The Screening, Characterization and Identification of Chitinolytic Actinomycetes Streptomyces sp. ANU5a.

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Research Article

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Abstract

Streptomyces sp. ANU5a is a potential plant growth-promoting Rhizobacteria and a highly effective biocontrol agent. It produces Chitinases in broth media containing Chitin. Earlier reports on the chitinase production revealed the key importance of casein hydrolysate and yeast extract as additional sources of carbon and nitrogen to increase the yield. 16S rRNA-based method was used to identification of a Chitinolytic Actinomycete a *Streptomyces sp.* ANU5a was completed from Mangrove soil samples. Ammonium sulphate precipitation method was used for partial purification of Chitinases. The Chitinases have optimum activity at a temperature of 50-55°C and pH 5-6. Three protein bands of 20kDa, 67kDa, and 240kDa are resolved by Native-PAGE of Ammonium Sulphate fractions; the smallest band of 20kDa belongs to Chitinase. Enzyme activity for chitinase was recorded by zymogram analysis. *Streptomyces sp.* ANU5a was found to have notable antifungal activity on culture plates. We correlate the antimicrobial activity of *Streptomycetes sp.* ANU5a with Chitinase activity and other PGPR traits.

Key Points

- 16S based identification of Chitinolytic actinomycetes *Streptomyces sp. ANU5a*.
- Antimicrobial activity of *Streptomycetes sp.* ANU5a with Chitinase activity and other PGPR traits.
- Charecterization of *Streptomycetes sp.* ANU5a by various physiochemical method/assays.

Introduction

Streptomycetes sp. in the phyla Actinomycetes are broadly identified for the commercial level production of secondary metabolites and enzymes in variety of applications viz. medicine, agriculture and environment sectors. *Streptomyces spp.* produces several chitinolytic enzymes to decompose chitins, some examples of these bacterial species includes *S. antibioticus*, *S. griseus*, *S. plicatus*, *S. lividans*, *S. aureofaciens* and *S. halstedii*. Chitinase producing actinomycetes are getting attention due to their plant promotion and biocontrol property. They are soil borne microorganism and this could be another reason for their exploited use in bio-production and agriculture. Chitinolytic enzymes have different applications in research fungal protoplast preparation that degrades cell wall of fungi or ophthalmic preparations in human health care. Chitinase containing hydrolytic enzyme cocktails are using in paper pulp industry for high quality product formulation. In addition to immense application of chitinase and chitinolytic enzymes in medical and bio processing industry, they have major role in agricultural applications like PGPR, Bio-control and transgenic plant development against phytopathogens. Fungi represents a major group of phytopathogens which are aggressive in nature and typically treated chemically using fungicides. Exhaustive usage of such fungicides results as a major contributor to environmental pollution, increased pathogenicity in plants and increased resistance in species of veterinary importance such as arthropods and helminths (Sanborn et al. 2002).

Additionally, these chemicals are proven for serious health hazards to humans and deleterious effect on helpful insects in agriculture. The important consortium of bacteria is also disturbed in the affected ecological niche. This is one of the reasons to facilitate the use of biological control in future generation agriculture strategies. To follow up advancements in this field of research, chitinolytic bacteria or chitinases are proposed to be used as an alternative to chemical treatment for increased yield. These kind of approaches in agriculture can have direct impact on environment and human health.

Over the past few years streptomyces and certain bacillus were investigated as good plant growth promoters. Bacteria that directly or indirectly increases the plant growth via different mechanisms are known as *plant growth promoting bacteria* (PGPB). A recent inclination towards these bacteria in production is promising to increase the crop yield. Other inputs to dramatically increase the crop yield over the past 50 years primarily includes agricultural fertilizers enriched in nitrogen and phosphorus. There are limitations in the sustainability of crops cultivated through conventional methods.

At present, there are only few examples of commercially available chitinases from bacteria. *Bacillus*, *Serratia*, and *Streptomyces spp.* produces chitinolytic enzymes with limitations in optimal activity due to acidic pH (Kielak et al., 2013). As development of new and worth enzyme technology, researchers around the world are looking for ideal enzyme producers, for that point of view they are screening millions of isolates and cultures and another side few are trying to develop new strain via recombinant DNA

technology or by mutagenesis. In few screening programs, researchers are trying to correlate lytic function of bacterial exolate with their hydrolytic enzyme activity. In doing so, many studies divulge that antifungal activity is associated with chitinases as well as antifungal metabolites like 2-furancarboxaldehyde (S. Y. Lee et al., 2012).

Advancement on biocontrol and plant growth promotion strategies will be beneficial for commercial and agricultural means. This particular area of research is needed for exploratory and sustainable development. Therefore, further investigation on this area will be a great achievement for the researchers in coming near future. In this study, we did optimisation in the culture conditions for better yield of chitinase, also the screening and characterisation of metabolites by the *Streptomyces* strains (Table 1).

1. Materials And Methods

1.1 Materials

Bushnell and Haas Medium (BHM), Luria-Bertani Broth, Actinomycetes Isolation Agar gelatin, casein hydrolysate, yeast extract, indole3-acetic acid and N-acetylglucosamine were obtained from Hi-Media, India. All other solvents and chemicals used during the experiment were of analytical grade. The polymeric substrates α -chitin (extracted from shrimp shells), β -chitin (extracted from squid pen) and chitin flakes (milled shrimp shell chitin) procured from Mahatani Chitosan Pvt. Ltd. (Veraval, India) were added to BHM as a substrate for enzyme production. Phytopathogenic fungi namely, *Aspergillus flavus* MTCC 16404, *Fusarium oxysporum* NCIM1072 and *Magnaporthe oryzae* were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh and National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune

1.2 Preparation of Colloidal chitin

Colloidal chitin was prepared as described by Aida et al. (2014) with minor modification. A known amount of dried chitin was suspended in concentrated hydrochloric acid and kept for overnight at 4°C, followed by precipitation of dissolved chitin by chilled distilled water and centrifuged at 10,000 rpm for 20 minutes at room temperature. The pellet was washed repeatedly with distilled water until the pH of the chitin suspension was neutralized.

1.3 Screening of chitinolytic actinobacteria

Pre-obtained Actinobacterial cultures of mangrove soil sediment were screened for their chitinolytic activity. Cultures were inoculated on colloidal chitin (0.1%, w/v) containing agar plate, followed by cultivation at 30°C for 72 h. Individual cultures with clear zone resulting from hydrolysis of chitin were selected for further screening and characterization. In order to get better visibility, plates were stained with Calcofluore White and observed under UV (Anil et al 2007). Clear zone against fluorescent background could be considered as positive chitinase producer.

1.4 *In vitro* screening for plant growth promoting traits

1.4.1 Indole-3-acetic acid production

Production of Indole-3-Acetic Acid (IAA) by actinobacterial isolates was investigated using Actinomycetes broth (Sodium caseinate, K₂HPO₄, MgSO₄, FeSO₄, L-Asparagine,) supplemented with yeast extract (1g/L) and Tryptophan (1g/L). Loopful of actively growing culture was inoculated and incubated for 72 h at 30°C and 120 rpm (Worsley et al., 2020). After 72 h of incubation, culture supernatant was assayed for IAA using Salkowski's reagent (50 mL of 35% perchloric acid and 1 mL of 0.5M FeCl₃) as per the method Brick et al (1991).

1.4.2 Phosphate solubilization

Phosphate solubilization by the actinobacterial isolates was checked using Pikovaskya's agar plate (Yeast extract 0.5g/l, Dextrose 10 g/l, Tri-calcium phosphate 5g/l, Ammonium sulphate 0.5 g/l, Potassium chloride 0.2g/l, Manganese sulphate 0.1 g/l, Manganese sulphate 0.0001g/l, Ferrous sulphate 0.0001g/l, Agar 20g/l). The cultures were spot inoculated on the

Pikovaskya's Agar plate and incubated for 7 days at 30°C. Positive phosphate solubilising bacteria was identified as a colony with clear halo.

1.4.3 Siderophore production

Production of siderophore by the actinobacterial isolates was determined using Chrome Azurol S (CAS) as per the method of Schwyn and Neiland (1991) with minor modifications. The Actinomycetes broth was deferrated before autoclaving using 8-hydroxyquinoline (0.1% w/v) as a iron-chelating agent. Sterilized 10% (v/v) CAS dye solution was added to the sterilized Actinomycetes medium and mixed properly before pouring into petridish. Actinobacterial isolates were spot inoculated onto these plates and incubated for 96 h at 30°C. Siderophore production by bacterial isolates was indicated by an orange halo around the colony.

1.4.4 Screening for organic solvent tolerance

The actinobacterial cultures were spot inoculated on BHM plates using sterile tooth picks, after which each plate was flooded with 10 mL of different solvents [iso-octane (log Pow=4.5), n-heptane (log Pow=4.39), n-hexane (log Pow=3.86), cyclohexane (log Pow=3.2), xylene (log Pow=3.1), toluene (log Pow=2.64), benzene (log Pow=2.13), chloroform (log Pow=2.0)] and incubated at 30°C in an air-tight canister inorder to prevent the evaporation of the solvent from the plates. After incubation, the plates were observed for growth.

1.4.5 Antifungal activity

A co-culture technique (Trivedi et al 2008) was used to test the ability of actinobacterial isolates to inhibit the growth of several phytopathogenic fungi (*Aspergillus flavus* MTCC 16404, *Fusarium oxysporum* NCIM 1072 and *Magnaporthe oryzae*). The cultures were spot inoculated on one side of oat meal agar plate and incubated at 30°C for 48 h after which each fungal strain was inoculated next to the bacterial culture and incubated at 30°C(Hrdý et al., 2020). The plates were incubated up to 5 days for monitoring antifungal activity.

1.5 Extracellular enzyme production

1.5.1 Amylase

Bacterial isolates were inoculated on BHM containing (1%, w/v) starch and incubated at 30°C for 48h. After incubation, plates were flooded with iodine solution. Positive culture exhibited clear zone around the colony against the blue black background due to hydrolysis of starch.

1.5.2 Cellulase

Culture were inoculated on on BHM containing (1%, w/v) CMC and incubated at 30 °C for 72 h. After incubation, plates were flooded with (0.1%, w/v) Congo red for 1h at room temperature followed by destaining with 1M NaCl till suitable contrast is obtained. The clear zone around the culture indicated production of cellulase enzyme.

1.5.3 Protease

In order to screen for protease producers, BHM amended with (1%, w/v) gelatine agar plates were used. Cultures were spot inoculated and incubated for 72h at 30°C. Then agar plates were flooded with 10mL of Frazeir's reagent (15% HgCl₂ in 2N HCl). Clear zone around colony were marked as protease producers.

1.5.4 Lipase

Actinobacterial isolates producing lipase were screened using BHM agar plates amended with Tributyrine (1% v/v). Bacterial cultures were spot inoculated and incubated at 30°C for 120 h. Zone of clearance around the colony indicated production of lipase enzyme.

1.5.5 Maintenance of isolates

The potential cultures were sub-cultured and maintained on Actinomycetes isolation agar slants and preserved in refrigerator at 4°C.

1.6 Identification of bacterial isolate

1.6.1 16s rRNA based molecular identification of ANU5a

Genomic DNA was extracted from the selected isolates according to Ausubel et al. (1997). PCR was done using 16S rRNA universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (MWG Biotech Pvt. Ltd., Bangalore, India) using 20-25 ng template DNA and 30 µl of each PCR reaction for 35 cycles. The thermocycler was set up for each PCR cycle of initial denaturation at 94°C for 5 mins followed by denaturation at 94°C for 2 mins, annealing at 55°C for 1 min, extension at 72°C for 15 mins (BioRad PCR cycler, USA). Agarose Gel electrophoresis was done for PCR products using 1.2% w/v agarose gel in TAE buffer (1X). 1kb DNA ladder (Bangalore Genei, Bangalore, India) was used to compare the resolution of bands. EtBr stained gel was visualized in Gel Documentation system (Alpha-Inotech, USA). Agarose gel purified PCR products were used in sequencing by automated DNA analyzer 3730 using ABI PRISM's BigDye™ cycle sequencing kit (Applied Biosystems, FosterCity, CA). The sequencing results produced approximately 1400bp of sequences, sequence data was analysed using NCBI servers and submitted to NCBI Genbank.

1.7 Inoculum preparation

The inoculum was prepared by method of Butterworth (1984) with minor modification. Chitinolytic actinobacterium ANU5a was inoculated on Actinomycetes isolation agar plate and incubated at 30°C for 48 h after which the spores were harvested using Tween 20 (0.1%, w/v) amended (20%, w/v) glycerol and stored at -20°C. Henceforth, this seed culture will be used as inoculum.

1.8 Profile of chitinase production by ANU5a

The 250 ml Erlenmeyer flasks containing 100 mL of BHM amended with (0.5%, w/v) chitin flakes was inoculated with activated seed culture of ANU5a and incubated on orbital shaker (120 rpm) at 30°C. The aliquots of 2 mL were withdrawn after 24, 48, 96, 120 and 144 h of inoculation and monitored for chitinase activity.

1.9 Medium optimization for chitinase production by ANU5a

The optimization of medium components for chitinase production by isolate ANU5a was done following one factor at a time approach. Here, the components were varied one at a time with the remaining factors held constant. The chitinase production by isolate ANU5a in BHM medium containing different chitinous source at (1%, w/v) as a sole carbon source viz. α-chitin, β-chitin, colloidal chitin (α and β). Similarly, 250ml Erlenmeyer flasks containing 100 ml of (1%, w/v) α-chitin in BHM with varying nitrogen sources were inoculated with ANU5a and incubated at 30°C. The influence of temperature (30°C and 37°C), pH (5 to 8) on chitinase production by isolate ANU5a was also investigated.

1.10 Analytical procedure:

1.10.1 Chitinase assay

Chitinase assay was performed using modified colorimetric method for the estimation of N acetyl amino sugar as described by Schales (Schales and Schales 1945). One unit of chitinase activity was defined as the amount of enzyme required to liberate 1ng of N-acetylglucosamine/mL.min under specified assay conditions.

1.11 SEM analysis of chitin degradation by ANU5a

To visualize the extent of polymer degradation, the degraded chitin flakes were immersed in iso-amylacetate, dried using liquefied carbon dioxide and coated with a layer of sublimated osmium tetroxide using an osmium plasma-coater (Itoh et al.,

2013). The osmium plasma coated specimens were glued onto carbon-aluminium stub and viewed under Scanning Electron Microscope (JSM-6320F; JEOL Ltd., Tokyo, Japan). Scanning was performed using at 10 kV.

1.12 Zymogram analysis

Chitinase activity was detected in polyacrylamide gels containing chitin as described by Escott et al. (1998).

2. Results

2.1 Identification and Screening of Chitinolytic bacteria

Bacteria that produces chitinase have shown poor chitinolytic activity, sometimes requires additional hydrolytic enzyme or even microorganism to boost up their chitinolytic property (Anné et al., 2014). Studies on chitinase production by bacteria have described the role of actinomycetes in natural environments e.g. soil where it contributes to recycle nutrients (Gopalakrishnan, Pande, et al., 2011). They are therefore potential source of hydrolytic enzymes and thus antagonist to major phytopathogens (Jayamurthy et al., 2014). Therefore, this study aims at screening Actinomycetes as a potential source of chitinase enzyme.

Twenty-five actinobacterial cultures were checked for antagonistic activity against phytopathogenic fungus and among them ANU5a, ANU5b, CAI155a, CAI155b, CAI127, CAI140b, KAI27b and KAI90 were found to effectively inhibit fungal growth (Figure 2.1).

Among these eight antagonistic actinobacteria ANU5a broadly exhibited antifungal activity against all the phytopathogenic fungi tested. The difference in extent of inhibition from one organism to other could be attributed to the difference in efficiency and specificity of the antagonistic agent (enzyme/metabolite) produced by the culture (Table 2.1)

2.2 *In vitro* plant growth promotion of actinobacterial isolates

The preliminary screening results for PGP traits have been tabulated in Table 2.2. Almost all the isolate possesses one or the other PGP trait. IAA production was the best trait being observed in the test cultures. From the present investigation, 56% isolates generate IAA in the approximate amount of 19.75 - 55 µg/mL. The ability of bacteria to produce IAA depends on the availability of precursor molecule i.e. L-tryptophan. Root exudates are one of the important natural source of tryptophan that probably accelerate the auxin biosynthesis in the rhizosphere soil (Khamna et al., 2009).

Production of siderophore was also detected by only 52% of isolates (Table 2.2). The isolates on CAS agar formed an orange halo around the colonies. Most of them exhibited high ability to produce siderophores. Usually, soil microbes produces siderophores that bind to Fe³⁺ ions in the iron deficient environment. These siderophores bind to the iron molecule and transport it back to the microbial cell and make it available for growth (W. Lee et al., 2012). These siderophores can also be used by plants to source iron as macronutrient. A possible mechanism to control the phytopathogens also includes competition for iron, inhibition of phytopathogens through hydroxamate-type siderophores by streptomycetes in plant rhizosphere soil is a good example for stimulating competition for iron in plant growth development (Khamna et al., 2009). Siderophore may increase the availability of minerals to soil bacteria that results into biosynthesis of antimicrobial compounds.

Among 25 cultures, 32% isolates were found to solubilize precipitated phosphate after 196h incubation (Figure 2.2). The bacteria could solubilize phosphorus either by organic acid and/or acid phosphatase production (Sharma et al., 2011). The action of organic acid synthesis by soil microbes is generally accepted for the mechanism involving mineral phosphate solubilization. Some of the examples of organic acids among phosphate solubilizers are glycolic, oxalic, lactic, fumaric, acetic, malonic, succinic acid (Sharma et al., 2011).

Therefore, among the above tested isolates, 3 isolates CAI 155a, CAI 155b, ANU 5b were showing maximum number of screened traits and ANU 5a exhibited almost all traits, which may promote plant growth directly or indirectly. Similar to our finding of multiple PGP activity, *Streptomycetes* have been reported to enhance plant growth (Khamna et al., 2009 , Ahmad, Ahmad, & Khan, 2008). While such reports from Indian subcontinent is very sparse.

2.3 Solvent tolerance on extracellular chitinase production

In order to screen for solvent tolerance, these four selected cultures were monitored for growth and chitinase activity on solvents flooded plates. Table 2.3, shows the solvent tolerance and chitinase production by 3 selected bacterial isolates in presence of iso-octane, n-Hexane, and iso-octane. Among these 3 isolates, ANU5a showed broad spectrum response toward solvent tolerance. However, growth of all isolates was inhibited Methanol, Acetone, Benzene, Toluene and Dichloromethane. Organic solvent tolerant chitinase in *Streptomyces* is less reported than till date.

2.4 Selection of chitinolytic strain for chitinase production

The chitinolytic activity of the four selected isolates (CAI155a, CAI155b, ANU5a and ANU5b) were further confirmed by growing them in 100 mL BHM broth amended with 0.5% of α -chitin. Chitinase activity was measured by using Schales reagent. Figure 2.3 represents the chitinase activity of these 4 isolates.

Soil isolates ANU5a showed maximum chitinase activity as 0.032 Units/mL. Thus, ANU 5a was selected for further investigation.

2.5 16SrDNA identification of ANU5a

The sequencing results for ANU5a shares 99% sequence identity with 16S rDNA of *Streptomyces cavourensis*. We have submitted the sequence to NCBI GenBank with accession number KR061434. The analyzed sequence was subsequently used to construct a phylogenetic tree to compare ANU5a and its genetically close relatives (Figure 2.4).

2.6 Influence of temperature and pH on ANU5a chitinase activity

The chitinase enzyme assay was performed at different pH (5, 6 and 7) and temperature (30 °C and 37 °C) to determine the optimal pH and temperature for the chitinase activity. *Streptomyces* sp. ANU5a chitinase exhibited optimal activity at 5.0 pH and 50 °C temperature, respectively (Figure 2.5A, 2.5B).

2.7 Profile of chitinase production by ANU5a

The duration of incubation period offers the potential for the cost effective production of enzymes. Time course study was carried out for the chitinolytic *Streptomyces* sp. ANU5a. The production of extracellular chitinases was detected by withdrawing the sample from each production flask (with additional supplement A without additional supplement B) at time interval of 24 h.

Chitinase activity in production medium without any additional source other than chitin and BHM showed maximum activity at 72 h of incubation and after which the activity declined. Whereas, the maximum activity for chitinase in production medium B supplemented with additional nutritional sources was detected to be at 120 h of incubation and after which the activity declined. Figure (2.6A) represents that *Streptomyces* sp. ANU 5a secretes chitinase with maximum activity of 110 U/mL in production medium B, whereas Figure (2.6B) exhibited 34 Units/mL in production medium A. This study indicates, additional nutritional sources are required for better enzyme production.

2.8 Optimization of chitinase production

Optimization of medium and production parameter for chitinase production by selecting the best nutritional and environmental condition is important to increase the chitinase yield (Saadoun et al., 2009). The traditional method of “optimizing one factor at a time” technique was used in this study. This method is determined by varying one factor while keeping the other factor at a constant level.

The aim of this investigation was to optimize the chitinase production medium for *Streptomyces* sp. ANU 5a. The cultural characters were optimized by amending with different concentration of chitin, various carbon and nitrogen sources, optimum pH, temperature and amino acid source supplementation were studied.

2.9 Effect of different chitin substrate on chitinase production by *Streptomyces* ANU5a

A study pertaining to the physiological requirement of any culture enables one to manipulate conditions so as to maximize product formation. There are difference in the performance of various Actinomycetes on the sources of carbon and energy depending upon their physiology and preference for substrate. The production of chitinase is inducible and affected by nature of substrate used in production. Therefore, the choice of an appropriate inducing substrate is of great importance. These solid substrates serve as support and/or nutrient source. As shown in Figure 2.7, different chitin substrate materials like α chitin, β chitin, colloidal chitin as well as varying concentration of α chitin was tested for their successful utilization as substrate. Among different substrate tested with variable concentration, α chitin showed maximum enzyme activity with 0.5g % (w/v) chitin concentration. The activity of chitinase was found to be maximum i.e 100 U/ml. As α chitin gave maximum activity for the enzyme, hence it was used for further experiment with their particular concentration 0.5g %.

2.10 Effect of pH on chitinase production by *Streptomyces* ANU5a

The hydrogen ion concentration has a marked effect on enzyme production. This may be due to stability of extracellular enzyme at this particular pH and the rapid denaturation at lower or higher pH values, which ultimately lowers the enzyme activity. Chitinases are fairly stable over broad pH range (Saber et al., 2015).

The pH stability of chitinase varies from organism to organism. The effect of different pH on chitinase production is depicted in Figure 2.8. Among the different pH checked, the maximum enzyme production (61U/ml) was observed on 7th day at pH 7 which declined with further increasing initial pH. Similar results were observed by Sowmya et al. (2012) and Thiagarajan et al., (2011), who investigated that the chitinase from *Streptomyces* to be stable over a pH range of 4.0 to 10.

2.11 Effect of temperature on chitinase production by *Streptomyces* ANU5a

The selection of temperature depends on the optimum growth temperature of the selected culture. As enzymes are primary metabolites, so their production is mainly related to growth. More the growth more will be the enzymes produced. The production of enzyme at different temperature 30°C and at 37°C was studied. As depicted in Figure 2.9 the optimum temperature for chitinase production is at 30 °C. It is found to be in mesophilic range. The chitinase was found to maximum as 42 U/mL at 30 °C supplemented with α chitin.

2.12 Effect of nitrogen source on chitinase production by *Streptomyces* ANU5a

The requirement of nitrogen source in the production medium of chitinases has been reported to vary considerably by various researchers. Production of chitinases is sensitive to nitrogen sources and nitrogen level in the medium. Few reports on nitrogen supplement in chitinase production medium indicates inhibitory effect (Suresh & Chandrasekaran, 1998). In this study yeast extract and casein hydrolysate were used to optimize nitrogen sources for maximum chitinase production. BHM broth supplemented with varying concentration (0.05g % to 0.2 g %) of yeast extract and casein hydrolysate were used. As shown in Fig.2.10 A & B, the enzyme was found to be enhanced when 0.05g % yeast extract and 0.2g % casein hydrolysate were applied as external source of nitrogen.

Present study indicates higher concentration of yeast extract and casein hydrolysate has inhibitory effect on chitinase production. They may contribute to fulfill the bacterial nitrogen demand much easily than chitin; it could be a reason for decrease in enzyme production at higher concentration.

2.13 Effect of media component on chitinase production

In this study, different media component were examined for maximum enzyme production. Result of this experiment as depicted in table (2.4) indicates; followed by addition of different media component into chitinase production medium, enzyme production was found to increase than without co-substrates. With one factor at a time approach, significant improvement in yield of enzyme could be achieved by media optimization which is summarized in Table 2.4.

2.14 ESEM Analysis

Chitin degradation and cell morphology of *Streptomyces* sp. ANU5a were analysed by using Environmental Scanning Electron Microscope (ESEM). The morphological examination of the degraded chitin flakes (Figure 2.10) using ESEM confirmed the degenerated native structure of the chitin flakes. Moreover, the microbial chitinases resulted in degradation of chitin fiber which is visible as increased porosity of the substrate (Hao et al. 2016).

2.15 Native PAGE and Zymogram Analysis

The purified chitinase exhibited a three protein bands on Native-PAGE. The relative molecular weight of enzymes was approximately 20kDa. Further identification of the target band was carried out by zymogram analysis as shown in Figure 2.11.

After subjecting the partial purified enzyme of the *Streptomyces* sp ANU5a to zymogram analysis, three bands were exhibited (240,67 & 20 kDa), in which two bands were showing chitinase activity. The size of the chitinase activity bands exhibited in the zymogram is relatively small compared to that observed by Saadoun et al., (2009). Who reported the molecular mass of chitinase from *Streptomyces* (strain 242) to be 55kDa to 97 kDa.

Discussion

The association of decomposition of chitin and antifungal properties is described in earlier reports (Huang et al., 2005). So, in the present study, we screened and determine twenty five different chitinolytic actinomycete strains exhibiting strong chitinolytic activity based on chitinolysis on BHM agar plates. Selected strains were further screened for antagonistic properties of *Aspergillus flavus*, *Fusarium oxysporium*, and *Magnaporthe oryzae*. The result indicates the significant inhibition of the growth of phytopathogenic fungi on OMA plates by ANU5a, ANU5b, CAI155 and CAI155b strains. The inhibition pattern and zones of inhibition on the OMA plates links to the production of water-soluble antifungal metabolites by the active *Actinomycetes* strains. There are numerous studies to support the critical role of chitinolytic enzymes for antifungal properties in various strains. S. Y. Lee et al. (2012) reported the inhibition of the growth of *Colletotrichum gloeosporioides*, a pathogen that causes a fungal disease known as anthracnose in black pepper by chitinases purified from *Streptomyces cavuerensis* SY224. Kishore, Pande, and Podile (2005) reported the biological potential in controlling late Leaf Spot disease in peanuts (*Arachis hypogaea*) by chitinolytic bacterial strains *B. circulans* GRS 243 and *S. marcescens* GPS. Recently, it was reported by Nguyen et al. (2012) that *Streptomyces griseus* H7602 have considerable potential as a biocontrol in papper against root rot disease (*Phytophthora capsici*). Hoster, Schmitz, and Daniel (2005) also highlighted the role of purified chitinase from *Streptomyces griseus* MG3 strain as possible antifungal against the phytopathogenic fungi such as *Aspergillus nidulans*, *Botrytis cinerea*, *Fusarium culmorum*, *Guignardia bidwellii* and *Sclerotia sclerotiorum*. In a same way selected strain were studied for antifungal as well as chitinolytic activity. ANU5a was found to be an efficient strain, as well as possesses plant growth promotion traits along with its ability to secrete a range of extracellular hydrolytic enzymes. This strain was identified as *Streptomyces* sp. ANU5a. It would be worth considering this organism for further investigation with respect to its plant growth promoting and agronomic practices (Aam et al., 2010).

Declarations

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Tables

Table 1: Major chitinase producers and their antifungal activity		
Source of chitinases	Antagonistic against	References
<i>Trichoderma harzianum</i> Rifai TM	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Sclerotium rolfsii</i>	(Okazaki et al., 2004)
<i>Trichoderma harzianum</i>	<i>Macrophomina phaseolina</i> , <i>Fusarium</i> sp. <i>R. solani</i> , <i>Aspergillus niger</i> (NCIM 563), <i>Aspergillus</i> , <i>Rhizopus</i> , <i>Mucor</i> sp.	(Swiontek Brzezinska et al., 2014)
<i>Trichoderma atroviride</i> PTCC5220	<i>Rhizoctonia solani</i>	(Agrawal & Kotasthane, 2012)
<i>Brevibacillus laterosporus</i>	<i>Fusarium equiseti</i>	(Gopalakrishnan, Humayun, et al., 2011)
<i>Bacillus thuringiensis</i> subsp. <i>colmeri</i> 15A3	<i>Rhizoctonia solani</i> , <i>B. cinerea</i> , <i>P. chrysogenum</i> , <i>P. piricola</i> , <i>Penicillium glaucum</i> , <i>Sclerotinia fuckeliana</i>	(Mehmood et al., 2011)
<i>Aeromonas hydrophila</i> SBK1	<i>Aspergillus flavus</i> , <i>F. oxysporum</i>	(Halder et al., 2014)
<i>Enterobacter</i> sp. NRG4	<i>Fusarium moniliforme</i> , <i>Aspergillus niger</i> , <i>Mucor rouxi</i> , <i>Rhizopus nigricans</i>	(Dahiya et al., 2005)
<i>Alcaligenes xylosoxydans</i>	<i>Fusarium</i> sp. <i>Rhizoctonia bataticola</i>	(Swiontek Brzezinska et al., 2014)
<i>Stenotrophomonas maltophilia</i>	<i>Fusarium solani</i> , <i>F. oxysporum</i> , <i>R. solani</i> , <i>A. alternata</i>	(Karaba et al., 2013)
<i>Rhizobium</i> sp	<i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Curvularia lunata</i> , <i>F. oxysporum</i> , <i>Fusarium udum</i>	(Wang et al., 2014)
<i>Serratia marcescens</i> strain B2	<i>Botrytis cinerea</i>	(Purushotham et al., 2012)
<i>Vibrio</i> spp.	<i>Mucor racemosus</i> , <i>Candida albicans</i> , <i>Trichoderma viride</i> , <i>Zygorhynchus heterogmus</i>	(Li & Roseman, 2004)
<i>Streptomyces hygroscopicus</i>	<i>Colletotrichum gloeosporioides</i> , <i>Sclerotium rolfsii</i>	(Swiontek Brzezinska et al., 2014)
<i>Streptomyces tendae</i> TK-VL_333	<i>Aspergillus niger</i> , <i>F. oxysporum</i>	(Gherbawy et al., 2012)
<i>Streptomyces</i> sp. S242	<i>Aspergillus niger</i> , <i>Candida albicans</i>	(Saadoun et al., 2009)
<i>Streptomyces roseolus</i> GH18	<i>Aspergillus</i> spp., <i>Rhizopus chinensis</i> , <i>Penicillium</i> spp., <i>Mucor</i> spp.	(<i>Streptomyces Roseolus</i> -GH18 (Jiang et Al 2012).Pdf, n.d.)
<i>Streptomyces sporovirgulis</i>	<i>Alternaria alternata</i>	(Swiontek Brzezinska et al., 2014)
<i>Streptomyces plicatus</i>	<i>Alternaria alternata</i> , <i>F. solani</i>	(Bonjar et al., 2001)
<i>Streptomyces</i> <i>griseus</i> H7602	<i>Phytophthora capsici</i>	(Nguyen et al., 2012)
<i>Streptomyces sudanensis</i>	<i>Magnaporthe oryzae</i>	(Zarandi et al., 2009)

Table 2.1: Inhibition of test fungi with bacterial isolates			
Bacterial isolates	<i>Aspergillus flavus</i>	<i>Fusarium oxysporium</i>	<i>Magnaporthe oryzae</i>
AC1	+	+	-
AC2	+	+	-
AC3	++	++	-
AC4	+	-	-
AC5	-	-	-
AC7	+	-	-
CAI 155a	+++	+	+
CAI 155b	+++	+	-
CAI 127	+	-	+
KAI 90	-	-	-
CAI 17	-	-	-
CAI 27B	-	-	-
CAI 140B	-	-	++
CAI 93B	-	-	-
CAI 13A	+++	+	-
ANU 18	+++	+	-
KAI 27B	+	-	-
KAI 180B	-	-	-
CAI 93C	-	-	-
ANU 5A	+++	+	++
ANU 5B	+++	+	++
ANU 3	+	-	-
ANU 7B	-	-	-
ANU 32	-	++	-
ANU 21	-	++	-
+++ strong antagonism, ++medium antagonism, +weak antagonism and - negative			

Table 2.2: Plant growth promoting traits of Bacterial isolates

PGPR traits					Extracellular lytic enzyme production				
Isolates	Indole acetic acid	Phosphate solubilization	Siderophore	HCN	Chitinase	Cellulase	Lipase	Protease	Amylase
AC1	-	-	-	-	++			+	+
AC2	+	-	+	+	-			++	++
AC3	+	-	-	-	-			++	++
AC4	++ (40µg/mL)	-	-	++	-			++	+
AC5	-	-	-	+	++			++	+
AC7	+	-	+	-	+			++	+
CAI 155a	+(27.7µg/mL)	-	++	++	++	+++	++	++	++
CAI 155b	++ (55µg/mL)	-	++	++	++	++	+	++	+
CAI 127	-	-	+	++	++			++	+
KAI 90	+	++	+	-	++			++	-
CAI 17	++ (30.5µg/mL)	-	-	++	-			++	-
CAI 27B	-	-	-	-	-			++	++
CAI 140B	+	+	-	-	-			++	-
CAI 93B	-	-	-	+	+				
CAI 13A	++ (30.7µg/mL)	+	+	+	+				
ANU 18	+	-	+	+	++				
KAI 27B	-	-	-	-	-				
KAI 180B	-	-	-	+	++				
CAI 93C	-	-	+	-	+				
ANU 5A	++ (24.5µg/mL)	+	++	++	++	+++	+	+++	++
ANU 5B	++ (19.75µg/mL)	+	+	+	++	++	-	++	+++
ANU 3	+	+	+	+	+			-	
ANU7B	-	++	-	-	++			-	
ANU 32	-	++	-	-	-			-	
ANU 21	-	-	-	-	-				

Table 2.3: chitinolytic Activity of the isolates in presence of solvents

Solvents	ANU5a	ANU5b	CAI 155a	CAI 155b
Control (without solvent)	+	+	+	+
Methanol	-	-	-	-
Acetone	-	-	-	-
Benzene	-	-	-	-
Toluene	-	-	-	-
Dichloromethane	-	-	-	-
Cyclohexane	+	+	+	+
N-Hexane	+	+	+	+
Iso-octane	++	+	+	+
Xylene	+	-	+	+

Table 2.4: Effect of media on enzyme production

S.No	Production medium	Concentration of media component (g %)	Incubation time (h)	Units/ml	Fold increase (X)
1	I	BHM + 0.1g chitin	120 h	5.2	1
2	II	BHM + 0.1g chitin + 0.1YE	144 h	31.3	6
3	III	BHM + 0.5g chitin + 0.1g YE + 0.1g CH	144 h	62	11
4	IV	BHM + 0.5g chitin + 0.1g YE + 0.2g CH	120 h	78	15
5	V	BHM + 0.5g chitin + 0.15g YE + 0.2g CH	144 h	96.5	18.55

Figures

Figure 1

Inhibition of test fungi by the actinobacterial cultures: inhibition of (a) *Fusarium oxysporum* by strain ANU 5a & ANU 5b, (B) *Aspergillus flavus* by strain ANU 5a & ANU 5b, (C) *A. flavus* by strain CAI 155a, (D) *A. flavus* by strain CAI 155b, (E) *Magnaporthe oryzae* by CAI 155a, CAI 127 & KAI 27b, (F) *Magnaporthe oryzae* by CAI 155b, CAI 140b & KAI 90.

Figure 2

Plates showing extracellular enzyme production, (A) CMC agar plate for cellulase activity, (B) Tributyrin agar plate for lipase activity, (C) Starch agar plate for amylase activity, (D) Gelatin agar plate for protease activity.

Figure 3

Chitinase Activity of promising Actinomycetes isolates.

Figure 4

Phylogenetic tree based on 16S rDNA gene sequences from strain ANU5a and other related organisms constructed using the neighbor-joining algorithm. (The tree was validated by bootstrap analysis (1,000 replications). Bar, 0.005 substitutions per 100 nucleotides.

Figure 5
Effect of pH (A) and temperature (B) on chitinase activity.

Figure 6
Effect of incubation period on chitinase (a) without additional supplement and (b) with additional supplements (Casein hydrolysate, yeast extract).

Figure 7

Effect of different chitin substrate material (A: 0.1g% α-chitin, B: 0.25g% α-chitin, C 0.5g% α-chitin, D: 1 g% α-chitin, E:α-chitin, F:colloidal chitin) on chitinase production by *Streptomyces* sp. ANU5a.

Figure 8

Effect of pH on chitinase production.

Figure 9

Effect of temperature on chitinase production.

Figure 10

Chitin degradation by *Streptomyces* sp. ANU5a. (A) Control sample: without inoculation (B) ANU5a treated β -chitin flaks, (C) Adhesion of *Streptomyces* sp. ANU5a on chitin flaks (D) Chitinolytic action of *Streptomyces* sp. ANU 5a on chitin flaks.

Figure 11

12% Native Page and zymogram analysis (agarose gel containing 0.1% Colloidal Chitin) of crude chitinase from culture filtrate of active isolate *Streptomyces* ANU5a. (A) Arrow indicates the size of crude enzyme (B) Lane 1 & 2 are the duplicates for enzyme and (M) is the broad range marker.