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Estimation of hydrolyzing potential of chickpea actinomycetes for degradation of complex compounds through enzymes and acid production

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Abstract
Extracellular enzymes and acids produced by different microorganisms in rhizosphere play important roles in degradation of complex agricultural wastes generated by various farming practices and agroindustries. In present study, total 40 actinomycete isolates previously retrieved from chickpea nodules and rhizosphere soil samples collected from fields of CCS Haryana Agricultural University, Hisar, were evaluated for their abilities to produce enzymes as well as acid. Out of 40 actinomycete isolates, 60%, 67.5% and 25% actinomycete isolates were able to produce protease, cellulase and acid, respectively, whereas only one isolate AK34 was able to show amylase activity. These actinomycete isolates having protease, cellulase, amylase and acid producing abilities seem to play a very significant role in degradation of agricultural wastes and can be explored for their bioremediation potential.

Keywords: Actinomycetes, agriculture, complex compounds, enzymes

Introduction
Agriculture field is facing a big challenge to feed substantial figure of population. In order to meet such a huge demand of increased food production, agriculture is being exercised with an unconditional frequency that leads to the generation of enormous amounts of agricultural wastes containing considerable quantities of lignocellulose, starch and protein contents [1]. Different enzymes like cellulases, amylases, proteases *etc*. are responsible for breakdown of these complex compounds into the simpler one which can be easily utilized by the plants and other soil dwelling organisms. Various enzymes are known to be produced by a diverse variety of soil resident microflora embracing bacteria, fungi, yeasts and nematodes [2]. Soil microbes are usually unable to transport complex compounds into their cell and therefore, rely on the production of extracellular enzymes. These extracellular enzymes are significant biocatalysts having immense applications in agriculture field. Actinomycetes comprise a large group of bacteria in soil microbial populations which are known to produce a vast variety of bioactive compounds including enzymes as well as acids and help in soil nutrient recycling and plant growth promotion [3]. Among actinomycetes, *Streptomyces* species alone are known to produce about 7,600 bioactive compounds [4]. Also, these microorganisms are recognized for the secretion of different acids. The production of such a huge number of bioactive compounds including extracellular hydrolytic enzymes and acids increases their importance in agriculture field as these enzymes aid in decomposition of organic wastes leading to nutrient recycling besides inhibition of growth of pathogens in the rhizosphere. Actinomycetes are predominantly present in various natural habitats such as plant tissues as well as soil, thereby, have been isolated from various niches and production of extracellular hydrolytic enzymes by theses actinomycetes confer them the properties of disease control and plant growth promotion. However, the applications of actinomycetes as enzyme producers in agriculture field are relatively less explored. Therefore, keeping in view the capabilities of actinomycetes in enzyme production and plant growth promotion, the present research work was directed to estimate hydrolysis of complex compounds through the production of enzymes and acids by actinomycete isolates retrieved from rhizosphere and nodules of chickpea.

Materials and Methods
Collection of isolates
Forty actinomycete isolates retrieved from chickpea nodules and soil samples collected from chickpea rhizosphere were obtained from Plant-Microbial Interactions Laboratory,

Department of Microbiology, CCS Haryana Agricultural University, Hisar [5].

Production of Enzymes for Hydrolysis of Complex Compounds

All the actinomycete isolates were grown in Kenknight and Munair's medium (KMM) broth and incubated at 28±2ºC for optimum growth. The cultures at their exponential phage of growth were used for spotting on different media to determine the production of various enzymes and acid.

Protease Production

All the actinomycete isolates were screened for their protein hydrolyzing capability through protease production on skim milk (SM) agar medium by using spot test method. The plates containing SM agar medium were spotted with an aliquot of about 3µl of log phage grown cultures of actinomycete isolates and incubated at 28±2ºC for 5 days. The clear zone observed around well grown colonies of actinomycete isolates was contemplated as an indication of protease production responsible for protein hydrolysis which led to the formation of clear zone. The protein hydrolyzing activity was determined by the following formula:

$$\text{Hydrolyzing index} = \frac{\text{Colony diameter (mm)} + \text{Zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

Cellulase Production

All the actinomycete isolates were tested for their ability to hydrolyze cellulose through the production of extracellular cellulase enzyme on carboxyl methyl cellulose (CMC) agar medium containing Carboxyl methyl cellulose (5.0g/l), glucose (20.0g/l), yeast extract (5.0g/l) and agar (20.0g/l) by using spot test method. Approximately 3µl of log phage grown cultures of actinomycete isolates was spotted on CMC agar medium plates and the plates were incubated at 28±2ºC for 5 days. The plates showing proper growth of actinomycete isolates were flooded with 0.1% aqueous solution of Congo red dye for 15-20 minutes. Then, the plates were destained with 1M NaCl. The formation of clear zone around the colonies of actinomycete isolates was considered as an indication of hydrolysis of cellulose due to the production of cellulase by the tested actinomycete isolates.

Amylase Production

The starch hydrolyzing ability of all the actinomycete isolates was estimated on starch agar medium [6] by using spot test method. About 3µl of well grown cultures of actinomycete isolates was spotted on starch agar medium plates and the plates were kept in an incubator at 28±2ºC for 5 days. After observing proper growth of actinomycete isolates on starch agar medium plates, the plates were flooded with Gram's iodine. Amylase producing isolates showed clear zone around the colonies due to the hydrolysis of starch whereas the development of blue to purple colour indicated no hydrolysis of starch, thereby confirmed that the isolates were not producing amylase.

Acid Production

The production of acids by actinomycetes confers them the property to mineralize complex compounds besides playing a significant role in growth inhibition of pathogens. All the actinomycete isolates were screened for their ability to produce acids on a medium containing tryptone (2.00g/l), sodium chloride (5.00g/l), dipotassium hydrogen phosphate (0.30g/l), glucose (10.00g/l), Bromo thymol blue (0.08g/l) and agar (20.00g/l) using spot test method. The pH of the medium was adjusted to 6.8±0.2. An aliquot of about 3µl of well grown cultures of actinomycete isolates was spotted on agar medium plates having composition as described above and the plates were incubated at 28±2ºC for 6-7 days. The appearance of yellow colour around the well grown colonies of actinomycete isolates indicated the production of acids by the tested actinomycete isolates.

Results and Discussion

Protease Production

Proteases possessing protein hydrolyzing potential are found in a variety of sources like plants, animals and microorganisms [7]. Among microorganisms, actinomycetes are potent candidates to produce proteases which play significant roles in agriculture field. For instance, a group of scientists has reported the production of various enzymes including proteases by *Streptomyces* sp. isolated form wheat rhizosphere and concluded that these enzymes help in growth promotion [8]. In present study, all the actinomycete isolates were screened for their protease producing ability on skim milk agar medium plates and the protein hydrolyzing index (considered as directly proportional to protease production) by actinomycete isolates was calculated. It was found that 60% out of all the tested actinomycete isolates had protease producing capability whereas 40% of actinomycete isolates were not having protease producing potential (Table 1). Actinomycete isolate AK11 was giving comparatively greater protein hydrolyzing index (5.00), thereby seemed as a potent protease producer **(**Fig.1**)**.

Table 1: Protein hydrolyzing index by different actinomycete isolates

Isolate	Hydrolyzing index	Isolate	Hydrolyzing index
AK1	2.66	AK21	2.86
AK2	-	AK22	-
AK3	2.81	AK23	3.00
AK4	2.58	AK24	-
AK5	-	AK25	3.33
AK6	2.8	AK26	-
AK7	3.05	AK27	3.00
AK8	-	AK28	3.00
AK9	-	AK29	-
AK10	3.14	AK30	3.00
AK11	5.00	AK31	-
AK12	2.50	AK32	-
AK13	2.30	AK33	-
AK14	-	AK34	3.04
AK15	-	AK35	3.22
AK16	-	AK36	3.10
AK17	2.60	AK37	2.53
AK18	2.95	AK38	3.00
AK19	2.21	AK39	-
AK20	-	AK40	3.22

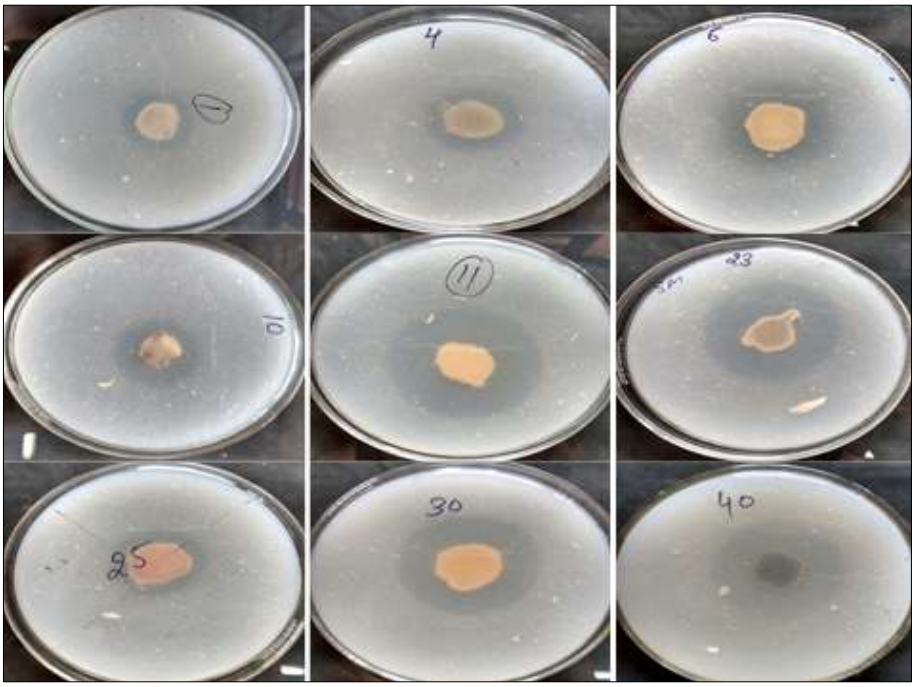

Fig 1: Formation of clear zone around the colonies of actinomycete isolates due to hydrolysis of protein

Cellulase Production

Cellulases are well known to be produced by a number of microorganisms and have attracted significant research interests due to their potential applications in various fields including agriculture. In current investigation, all the actinomycete isolates were tested for cellulase producing activity and it was observed that 67.5% actinomycete isolates were producing cellulase (Table 2) as evident from appearance of clear zone around the well grown colonies. Cellulase activity of actinomycetes has also been studied by various other scientists, for instance, cellulolytic activity of six actinomycete isolates had been analyzed on CMC agar medium and reported as potent cellulase producers [9].

Table 2: Qualitative estimation of enzymes and acid production by different actinomycete isolates

Isolate	Cellulase	Amylase	Acid	Isolate	Cellulase	Amylase	Acid
AK1	+	-	-	AK21	+	-	-
AK2	+	-	-	AK22	-	-	-
AK3	-	-	+	AK23	+	-	-
AK4	+	-	+	AK24	-	-	-
AK5	+	-	-	AK25	+	-	-
AK6	+	-	-	AK26	-	-	-
AK7	+	-	-	AK27	+	-	+
AK8	+	-	-	AK28	+	-	+
AK9	-	-	-	AK29	+	-	-
AK10	+	-	-	AK30	+	-	+
AK11	+	-	+	AK31	-	-	-
AK12	+	-	-	AK32	-	-	-
AK13	+	-	-	AK33	-	-	-
AK14	-	-	-	AK34	+	+	+
AK15	-	-	-	AK35	+	-	-
AK16	-	-	-	AK36	+	-	-
AK17	+	-	+	AK37	-	-	-
AK18	+	-	-	AK38	+	-	-
AK19	+	-	-	AK39	+	-	+
AK20	-	-	-	AK40	+	-	+

Amylase Production

Starch is abundantly found carbon compound in nature which is degraded by amylases. Amylases are the enzymes known to be produced by a number of microorganisms including bacteria. Actinomycetes are also known as potent amylase producers and have attracted interests of various researchers. In present study, all the actinomycete isolates were screened for their amylase producing activity on starch agar medium. Out of the total tested actinomycete isolates, only one isolate, AK34, was found to be amylase positive isolate as indicated by appearance of clear zone around the colony upon flooding with Gram's iodine (Fig. 2a) whereas all the other 39 actinomycete isolates were found to be amylase negative (Table 2). Likewise, Stamford and his co-workers studied amylase producing activity of an endophytic actinomycete isolate *Nocardiopsis* sp. isolated from tubers of yam bean and concluded *Nocardiopsis* sp. as a potential candidate to produce amylase [10].

Acid Production

The production of acids by microorganisms in rhizosphere helps in plant growth promotion as well as mineralization of complex compounds into simpler one by creating acidic environment. In present research work, all the actinomycete isolates were screened for their ability to produce acids and it was found that 25% out of total actinomycete isolates were able to produce acids (Table 2) as evident from appearance of yellow colour around well grown colonies of actinomycete isolates (Fig. 2b). Ruanpanun and his co-workers corroborated the production of acid responsible for plant growth promotion by *Streptomyces* sp. as well as growth inhibition of pathogens [11].

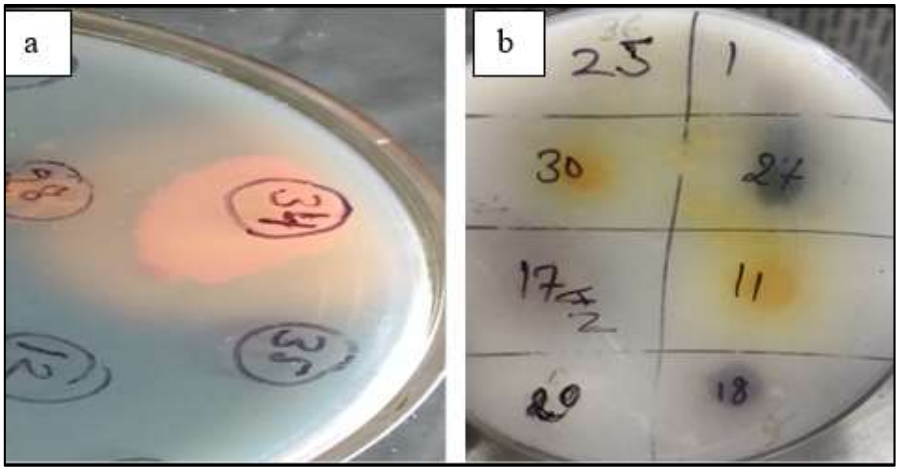

Fig 2.a: Appearance of clear zone around the colony of AK34 on Gram's iodine flooded plate due to hydrolysis of starch, b) appearance of yellow colour due to production of acid by actinomycete isolates

Conclusion

Out of total tested actinomycete isolates, 60% actinomycete isolates were observed to show protease activity, 67.5% isolates were recorded as cellulase producers and 25% isolates were found to produce acid whereas only one isolate AK34 was able to show amylase activity. The different actinomycete isolates possessing enzymes and acid producing abilities can be explored further for bioremediation of complex compounds. Furthermore, actinomycete isolate AK11 showing greater protein hydrolyzing index (5.00) can be optimized for higher protease producing ability and may be suggested as a potent protease producer after optimization and its applications in various fields can be examined.

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