IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Screening Of Extracellular Enzymes From Endophytic Fungi Associated With *Withania Somnifera*

Khushbu H. Patel[1] and Meghna R. Adhvaryu[1*]

[1]Government Arts, Commerce & Science College, Limbayat, Surat, India.

Abstract

Endophytic fungi reside inside plant tissues without causing any harmful effects and any noticeable symptoms to the plants. Endophytic fungi are found within medicinal plants and have shown strong biological activities, such as anticancer, antioxidant, antimicrobial as well as producing extracellular enzymes. In this study, different fungal species were isolated from the medicinal plant *Withania somnifera* such as *Aspergillus glaucus, Aspergillus sp., Coprinellus radians, Fusarium moniliformae, Aspergillus nidulans, Cladosporium sp., Chaetomium globosum, Alternaria alternata, Alternaria raphani, Penicillium citrinum, Penicillium griseofulvin* and *mycelia sterilia*. Extracellular enzymes have been quantified using agar plate-based methods in which fungi were grown in specified growth media to detect the enzymes produced. Results showed that *Penicillium griseofulvin* have highest ability to produce amylase, cellulase and lipase while, *Coprinellus radians* have highest ability to produce protease and laccase. Endophytic fungi isolated from *Withania somnifera* have ability to produce extracellular enzymes which have great therapeutic potential in clinical microbiology and could be used in a variety of applications.

Key words: Endophytic fungi; Enzyme; *Withania somnifera*

1. Introduction

Extracellular enzymes secreted by Endophytic fungi are currently attracting an enormous attention due to their biotechnological applications. Some have potential applications in a wide variety of medical, agricultural, and industrial areas. Among the widely used important enzymes are Amylase, protease, lipase, cellulase etc. Amylase able to digest starch molecule is widely used in food industry, fermentation and pharmaceutical industry. Versatility of microbial lipases makes it biotechnologically valuable enzyme in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Houde, A., Kademi, A., & Leblanc, 2004). However, very few endophytic fungi have been reported for Lipase production. Cellulases are the most successful enzymes have been widely used in many industries including paper and pulp, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (Kuhad, R. C., Gupta, R., & Singh, 2011). Proteases of microbial origin are most important hydrolytic enzymes have been extensively used in a wide range of applications, mainly in detergent and food industries. As new tools for medicine they are also used in pharmaceutical industries (Poza, M et al., 2007).

Enzymes from endophytes help to understand the lifestyle and fungal diversity from endophytes to pathogens in host. Importance of microbes in enzyme production was often more dominant than enzymes of derived from plants or animals. This is due to regular supply without any seasonal variation, easy genetic manipulation susceptibility, more stability, convenient production, comparatively high yield and rapid growth on reliable inexpensive media as well as low cost (Wiseman, 1995).

2. Material and Methods

2.1 Collection of plant materials

The plant samples of *Withania somnifera* were collected from cultivated species in premises of Government science college Vankal. Fresh, healthy leaf, stem and root segments of *Withania somnifera* were collected, cut them with sterile scalpel and stored in sterile polythene bag at 4^0 C.

2.2 Isolation and identification of endophytic fungi

Healthy Leaves and stem samples of the plant without any symptoms were chosen for an experiment. They were washed in running tap water for 5 minutes to remove traces of dust particles adhering to them. Leaves and stem segmented measuring about 0.5x0.5 cm^2 of *W. somnifera* were cut from the plant samples using sterile scalpel and washed thoroughly with sterile distilled water, dry and then employing for sterilization treatments immediately to reduce the risk of contamination. Different sterilizing agents were used for plant organs like leaves, stem and roots. They were initially treated with 70% Ethanol for 3 minutes. After then deeped in 4% Sodium hypochlorite for 2 minutes to remove the surface contaminants of microorganisms and finally they were sterilized with 0.1% $HgCl_2$ for 1 minute and final wash with sterile distilled water for 3 times to remove traces of mercury chloride. As stem segments especially nodes and internodes were cut into equal halves vertically so that their xylem and phloem can directly exposed to the media. Leaves were scrapped at ventral and dorsal sides to expose the way to emerge the endophytes from veins and veinlets directly to the media. They were inoculated in petri dishes containing Potato dextrose agar (PDA) supplemented with (50 microgram/ml) streptomycin to inhibit the bacterial growth. 4-5 segments were placed in each plate, sealed with parafilm and incubated at 27±2°C in dark for 1-2 weeks under dark conditions and monitored frequently to check the endophytic fungal colonial growth every day. Fungi emerging out from the explants were subcultured on separate PDA plates to obtain pure cultures.

The fungi were identified on the basis of colony morphology, spore structure, and spore attachment. Fungi were microscopically examined after staining with lactophenol cotton blue. Fungal cultures that failed to sporulate were recorded as sterile form. The endophytic fungi were authenticated by National Fungal Culture Collection of India (NFCCI), Agharkar Institute, Pune, India.

2.3 Screening the isolated fungi for their biological Activity

2.3.1 Extracellular enzyme assay

The present study involves isolation and identification of endophytic colonies from *Withania somnifera* and qualitative analysis of five different extracellular enzymes such as amylase, protease, lipase (Hankin and Anagnostakis 2012), cellulase, and laccase *(*Kannan et al., 2012). The clear zones formed around the colonies in the agar medium supplied with appropriate substrate indicate production of particular extracellular enzyme after incubation at room temperature for 3-5 days.

2.3.1.1 Amylase production

Decomposition of starch was used as a criterion for analysis of amylolytic activity of selected fungal isolate which is evaluated by growing the isolate on Glucose Yeast Peptone (1 gm glucose,0.1 gm Yeast extract,0.5 gm Peptone,16 gm Agar ,1000ml distilled water, pH 6) amended with 2% soluble starch as a substrate. The fungi to be tested were point inoculated this media and allowed to grow for 5-7 days. The plates were flooded with 1% iodine dissolve in 2% KI. The clear zone around the colony indicated the production of Amylase.

2.3.1.2 Protease production

The proteolytic activity of fungal endophyte was determined by growing fungal isolate on Glucose Yeast Peptone media (1g glucose, 0.1g yeast extract, 0.5g peptone, 16g agar, 1000ml distilled water, pH 6) supplemented with 0.4% gelatin. After incubation plates were flooded with saturated ammonium sulphate (NH_4SO_3). Clear zone around the colony indicates hydrolysis of gelatin while unhydrolyzed gelatin is precipitated by ammonium sulphate.

2.3.1.3 Cellulase production

Cellulase producing isolated were screened by inoculating selected isolates on Glucose Yeast Peptone agar media (0.1g yeast extract, 0.5g peptone, 16g agar, and 1000 ml distilled water) emended with 0.5% Cellulose powder. Plates were stained by flooded with 0.1 % Congo red for 15 mins and subsequently destained with 1 M NaCl for 15 mins. Formation of clear zone around the colony indicates presence of cellulase enzyme.

2.3.1.4 Lipase production

Lipolytic activity of isolate was determined by growing the fungal endophyte on the Peptone Agar media (10 g peptone; 5 g NaCl; 0.1 g $CaCl_2$ $2H_2O$; 15 g agar; distilled water 1000 ml; pH 6.0) supplemented with sterile 1% (v/v) Tween 20. After incubation the clear halos produced around the colony is the positive indication for Lipase production.

2.3.1.5 Laccase production

The ability to decompose naphthol was used as a criterion for analysis of laccase activity of fungi. Laccase production was screened on Glucose Yeast Peptone agar media (0.1g yeast extract, 0.5g peptone, 16g agar, and 1000ml distilled water) emended with 0.005% 1 naphthol. Change the media from colorless to blue indicates production of Laccase due to oxidation of 1 naphthol.

2.3.2 Phosphate solubilization activity

Fungal ability for phosphate solubilization was tested on Pikovaskaya (PVK) media (glucose, 10 g; $Ca_3(PO4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_20$, 0.1 g; KCl, 0.2 g; yeast extract 0.5 g; $MnSO_4.H_2O$, 0.002 g, $FeSO_4.7H_2O$ 0.002 g and Agar 15 g, distilled water 1 L) with bromophenol blue at a concentration of 0.003%. After incubation period of 3-5 days, clear zones produce around the colony indicates phosphate solubilization (Iman 2008).

3. Results and Discussion

In the present study, 13 endophytic fungal isolates were screened for the presence of extra cellular enzymes such as Amylase, Cellulase, Laccase, Lipase and Protease based on growth on a specific medium discussed earlier in material and methods (Table 1; Figure 2). From the isolated endophytic fungi eleven fungal isolates showed amylase activity (Table 1) while only one endophytic fungus was involved in laccase production (i.e., *Coprinellus radians*).

Table 1 Production of extracellular enzymes and phosphate solubilizing activity of endophytic fungi isolated from of *W. somnifera*

Zone of hydrolysis (mm)						
Endophytic Fungi	**Amylase**	**Lipase**	**Cellulase**	**Protease**	**Laccase**	**Phosphate solubilization activity**
Aspergillus glaucus	1.03±0.05	-	1.06±0.05	3.96±0.05	-	8±0.00
sterile mycelia	-	-	1.1±0.1	0.53±0.05	-	
Aspergillus spp.	1.16±0.05	1.93±0.11	0.56±0.05	4.93±0.11	-	3.93±0.11
Alternaria raphani	1.1±±0.1	0.53±0.05	-	1.06±0.05	-	
Coprinellus radians	1.03±0.05	-	-	8.03±0.05	0.76±0.05	0
Fusarium moniliformae	1.06±0.05	2.0±0.05	-	3.16±0.15	-	0.53±0.05
Aspergillus nidulans	-	0.23±0.05	-	3.03±0.05	-	1.03±0.05
Cladosporium sp.	3.93±0.11	1.1±0.05	0.53±0.05	0.66±0.05	-	3.06±0.11
Non sporulating hyaline form	2.73±1.10	-	0.93±0.05	1.03±0.05	-	
Chaetomium globosum	1.03±0.05	-	1.43±0.05	1.06±0.11	-	3.96±0.05
Alternaria alternata	3.06±0.05	0.53±0.05	-	3.13±0.05	-	0.53±0.05
Penicillium griseofulvin	9.96±0.05	5.66±0.57	1.93±0.11	1.13±0.05	-	5.03±0.05
Penicillium citrinum	5.93±0.11	5.06±0.05	1.06±0.11	1.06±0.05	-	5.03±0.05
Percentage	84.62%	61.54%	61.54%	100%	7.69%	69.23%

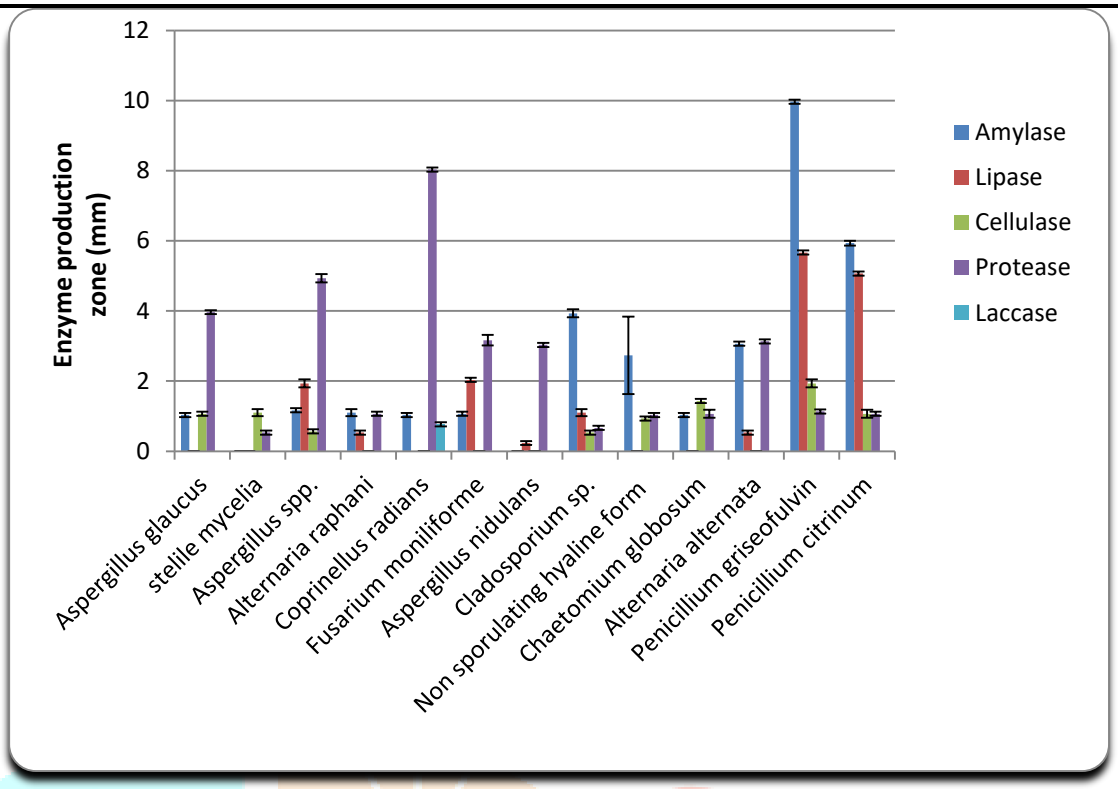


Fig.1 Extracellular enzyme production by endophytic fungi isolated from *W. somnifera*

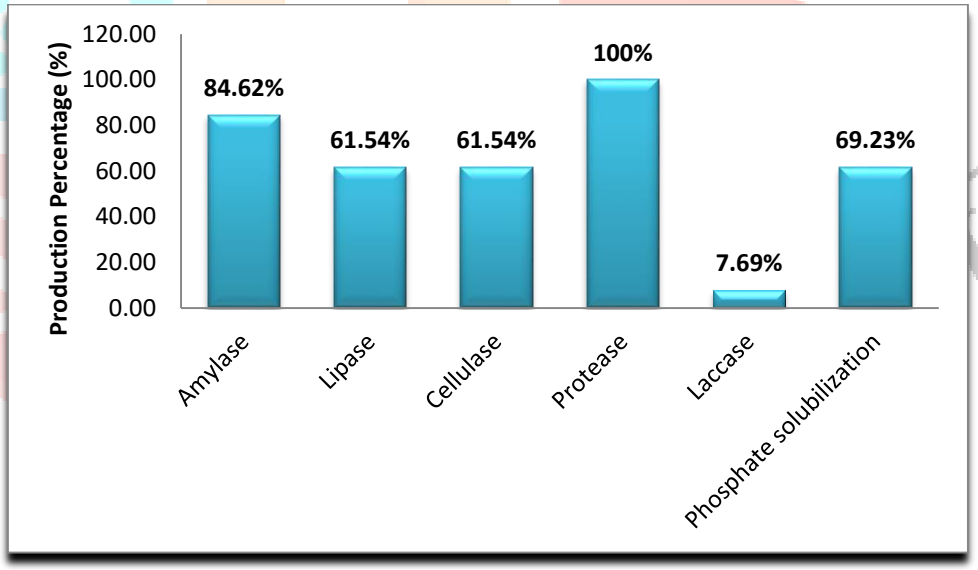
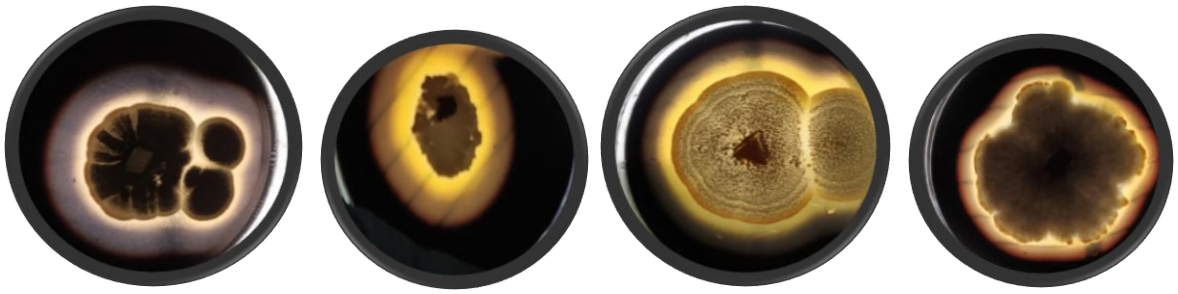


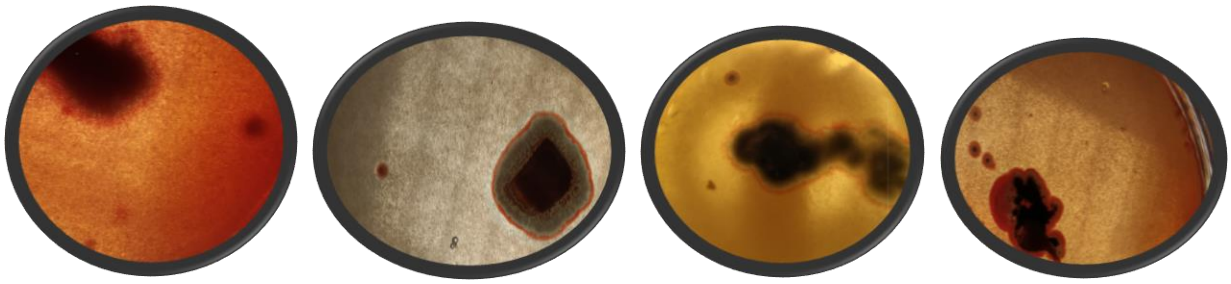
Fig.2 Extracellular enzyme production percentage of fungal isolates from *W. somnifera*

(A) Assay for the detection of Amylase



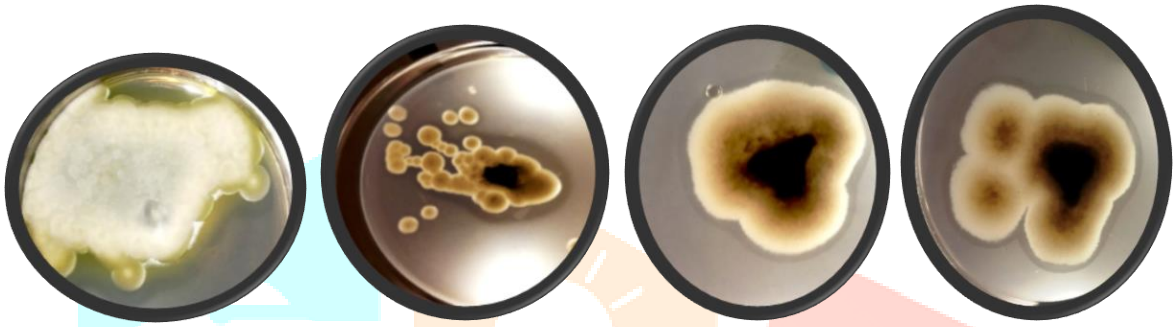
Penicillium citrinum *Aspergillus glaucus* *Aspergillus sp.* *Alternaria raphani*

(B) Assay for the detection of Cellulase



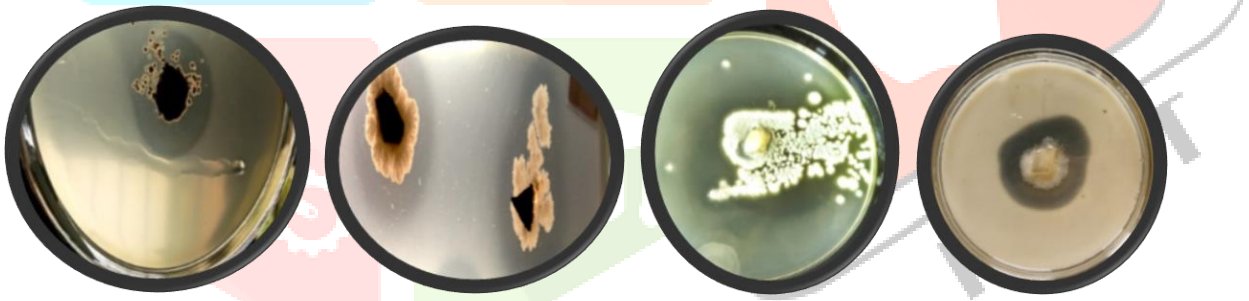
Alternaria raphani *Aspergillus sp.* *Penicillium griseofulvin* *Penicillium citrinum*

(C) Assay for the detection of Lipase



Penicillium citrinum *Penicillium griseofulvin* *Aspergillus sp.* *Alternaria raphani*

(E) Assay for the detection of Protease



Cladosporium sp. *Aspergillus glaucus* *Penicillium citrinum* *Coprinellus radians*

Fig.3**:** Typical view of the plates with fungal endophytes tested for different enzyme activities (**A**) Amylase (**B**) Cellulase (**C**) Lipase (**D**) Protease

From present study, it was observed that the except *Aspergillus nidulans* (E9), all fungi were producing Amylase; *Aspergillus nidulans did not show the ability to produce amylase*. Moreover, data showed that the most highly effective endophytic fungi for amylase were *Penicillium citrinum, Penicillium griseofulvin followed by Cladosporium sp.* and *Alternaria alternata,* while the remaining strains had poor productivity; only 84.62% of the endophytes were able to produce amylase. The maximum production of amylase was from the isolates of *Penicillium sp.* From All the Thirteen isolates only eight strains will able to produce lipase enzyme. *Penicillium sp.* were the most productive strains, followed by *Fusarium moniliformae* sp. while, *Alternaria sp. (Alternaria raphani* and *Alternaria alternata*) and *Aspergillus nidulans* showed weak productivity of lipase. Only 61.54% strains were producing lipase (Fig.1).

All the isolated endophytes (100%) were able to produce protease/were positive for the protease production from which the most protease producing strains were *Coprinellus radians (*8.03±0.05)/maximum production of protease was observed in *Coprinellus radians* (E7). Whereas some of them, such as *Aspergillus* and *Fusarium* had medium activity for this enzyme, while *Penicillium sp.* have lowest zone of enzyme

production. Unlike Lipase, only eight strains out of thirteen have ability for production of cellulase. *Penicillium griseofulvin* and *Chaetomium globosum* were highly efficient producers of cellulase enzyme, and the remaining strains exhibited a weak ability to produce this enzyme (*Aspergillus sp.* and *Cladosporium sp.*) while *Alternaria sp.* could not produce the cellulase. Results found that some but not all endophytic fungal isolates are capable of producing cellulase. Study showed that only 61.54% of the endophytic fungi were able to produce cellulase.

Findings of this study showed that *Coprinellus radians* was the only fungi among the isolated fungi from *Withania somnifera* that showed an ability to produce laccase enzyme, while the other isolates did not show any ability to produce it. Only 7.69% of the endophytic fungi were positive in terms of production of laccase.

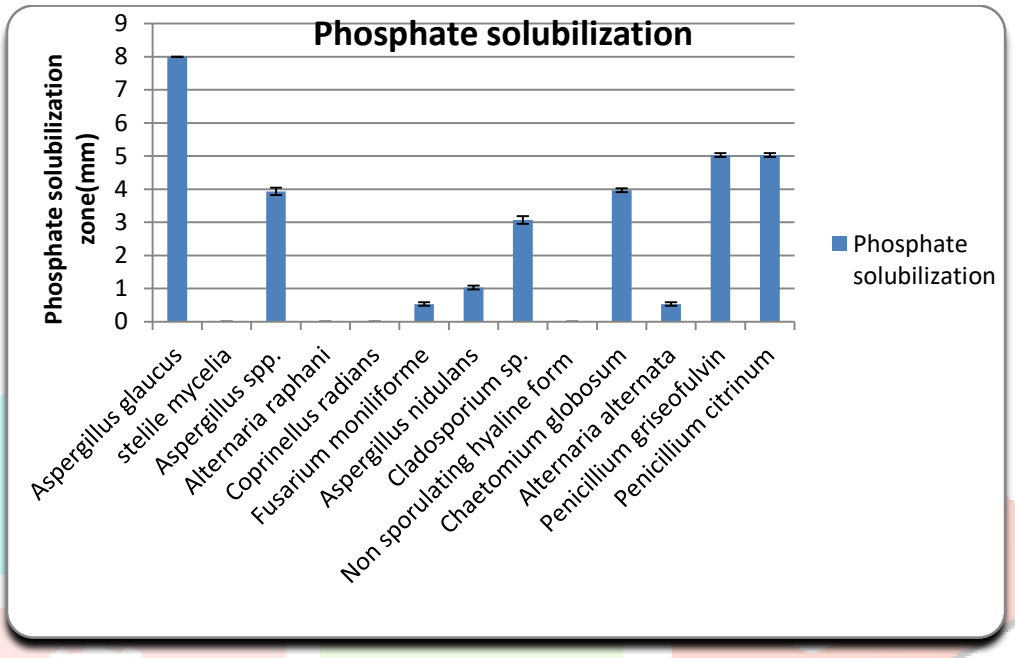


Figure 3. phosphate solubilization activity of endophytic fungi isolated from *W. somnifera*

Aspergillus glaucus *Cladosporium sp.* *Aspergillus sp.*

Phosphate solubilizing fungi can convert insoluble forms of phosphorus to accessible forms which are routinely screened by a plate assay method using Pikovaskaya agar. The findings of the present study suggested that *Penicillium griseofulvin* is capable of solubilize inorganic phosphorous from insoluble compound and ultimately promote the plant growth. *Alternaria raphani* and *Penicillium citrinum* have the same phosphate solubilization ability. In the same way, *Aspergillus nidulans* and *Aspergillus sp.* have the same ability. *Cladosporium sp.* has lowest phosphate hydrolyzing ability.

Conclusion

Withania somnifera, the medicinal plant contains different endophytic fungi that possess the ability to produce extracellular enzymes. High enzyme activity of extracellular enzymes indicates the possibility of exploiting the enzymes of these fungi in many applications after their separation and characterization. Enzymes of fungal origin are more stable than those of plants and animal origin, as they can be easily helpful in food processing, beverages, lather and textile industries (Maria et al. 2005). They also show high therapeutic potential in many clinical microbiological and biotechnological applications. However, knowledge of the types, amounts and characteristic features of enzymes produced by endophytic fungi cited above would be useful for selecting the best organisms suited for industrial requirements. The potential endophytic fungi are being investigated quantitatively for extracellular enzymes.

References

Houde, A., Kademi, A., & Leblanc, D. (2004). Lipases and their industrial applications: an overview. *Applied Biochemistry and Biotechnology, 118*, 155–170.

Kuhad, R. C., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Research*, 1–10.

Poza, M., Ageitos, J. M., Vallejo, J. A., De Miguel, T., Veiga-Crespo, P., & Villa, T. G. (2007). Industrial applications of microbial proteases and genetic engineering. *Hot Spots in Applied Microbiology, 661*, 91–125.

Wiseman, A. (1995). Introduction to principles, Handbook of enzyme biotechnology. *3rd Ed. Padstow, Cornwall, UK: Ellis Horwood Ltd. T.J. Press Ltd.;*, 3–8.

Hankin, Lester, and S L Anagnostakis. 2012. "Mycological Society of America The Use of Solid Media for Detection of Enzyme Production by Fungi" 67 (3): 597–607.

A. Amirita, P. Sindhu, J. Swetha, N. S. Vasanthi, And, and K. P. Kannan. 2012. "'Enumeration of Endophytic Fungi from Medicinal Plants and Screening of Extracellular Enzymes,.'" *World Journal of Science and Technology* 2: 13–19.

Iman, M. 2008. "Effect of Phosphate Solubilizing Fungi on Growth and Nutrient Uptake of Soybean (Glycine Max L.) Plants." *J. Appl. Sci. Res* 4: 592–598.

Maria GL, Sridhar KR, Raviraja NS. (2005) Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *J Agric Technol*. 1:67-80.