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# Isolation and morphological study of *Azotobacter* and phosphate solubilizing fungal isolates

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## Abstract
Fifteen *Azotobacter* and five phosphate solubilizing fungal (PSF) were isolated from chilli rhizosphere of Kolhapur District and characterized by using both morphological and biochemical tests for cell shape, colony size, gram reaction and catalase activity were identified as *Azotobactor*. All isolates of *Azotobactor* were gram negative. Eleven *Azotobactor* isolates were cocci in shape and three were bacillus. Cell arrangement was observed as scattered in chain in 8 isolates and 7 isolates were singly scattered. Thirteen isolates showed positive results for the methyl red test and catalase test except two isolate. The results of starch hydrolase test were showed positive by fourteen isolates except one isolate whereas all isolates indicated positive results for the gelatine hydrolysis test, gas production test and $H_2S$ production test. Fourteen isolates showed positive results for oxidase test except one isolate. Five phosphate solubilizing fungal isolates were showed white and black colonies producing brown, black and green spores with smooth and rough colony surface. Mycelium hyaline and septate which producing conidia with globuse black, brown brush like phialides with green conidia. Finally from above characteristics five isolates, three *Aspergillus* sp. and two *Penicillium* sp. were identified as phosphate solubilizing fungal isolates.

**Keywords:** Plant rhizosphere, morphological, Penicillium, phosphate solubilizing fungal (PSF)

## Introduction
Plant rhizosphere is a very complex place in which soil, plant and microbe interaction occurs, and plant roots harbours a multitude of different beneficial and deleterious microorganisms. In this place, aerobic bacteria belonging to genus *Azotobacter* play a key role in maintaining soil fertility through several beneficial effects in the rhizosphere. *Azotobacter* is a free living, aerobic, nitrogen fixing bacteria found in soil. They are non-symbiotic heterotrophic bacteria that can fix an average 20 kg N/ha/year. Some species of *Azotobacter* are associated with some plants (Kass *et al.,* 1971) [7]. *Azotobacter* fixes nitrogen with the help of enzyme 'nitrogenase'. *Azotobacter* also produces biologically active compounds such as phytohormones like auxins (Ahmad *et al.,* 2005) [1] thereby stimulating plant growth (Oblisami *et al.,* 2005) [12], (Rajaee *et al.*, 2007) [14]. Despite the fact that, phosphorus is abundant in soils in both organic and inorganic form but it is unavailable to plants. Under *in vitro* conditions, the dissolution of inorganic phosphorus by microbial communities such as fungi, bacteria and other is common. Mineral Solubilization, biological control, and production of secondary metabolites are the characteristics of fungi. As a result, their ability to enhance plant growth when present in association with the roots is undeniable. It has also been observed that the available P and aggregate stability levels, higher soil carbon levels, enzyme activities, and lower soil pH were reported due to inoculation of phosphate solubilizing fungi. (Malviya *et al.*, 2011) [10]. The present investigation was conducted for isolation, characterization and morphological study of *Azotobacter* and phosphate solubilizing fungi isolates.

## Material and Methods
Soil samples were collected by taking out rhizosphere soil of chilli crop along with plant root. The soil was carefully removed from plant roots and kept in fresh plastic bags after labelling and tagging. These samples were preserved in refrigerator at 4°C temperature for further use. Twenty-one soil samples were collected from the chilli growing area of Gadhinglaj, Shirol and Hatkanangale tehsils of Kolhapur District.
The isolation was carried out by serial dilution and pour plate technique using Ashby's medium and Pikovskaya's agar medium (PKV).

**Serial dilution and pour plate technique**

One gram of well mixed soil sample was added in 9 ml of distilled water blank. Tenfold serial dilutions were prepared up to $10^7$ dilutions. One ml aliquot was transferred from $10^4$ to $10^6$ in sterilized petriplates under aseptic conditions. After that each petriplates with aliquot was filled with sterilized Ashby's and Pikovskaya's agar medium (15-20 ml) and mixed gently. After solidification of the medium, plates were incubated at 28±2 ˚C for 4 to 5 days. Later on, the morphological and biochemical characteristics were compared with those defined in Bergey's manual (Krieg *et al*. 1994) [9] to confirm them as *Azotobacter* strains. The strains with similar characters of *Azotobacter* were streaked onto another medium plate and were purified by subsequent streaking after each growth till all the colonies in petriplates appeared similar in morphology and characters. For Phosphate solubilizing fungus, identification was done on basis of colony characteristics and microscopic features. Surface appearance, texture and colour of colonies were considered in colonial characteristics. In addition, arrangement of spores, conidiophores, conidia and vegetative structure was determined by microscopy.

**Morphological characteristics**

Morphological characteristics such as gram staining, cell shape, stain colour, cell morphology, motility test, 3% KOH test were studied. Gram staining was done as per standard procedure described by Cerny (1976) [3].

**KOH (Potassium Hydroxide) test:** A loop full culture of bacteria was taken from a week-old colony and mixed with a drop of 3 % aqueous KOH solution on a clean glass slide, then stirred for 5-10 seconds in a rapid circular motion with a needle. To confirm the gram response, the needle was lifted a few centimeters above the glass slide and watched for the creation of viscid material stands. When a needle was used to pick up a thread-like slime, it showed the presence of gram negative bacteria (Suslow *et al.*, 1982 [17] and Schaad, 1980) [16].

**Motility Test:** The test tubes were filled with 5 ml SIM agar medium and sterilized by autoclaving for 20 min. at 121 $^0$C. Fresh cultures of isolated colony were taken with a sterile needle and puncture the medium to within 1 cm of the tube's bottom to test for motility. When inserting and removing the needle from the medium, make sure it stays in the same line. Incubate for 18-48 hours at 27 °C, or until growth is visible. A dispersed cloud of growth away from the inoculation line indicates a positive motility test (Tittsler and Sandholzer, 1936) [18].

**Cultural characteristics:** Colony size and colony characters (colour, shape and surface) were studied on Potato Dextrose Agar media, Nutrient Agar media, Burk's media, Jensen's media and Ashby's media.

**Biochemical characteristics:** Biochemical tests were studied *viz*. methyl red test, catalase test, starch hydrolyse test, gelatine hydrolysis test, gas production, $H_2S$ production and oxidase test.

**Methyl red (MR) test:** Fresh isolates of *Azotobacter* were inoculated in test tubes containing MR-VP broth under aseptic conditions and were incubated at 28±2 ºC for 48 hours. 5 ml of methyl red solution was added to each after the incubation period. The formation of red colour in the broth indicated the positive result and the formation of yellow colour indicated negative result for the test.

**Catalase test:** In order to determine the catalase activity of the isolates, the 24 hour old cultures were placed on a clean labeled glass slide. A drop of 3% $H_2O_2$ was added on the samples on the glass slide. The development of gas bubbles indicated the positive catalase test.

**Starch hydrolysis test:** The isolates of *Azotobacter* were inoculated on the plates containing sterilized starch agar medium by quadrant method and were incubated at 28±2 ºC for two days. After incubation, the plates containing the bacterial growth were flooded with Lugol's iodine solution. The presence of clear zone around the bacterial colony indicated that starch hydrolysis test was positive.

**Gelatin hydrolysis test:** The gelatin medium stabs were prepared and the fresh grown cultures of *Azotobacter* were inoculated on the stabs under aseptic conditions. The stabs were then incubated for 48 hours at 28 ºC.

After incubation of 2 days, the gelatin dip tubes were placed in refrigerator at 4 ºC for about 20 minutes. The culture was then examined whether the medium was solid or liquid. The liquefaction of the medium indicated positive test.

**$H_2S$ production test:** The isolates of Azotobacter were inoculated to the test tubes containing SIM agar medium under aseptic conditions. The test tubes were incubated at 28±2 ºC for 4 days. The production of $H_2S$ was observed by change in color of medium.

**Oxidase test:** The isolates were inoculated on the sterilized petriplates containing trypticase soy agar and incubated at 28±2 ºC for 2 days. After the incubation for 2 days, the oxidase reagent was added to the surface of colonies and observed for colour change of the surface. The colour formation indicates positive test.

**Gas production test:** Growing the organism in nutrient broth containing 2% glucose was used to examine the culture's ability to create gas. The inverted Durham's tube was placed in a test tube within the nutrient broth and sterilized by autoclaving for 20 minutes at 121 $^0$C. The tubes were injected with 0.5 ml of bacterial suspension and cultured for seven days at room temperature (28±2 °C). Air bubbles in the inverted Durham's tube indicated gas production.

Ability for nitrogen fixation of *Azotobacter* isolates was determined in terms of the quantity of nitrogen accumulated in seven days old culture of each isolate developed on 50ml in Ashby's broth medium. Nitrogen in broth culture was estimated by Kjeldhal method.

**Kjeldhal method:** The fresh culture of all isolate were transferred in Ashby's broth and incubated for seven days. After incubation the 5ml from each broth were taken in digestion tubes which contain digestion mixture. Then sulphuric acid was added and kept for digestion in digestion machine. After completion of digestion the clear liquid were obtained. After that nitrogen was estimated by Automatic N analyzer.

Screened phosphate solubilizing activity of Phosphate solubilizing fungus. Transparent halo zone around the colony indicated the phosphate solubilizing activity of the fungus. Phosphate solubilizing index of the isolates was calculated by using the formula:

$$\text{Phosphate Solubilization Index (SI)} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

**Total Phosphate Solubilization:** The isolates showed zone of Solubilization on Pikovskaya's medium were transferred to 100 mL Pikovskaya's liquid medium with pH 7.0 and incubated for 10 days along with one flask containing liquid medium without inoculation of fungus which served as control. The broth were subjected to centrifuge at 10,000 rpm for 10 minutes. One ml of the supernatant was used for estimation of available P content by phosphomolybdic blue colour method (Jackson, 1971) [5]. The phosphate solubilizing activity was measured at 830 nm wavelength.

## Results and Discussion

Twenty-one rhizosphere soil samples of chilli were collected from different fields of Kolhapur District in year 2020-21. From twenty one soil samples of chilli, fifteen isolates of *Azotobacter* and five phosphate solubilizing fungal (PSF) were obtained.

### Morphological characteristics of *Azotobacter* isolates

Morphological characteristics revealed that all the isolates of *Azotobacter* were gram negative, motile and positive for KOH test. Most of the *Azotobacter* isolates were cocci in shape *viz.* AzoCGH$_2$, AzoCGG$_4$, AzoCGJ$_7$, AzoCSNd$_8$, AzoCSJ$_{10}$, AzoCSU$_{11}$, AzoCSD$_{12}$, AzoCHHe$_{16}$, AzoCHRu$_{18}$, AzoCHRe$_{19}$, AzoCHM$_{20}$ and AzoCHM$_{21}$ except AzoCGK$_6$, AzoCSNa$_{13}$ and AzoCHHa$_{17}$ which were bacillus. Cell arrangement was observed as scattered in chain *viz.* AzoCGH$_2$, AzoCGK$_6$, AzoCGJ$_7$, AzoCSJ$_{10}$, AzoCSU$_{11}$, AzoCHRu$_{18}$, AzoCHRe$_{19}$, AzoCHM$_{21}$ and in single *viz.* AzoCGG$_4$, AzoCSNd$_8$, AzoCSD$_{12}$, AzoCSNa$_{13}$, AzoCHHe$_{16}$, AzoCHHa$_{17}$, AzoCHHa$_{17}$. All isolates of *Azotobacter* were gram negative, motile and showed positive KOH test (Table 1).

The results are in agreement with Martinez-Toledo *et al.,* (1988), reported that the isolates were motile, gram negative and rod shape.

Upadhyay *et al.,* (2015) [19], observed the arrangement of cells was cocci in single, cocci in chain or cocci in clumps. Roychowdhury *et al.* (2017) [21], observed that the isolate were gram negative and colonies were slimy, flat, yellow and spherical in shape.

**Table 1:** Morphological characterization of *Azotobacter* isolates

| Sr. No. | *Azotobacter* Isolates | Cell morphology | Cell arrangement | Gram reaction | Stain colour | Motility | KOH test |
|---|---|---|---|---|---|---|---|
| 1 | AzoCGH$_2$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 2 | AzoCGG$_4$ | Cocci | Scattered single | - Ve | Pink | + Ve | + Ve |
| 3 | AzoCGK$_6$ | Bacillus | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 4 | AzoCGJ$_7$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 5 | AzoCSNd$_8$ | Cocci | Scattered single | - Ve | Pink | + Ve | + Ve |
| 6 | AzoCSJ$_{10}$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 7 | AzoCSU$_{11}$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 8 | AzoCSD$_{12}$ | Cocci | Scattered single | - Ve | Pink | + Ve | + Ve |
| 9 | AzoCSNa$_{13}$ | Bacillus | Scattered single | - Ve | Pink | + Ve | + Ve |
| 10 | AzoCHHe$_{16}$ | Cocci | Scattered single | - Ve | Pink | + Ve | + Ve |
| 11 | AzoCHHa$_{17}$ | Bacillus | Scattered single | - Ve | Pink | + Ve | + Ve |
| 12 | AzoCHRu$_{18}$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 13 | AzoCHRe$_{19}$ | Cocci | Scattered in chain | - Ve | Pink | + Ve | + Ve |
| 14 | AzoCHHa$_{17}$ | Cocci | Scattered single | - Ve | Pink | + Ve | + Ve |
| 15 | AzoCHM$_{21}$ | Cocci | Scattered chain | - Ve | Pink | + Ve | + Ve |

### Cultural characteristics of *Azotobacter* isolates

The colour of colony was recorded as white, creamy or yellowish with irregular or round in shape whereas colonies were cuboidal or slime texture. The colonies developed on different media were wavy, smooth or irregular margin moreover colony elevation varied from unbonate to flat (Table 2). The colony size varied from 6.5 mm to 25 mm in seven days.

The maximum 25 mm colony growth of *Azotobacter* was observed in Ashby's medium followed by Potato dextrose agar Modified Hayward media and Jensen's medium. Isolate AzoCHRu$_{18}$ showed highest colony diameter in the all medium. The findings of present investigation are in support with the results of Upadhyay *et al.,* (2015) [19] examine the cultural characteristics of *Azotobacter* isolates and found that majority of isolates were spherical, flat, raised with irregular margins having mucoid to slime consistency and dull white to yellow colour.

### Biochemical characteristics of *Azotobacter* isolates

The results of biochemical tests were tabulated in Table 3 & Plate 2. Thirteen isolates *viz.* AzoCGH$_2$, AzoCGG$_4$, AzoCGK$_6$, AzoCGJ$_7$, AzoCSNd$_8$, AzoCSJ$_{10}$, AzoCSU$_{11}$, AzoCSNa$_{13}$, AzoCHHa$_{17}$, AzoCHRu$_{18}$, AzoCHRe$_{19}$, AzoCHM$_{20}$ and AzoCHM$_{21}$showed positive results for the methyl red test and catalase test except isolate AzoCSD$_{12}$ and AzoCHHe$_{16}$. The results of starch hydrolase test were showed positive by 14 isolates *viz.* AzoCGH$_2$, AzoCGG$_4$, AzoCGK$_6$, AzoCGJ$_7$, AzoCSNd$_8$, AzoCSJ$_{10}$, AzoCSU$_{11}$, AzoCSD$_{12}$, AzoCHHe$_{16}$, AzoCHHa$_{17}$, AzoCHRu$_{18}$, AzoCHRe$_{19}$, AzoCHM$_{20}$ and AzoCHM$_{21}$ except isolate AzoCSNa$_{13}$ whereas all isolates indicated positive results for the gelatine hydrolysis test, gas production test and $H_2S$ production test.

Fourteen isolates *viz.* AzoCGH$_2$, AzoCGG$_4$, AzoCGK$_6$, AzoCGJ$_7$, AzoCSNd$_8$, AzoCSJ$_{10}$, AzoCSD$_{12}$, AzoCSNa$_{13}$, AzoCHHe$_{16}$, AzoCHHa$_{17}$, AzoCHRu$_{18}$, AzoCHRe$_{19}$, AzoCHM$_{20}$ and AzoCHM$_{21}$ showed positive results for oxidase test except isolate AzoCSU$_{11}$. The results of

biochemical tests obtained from present investigation showed similarity with Jimenez *et al.,* (2011) [6], Akhter *et al.,* (2012) [2], reported that all isolates showed positive results for the catalase test, oxidase test and starch hydrolysis test. Patil *et al.,* (2014) [13] examine biochemical characteristics of *Azotobacter* such as catalase test, oxidase test, $H_2S$ production test. The amount $N_2$ fixed by different strains of *Azotobacter* ranged from 9.34 to 16.35 mg/ml. Highest amount of nitrogen was fixed by *Azotobacter* isolate AzoCHRu$_{18}$ while lowest by *Azotobacter* isolate AzoCGG$_4$. All the *Azotobacter* strains fixed nitrogen which was shown in Table 4. All the obtained results are in agreement with Kizilkaya (2009) [8], analysed the N fixation capacity of 3 days old *Azotobacter chroococcum* on Ashby's medium by Microkjeldhal method.

**Table 2:** Cultural characterization of *Azotobacter* isolates on different solid media

| Media | Isolates | Colony characters of *Azotobacter* isolates | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Colour** | **Shape** | **Texture** | **Margin** | **Elevation** | **Size (mm)** |
| **Ashby's media** | AzoCGH$_2$ | Transparent white | Circular | Slime | Smooth | Hilly | 17.0 |
| | AzoCGG$_4$ | White | Round | Slime | Wavy | Unbonate | 13.0 |
| | AzoCGK$_6$ | White | Irregular | Mucoid | Smooth | Flat | 12.0 |
| | AzoCGJ$_7$ | White creamy | Irregular | Mucoid | Wavy | Flat | 15.0 |
| | AzoCSNd$_8$ | White | Round | Mucoid | Smooth | Flat | 11.0 |
| | AzoCSJ$_{10}$ | White | Circular | Slime | Wavy | Flat | 23.0 |
| | AzoCSU$_{11}$ | Dull white | Round | Mucoid | Wavy | Drop like | 13.0 |
| | AzoCSD$_{12}$ | Opaque white | Irregular | Mucoid | Wavy | Convex | 11.0 |
| | AzoCSNa$_{13}$ | White | Round | Slime | Smooth | Convex | 16.0 |
| | AzoCHHe$_{16}$ | White creamy | Irregular | Mucoid | Smooth | Flat | 14.5 |
| | AzoCHHa$_{17}$ | Creamy yellow | Irregular | Mucoid | Wavy | Flat | 11.5 |
| | AzoCHRu$_{18}$ | White | Round | Slime | Wavy | Flat | 25.0 |
| | AzoCHRe$_{19}$ | White | Irregular | Slime | Wavy | Flat | 9.0 |
| | AzoCHM$_{20}$ | White | Irregular | Slime | Smooth | Unbonate | 10.5 |
| | AzoCHM$_{21}$ | White creamy | Round | Mucoid | Wavy | Unbonate | 15.5 |
| **Burk's media** | AzoCGH$_2$ | White | Complex | Slime | Wavy | Flat | 7.5 |
| | AzoCGG$_4$ | Transparent white | Circular | Slime | Scalloped | Crateriform | 6.5 |
| | AzoCGK$_6$ | Creamy white | Circular | Mucoid | Wavy | Unbonate | 7.0 |
| | AzoCGJ$_7$ | Creamy white | Irregular | Slime | Wavy | Unbonate | 7.5 |
| | AzoCSNd$_8$ | White | Wrinkled | Mucoid | Smooth | Unbonate | 7.0 |
| | AzoCSJ$_{10}$ | White | Round | Slime | Smooth | Unbonate | 10.0 |
| | AzoCSU$_{11}$ | Transparent white | Irregular | Slime | Wavy | Flat | 6.5 |
| | **Isolates** | **Colony characters of *Azotobacter* isolates** | | | | | |
| | | **Colour** | **Shape** | **Texture** | **Margin** | **Elevation** | **Size (mm)** |
| | AzoCSD$_{12}$ | Whitish yellow | Irregular | Slime | Wavy | Flat | 7.0 |
| | AzoCSNa$_{13}$ | Whitish creamy | Round | Mucoid | Scalloped | Convex | 8.5 |
| | AzoCHHe$_{16}$ | White | Irregular | Mucoid | Wavy | Crateriform | 8.0 |
| | AzoCHHa$_{17}$ | White | Irregular | Mucoid | Wavy | Unbonate | 7.5 |
| | AzoCHRu$_{18}$ | White | Irregular | Mucoid | Smooth | Unbonate | 12.5 |
| | AzoCHRe$_{19}$ | White | Round | Slime | Smooth | Flat | 6.0 |
| | AzoCHM$_{20}$ | White | Round | Slime | Wavy | Flat | 6.5 |
| **Burk's media** | AzoCHM$_{21}$ | White creamy | Irregular | Slime | Wavy | Crateriform | 9.0 |
| **PDA media** | AzoCGH$_2$ | White creamy | Round | Mucoid | Wavy | Unbonate | 16.0 |
| | AzoCGG$_4$ | Yellowish | Irregular | Mucoid | Wavy | Flat | 12.0 |
| | AzoCGK$_6$ | Yellowish creamy | Round | Slime | Smooth | Flat | 11.0 |
| | AzoCGJ$_7$ | White | Irregular | Mucoid | Wavy | Flat | 13.0 |
| | AzoCSNd$_8$ | White creamy | Irregular | Slime | Smooth | Flat | 9.0 |
| | AzoCSJ$_{10}$ | White | Round | Slime | Smooth | Flat | 22.0 |
| | AzoCSU$_{11}$ | Yellowish | Round | Mucoid | Smooth | Convex | 11.5 |
| | AzoCSD$_{12}$ | Dull white | Irregular | Slime | Wavy | Flat | 10.5 |
| | AzoCSNa$_{13}$ | Creamy yellow | Round | Mucoid | Smooth | Flat | 14.5 |
| | AzoCHHe$_{16}$ | Yellowish creamy | Irregular | Mucoid | Wavy | Flat | 14.0 |
| | AzoCHHa$_{17}$ | Creamy white | Irregular | Slime | Wavy | Flat | 10.0 |
| | AzoCHRu$_{18}$ | White | Round | Slime | Smooth | Flat | 24.0 |
| | AzoCHRe$_{19}$ | White | Irregular | Mucoid | Wavy | Flat | 8.5 |
| | AzoCHM$_{20}$ | Whitish creamy | Round | Mucoid | Smooth | Crateriform | 9.5 |
| | AzoCHM$_{21}$ | White | Round | Mucoid | Smooth | Flat | 14.0 |
| **Nutrient Agar Media** | AzoCGH$_2$ | Whitish creamy | Irregular | Mucoid | Wavy | Flat | 8.0 |
| | AzoCGG$_4$ | Yellowish creamy | Irregular | Mucoid | Wavy | Unbonate | 7.5 |
| | AzoCGK$_6$ | Dull white | Irregular | Slime | Wavy | Flat | 8.0 |
| | AzoCGJ$_7$ | White | Irregular | Slime | Smooth | Unbonate | 8.5 |
| | AzoCSNd$_8$ | Whitish creamy | Round | Slime | Wavy | Unbonate | 8.0 |
| | AzoCSJ$_{10}$ | Whitish creamy | Round | Slime | Irregular | Flat | 11.5 |
| | AzoCSU$_{11}$ | White | Irregular | Mucoid | Smooth | Flat | 7.0 |
| | AzoCSD$_{12}$ | White | Irregular | Slime | Smooth | Unbonate | 8.0 |

| | Isolates | Colour | Shape | Texture | Margin | Elevation | Size (mm) |
|---|---|---|---|---|---|---|---|
| | AzoCSNa$_{13}$ | Yellowish creamy | Irregular | Mucoid | Wavy | Flat | 10.0 |
| | **Isolates** | **Colony characters of *Azotobacter* isolates** | | | | | |
| | | **Colour** | **Shape** | **Texture** | **Margin** | **Elevation** | **Size (mm)** |
| **Nutrient Agar Media** | AzoCHHe$_{16}$ | White | Round | Slime | Wavy | Flat | 9.0 |
| | AzoCHHa$_{17}$ | Whitish creamy | Irregular | Slime | Smooth | Flat | 8.5 |
| | AzoCHRu$_{18}$ | Whitish creamy | Irregular | Mucoid | Wavy | Unbonate | 14.0 |
| | AzoCHRe$_{19}$ | White | Round | Mucoid | Wavy | Flat | 7.0 |
| | AzoCHHa$_{17}$ | Yellow | Irregular | Slime | Wavy | Flat | 7.0 |
| | AzoCHM$_{21}$ | White | Irregular | Slime | Wavy | Convex | 11.0 |
| **Modified Hayward media** | AzoCGH$_2$ | Transparent white | Irregular | Mucoid | Wavy | Flat | 12.5 |
| | AzoCGG$_4$ | White | Irregular | Mucoid | Scalloped | Crateriform | 10.5 |
| | AzoCGK$_6$ | White | Round | Mucoid | Wavy | Flat | 11.0 |
| | AzoCGJ$_7$ | White creamy | Irregular | Slime | Wavy | Unbonate | 11.5 |
| | AzoCSNd$_8$ | Whitish creamy | Round | Slime | Smooth | Flat | 10.0 |
| | AzoCSJ$_{10}$ | Whitish creamy | Round | Slime | Smooth | Flat | 15.0 |
| | AzoCSU$_{11}$ | White | Irregular | Mucoid | Wavy | Flat | 11.0 |
| | AzoCSD$_{12}$ | White | Irregular | Mucoid | Wavy | Unbonate | 10.5 |
| | AzoCSNa$_{13}$ | Yellowish creamy | Round | Slime | Scalloped | Flat | 14.0 |
| | AzoCHHe$_{16}$ | White | Irregular | Slime | Wavy | Wavy | 13.0 |
| | AzoCHHa$_{17}$ | Creamy yellow | Irregular | Slime | Wavy | Smooth | 12.0 |
| | AzoCHRu$_{18}$ | White | Round | Slime | Smooth | Smooth | 19.0 |
| | AzoCHRe$_{19}$ | White | Irregular | Mucoid | Smooth | Wavy | 9.0 |
| | AzoCHM$_{20}$ | White | Round | Slime | Wavy | Wavy | 10.5 |
| | AzoCHM$_{21}$ | White creamy | Round | Mucoid | Wavy | Scalloped | 12.5 |
| **Jensen's agar media** | AzoCGH$_2$ | Creamy yellow | Irregular | Slime | Wavy | Unbonate | 10.5 |
| | AzoCGG$_4$ | Creamy yellow | Irregular | Slime | Wavy | Crateriform | 8.5 |
| | AzoCGK$_6$ | Creamy white | Round | Slime | Smooth | Crateriform | 8.0 |
| | AzoCGJ$_7$ | White | Irregular | Slime | Smooth | Unbonate | 10.0 |
| | AzoCSNd$_8$ | Transparent white | Round | Mucoid | Smooth | Unbonate | 7.5 |
| | AzoCSJ$_{10}$ | White | Round | Slime | Wavy | Flat | 18.0 |
| | AzoCSU$_{11}$ | White | Irregular | Mucoid | Wavy | Crateriform | 9.0 |
| | AzoCSD$_{12}$ | White | Irregular | Slime | Wavy | Unbonate | 8.0 |
| | AzoCSNa$_{13}$ | White | Irregular | Slime | Wavy | Crateriform | 12.0 |
| | AzoCHHe$_{16}$ | White | Round | Slime | Smooth | Crateriform | 11.0 |
| | AzoCHHa$_{17}$ | Creamy white | Round | Slime | Smooth | Hilly | 9.0 |
| | **Isolates** | **Colony characters of *Azotobacter* isolates** | | | | | |
| | | **Colour** | **Shape** | **Texture** | **Margin** | **Elevation** | **Size (mm)** |
| | AzoCHRu$_{18}$ | White | Round | Mucoid | Wavy | Unbonate | 20.5 |
| | AzoCHRe$_{19}$ | White | Irregular | Slime | Wavy | Flat | 7.0 |
| | AzoCHM$_{20}$ | Creamy white | Irregular | Mucoid | Wavy | Unbonate | 8.5 |
| **Jensen's agar media** | AzoCHM$_{21}$ | White | Irregular | Mucoid | Wavy | Unbonate | 11.5 |

Based on morphological, cultural characteristics (texture, colour, surface of colony) and microscopic observations the fungal isolates were identified as *Aspergillums* sp. (PSFCGG$_4$, PSFCGJ$_7$ and PSFCSD$_{12}$) and *Penicillium* sp. (PSFCHH$_{15,}$ PSFCHR$_{18}$).

PSFCGG$_4$ isolate colonies was black which produced black spores, colony surface rough, conidial head globose black, septate mycelium with hyaline colour. Whereas, PSFCGJ$_7$ and PSFCSD$_{12}$ isolates colonies were white in colour which produced brown spores, colony surface rough, globose black brown conidial head with hyaline and septate mycelium while PSFCHH$_{15}$ andPSFCHR$_{18}$ isolate produced white colonies, septate, hyaline mycelium which produced pale green and grayish green colour spores i.e. conidia, respectively besides brush like phialides. Colony surface was smooth in PSFCHH$_{15}$ isolate whereas rough in PSFCHR$_{18}$ isolate. Colony size ranged from 1.08 cm to 2.2 cm on Pikovskaya's agar medium and 0.70 cm to 2.75 cm on Potato dextrose agar medium whereas poor growth on NA medium (Table 5). Verma and Ekka (2015) [20] identified the fungal isolate based on colony morphology and microscopic observations.

**Table 3:** Biochemical characterization of *Azotobacter* isolates

| **Biochemical characters of *Azotobacter* isolates** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sr. No.** | **Biochemical tests** | | | | | | | |
| | **Isolates of *Azotobacter*** | **Methyl red** | **Catalase** | **Starch hydrolase** | **Gelatine hydrolase** | **Gas Prod$^n$.** | **$H_2S$ Prod$^n$.** | **Oxidase** |
| 1 | AzoCGH$_2$ | + | + | + | + | + | + | + |
| 2 | AzoCGG$_4$ | + | + | + | + | + | + | + |
| 3 | AzoCGK$_6$ | + | + | + | + | + | + | + |
| 4 | AzoCGJ$_7$ | + | + | + | + | + | + | + |
| 5 | AzoCSNd$_8$ | + | + | + | + | + | + | + |
| 6 | AzoCSJ$_{10}$ | + | + | + | + | + | + | + |
| 7 | AzoCSU$_{11}$ | + | + | + | + | + | + | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | AzoCSD12 | - | - | + | + | + | + | + |
| 9 | AzoCSNa13 | + | + | - | + | + | + | + |
| 10 | AzoCHHe16 | - | - | + | + | + | + | + |
| 11 | AzoCHHa17 | + | + | + | + | + | + | + |
| 12 | AzoCHRu18 | + | + | + | + | + | + | + |
| 13 | AzoCHRe19 | + | + | + | + | + | + | + |
| 14 | AzoCHM20 | + | + | + | + | + | + | + |
| 15 | AzoCHM21 | + | + | + | + | + | + | + |

**Table 4:** Nitrogen fixing ability of *Azotobacter* isolates in Ashby's broth (mg/ml)

| Sr. No. | Isolates of *Azotobacter* | N fixation in broth (mg/ml) |
|---|---|---|
| 1 | AzoCGH2 | 10.27 |
| 2 | AzoCGG4 | 9.34 |
| 3 | AzoCGK6 | 13.45 |
| 4 | AzoCGJ7 | 12.98 |
| 5 | AzoCSNd8 | 12.14 |
| 6 | AzoCSJ10 | 15.88 |
| 7 | AzoCSU11 | 14.48 |
| 8 | AzoCSD12 | 11.68 |
| 9 | AzoCSNa13 | 10.97 |
| 10 | AzoCHHe16 | 10.97 |
| 11 | AzoCHHa17 | 10.83 |
| 12 | AzoCHRu18 | 16.35 |
| 13 | AzoCHRe19 | 11.21 |
| 14 | AzoCHM20 | 12.80 |
| 15 | AzoCHM21 | 13.73 |

The highest P Solubilization was recorded by isolate $PCHH_{15}$ (691.33 µg/ml) followed by isolate $ACGJ_7$ (457.46 µg/ml). The P Solubilization of remaining isolates *viz*. $PCHR_{18}$ and $ACGG_4$ were 429.19 µg/ml and 274.99 µg/ml, respectively. The lowest P Solubilization was recorded by isolate $ACSD_{12}$ (308.4 µg/ml). The pH of medium was adjusted to 7, all five isolates showed decrease in pH after seven days of incubation period. Elias *et al.,* (2016) [4] determine the phosphate solubilizing potential of isolates using Pikovskaya's broth with 0.5% tricalcium phosphate.

**Table 5:** Morphological and cultural characteristics of Phosphate solubilizing fungal isolates

| Sr. No. | Character | Fungal isolates | | | | |
|---|---|---|---|---|---|---|
| | | **ACGG4** | **ACGJ7** | **ACSD12** | **PCHH15** | **PCHR18** |
| 1. | Colony character | Black colonies producing black spore | White colonies producing brown spores | White colonies producing brown spore | White colonies producing green spores | White colonies producing green spores |
| 2. | Colony surface | Rough | Rough | Rough | Smooth | Rough |
| 3. | Conidial head | Globose black | Globose black brown | Globose black brown | Brush like phialides with pale green conidia | Brush like phialides with grayish green conidia |
| 4. | Mycelial septation | Septate | Septate | Septate | Septate | Septate |
| 5. | Mycelial colour | Hyaline | Hyaline | Hyaline | Hyaline | Hyaline |
| 6. | Spore formation | Present | Present | Present | Present | Present |
| 7. | Spore colour | Black | Brown | Brown | Pale green | Grayish green |
| 8. | Colony size on Pikovskaya's agar medium (cm) | 1.43 | 1.71 | 1.83 | 2.2 | 1.08 |
| 9. | Colony size on PDA (cm) | 0.70 | 2.70 | 2.75 | 1.91 | 1.07 |

**Table 6:** Phosphate Solubilization by the fungal isolates in liquid medium using Tricalcium phosphate

| Sr. No. | Isolates of PSF | Designation of Isolates | Final pH | Total soluble P (µg/ml) |
|---|---|---|---|---|
| 1 | *Aspergillus* sp. | PSFCGG4 | 5.14 | 274.99 |
| 2 | *Aspergillus* sp. | PSFCGJ7 | 4.5 | 457.46 |
| 3 | *Aspergillus* sp. | PSFCSD12 | 5.3 | 308.4 |
| 4 | *Penicillium* sp. | PSFCHH15 | 3.0 | 691.33 |
| 5 | *Penicillium*sp. | PSFCHR18 | 4.9 | 429.19 |

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