the activity of nettle insects. Journal of Invertebrate Pathology. 2006;93:121-126.
10. Nadeau MP, Dunphy GB, Boisvert JL. Development of *Erynia conica* (Zygomycetes: Entomophthorales) on the cuticle of the adult black flies *Simulium rostratum* and *Simulium decorum* (Diptera: Simuliidae). Journal of Invertebrate Pathology. 1996;68:50-58.
11. Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiology Letters. 1999;170:265-270.
12. Nguyen C, Yan W, Le Tacon F, Lapyire F. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) PD Orton. Plant and Soil. 1992;143:193-199.
13. Nosrati R, Owlia P, Saderi H, Rasooli I, Malboobi MA. Phosphate solubilisation characteristics of efficient nitrogen fixing soil *Azotobacter* strains. Iranian Journal of Microbiology. 2014;6(4):285-295.
14. Park MK, Cho MK, Kang SA, Park HK, Kim DH, Yu HS. *Canthamoeba* protease activity promotes allergic airway inflammation via protease-activated receptor 2. PLoS ONE. 2014;9(3).
15. Ruth C, Steven Y, Pauline C, Veronika IS, Tjandra P. Production and characterization of chitinases from thermophilic bacteria isolated from Prataan hot spring, East Java. Journal of Mathematical and Fundamental Sciences. 2016;48(2):149-163.
16. Sadia Almas, Abdul Hameed, Dennis Shelly, Priya Mohan. Purification and characterization of a novel protease from *Bacillus* strain SAL1. African Journal of Biotechnology. 2009;8(15):3603-3609.