

Isolation and Characterization of Microorganisms for Insect Biocontrol by Chitin Deacetylase

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ABSTRACT: Extensive use of synthetic chemical insecticides has led to many environmental, agricultural and health hazards. To overcome these problems and also to protect the crops from insect pest which are responsible for huge loss of crop yield, enzymatic biocontrol may be opted. As Chitin is an important component of the insect cuticle it can be used as a target to control insect pests. So enzyme chitin deacetylase (CDA), which alters chitin, is used in the present study. Chitin deacetylase producing microorganisms were isolated and screened. Out of 142 microbial isolates, 91 were found to be CDA positive using PN strip and PN agar method. After enzyme assay only 2 isolates, one fungal and one bacterial, were selected. Their morphological and cultural characteristics were studied and identified as *Aspergillus flavus* and *Bacillus thuringiensis*. Cuticle of insect pupa has been tested for CDA action in its crude form. In conclusion, these two microbial isolates may be considered as potent CDA producing sources.

Keywords: Chitin; Chitosan; *Aspergillus flavus*; *Bacillus thuringiensis*.

INTRODUCTION: Among crops, the total global potential loss due to pests ranged from about 50% in wheat to more than 80% in cotton production.¹⁻² Extensive use of synthetic chemical insecticides results in the development of insect pest resistance. Besides, these insecticides are carcinogenic, recalcitrant, environmentally unsafe and health hazardous. An impending alternative to chemical insecticides is biological control of insect pest via degrading insect cuticle. Insect cuticle is a composite material consisting of a lipid-protein and chitin. Chitin fraction ranging from 20.0%-85.0% is found in calcified cuticles, intersegment membranes and hardened cuticle. Microorganisms such as entomopathogens can be used for biological control of insect pests.

Entomopathogens secrete different types of enzymes such as chitinase, chitin deacetylase, protease and lipase that damage insect cuticle, however direct application of enzymes may show fast and efficient results. One such enzyme chitin deacetylase (CDA; E.C. 3.5.1.41) is known to be produced by many different types of microorganisms.³⁻⁶ It plays an important role in softening insect cuticular chitin by converting it into chitosan⁷ and latter can be degraded easily by chitosanase which further facilitates the entry of fungal conidia into the insect. Microorganisms are potential source for enzyme production due to their advantages in terms of availability; stability;

cost efficiency; labor; yield and easy downstream processing.⁸⁻⁹ Till date, presence of CDA enzyme has been reported in several microorganisms such as *Colletotrichum lindemuthianum*¹⁰, *Gongronella butleri*¹¹, *Metarhizium anisopliae*¹², *Scopulariopsis brevicaulis*¹³, *Mortierella* sp. DY-52¹⁴, *Clostridium aminovalericum*¹⁵, *Vibrio parahaemolyticus* KN1699¹⁶. However, there is still a need to explore competent microorganisms which hold potential to produce bio active CDA. Therefore the present study has been undertaken to isolate, screen and identify efficient CDA producing bacterial and fungal microorganisms followed by its application to digest insect pupa chitin into chitosan.

MATERIALS AND METHODS:

Isolation and Screening of CDA producing microorganisms: Samples of soil, water and faecal matter collected from various places were placed on nutrient agar and Yeast extract peptone dextrose agar medium (pH-5.0) supplemented with 1% chitin and incubated at 37°C and 25°C for 2-3 days respectively for bacteria and fungi. Different isolated colonies were screened qualitatively for CDA production using PN strips¹⁷ dipped in broth fermented with isolate. PN-agar plates were prepared by adding p-nitroacetanilide in nutrient agar & YPD agar medium, inoculated with bacterial & fungal isolates and incubated at 37°C and

25°C respectively. The isolates producing yellow coloration in both methods were considered as positive for CDA production.

Production of crude enzyme: All the isolates showing positive results were grown in their respective medium for CDA production. These media were incubated at same temperature and time condition as in the earlier experiments. After incubation, the fermented medium was centrifuged at 10000 rpm for 20 min. and supernatant was used as crude enzyme.

Enzyme assay: Crude enzyme was used for quantitative estimation of chitin deacetylase by the method given by Kauss & Bausch (1988) using glycol chitin as substrate.¹⁸ Enzyme activity was calculated as International enzyme units using glucosamine as reference standard. One International unit of chitin deacetylase activity was defined as the amount of enzyme which released 1 µmol of glucosamine under the reaction condition and the enzyme production was expressed as IU/ml.

One fungal and one bacterial isolate with maximum CDA production were selected and characterized by conventional methods.

Identification and Characterization of isolates: The selected fungal and bacterial isolates were stained with Lactophenol cotton blue and gram stains and examined under microscope for morphological characteristics.

Cultural Characterization: Colony characteristics were studied by streaking the bacterial isolate on nutrient agar and by placing the agar disc of fungal culture on YPD agar medium. These selected isolates were inoculated in their respective broths and incubated for other cultural characteristics.

Biochemical Characterization: Various biochemical tests, including nitrate reduction, ONPG, malonate utilization, urease, catalase and sugar fermentation of bacterial isolate were performed using Identification Kit from Hi media.

Identification of isolates: The identification of the bacterial isolate was carried out by 'Xcelris genomics' using 16S rRNA sequence analysis. The fungal isolate was identified on the basis of morphological and cultural characteristics and confirmed by Forest Pathology Division, Forest Research Institute, Dehradun (India).

Application of Chitin deacetylase on cuticle of insect pupae (*Helicoverpa*):

Procurement of *Helicoverpa* pupae: Third instars of *Helicoverpa* pupae were collected from CCS Haryana Agricultural University, Hisar. All the collected pupae

were washed with distilled water and dried at 55°C. The dried pupae were stored in refrigerator for further use.

Treatment with CDA to pupae's cuticle: Cuticle of *Helicoverpa* pupae was incubated for an overnight with 2 ml of crude CDA enzyme solution. After incubation the cuticles were removed from the enzyme solution and washed with distilled water followed by MBTH (0.5 %) staining for 30 minutes to stain the chitosan which was formed by enzymatic action of CDA on cuticle chitin. The staining was visualized using olympus microscope.

RESULTS AND DISCUSSION:

CDA producing isolates: A total of 142 microorganisms were isolated, 91 isolates were found to be positive for CDA production as indicated by development of yellow coloration in PN strips (Figure 1) and PN agar plates (Figure 2).



Figure1: Detection of CDA positive isolates by development of yellow coloration using PN strips.

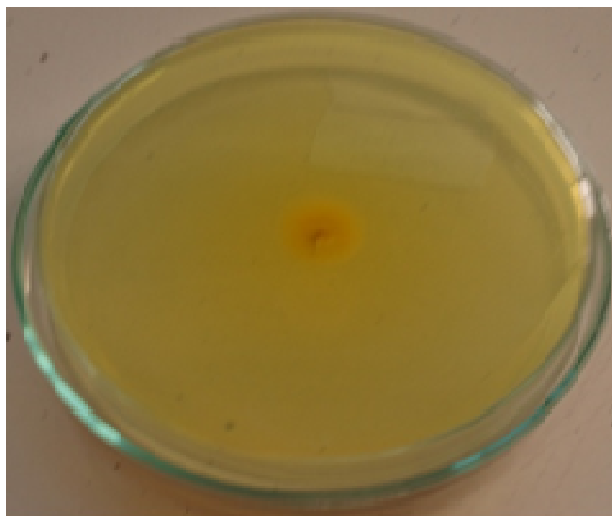


Figure 2: Detection of CDA positive isolate by development of yellow coloration on PN agar plate.

Screening of chitin deacetylase producing microorganisms: All the 91 isolates were screened for CDA production quantitatively. Highest CDA producing bacterial isolate NH74 with 79.88 IU/L and fungal isolate F3 with 74.07 IU/L were selected for identified and characterized.

Characterization of selected Isolates: The selected fungal isolate, F3 was grown on YPD agar plate and slide was prepared for microscopy. Fungal isolate F3 was identified as *Aspergillus flavus* from its colony morphology (Figure 3) and microscopic examination (Figure 4) according to manual of Kulwant Singh et.al. (1991) *Aspergillus flavus* and was further confirmed from Forest Research Institute, Dehradun (India).¹⁹ Some *Aspergillus* species such as *A. nidulans*²⁰, *A. niger*²¹ and *A. flavus*²² have been reported to produce CDA.

Colonies of bacterial isolate NH74 were large, flat, opaque, cream in color, spreading (Fig. 5a) and the cells were Gram positive rods in pairs and in short chains (Figure 5b). Results of biochemical tests are given in Table 1. Based on the biochemical and rRNA analysis, the bacterial isolate was identified as *Bacillus thuringiensis* and the rRNA data has been submitted to the NCBI database with accession number: **KP165532**. *Bacillus* is known for production of many extracellular enzymes. Some *Bacillus* species with CDA production have also found, before such as *Bacillus thermoleovorans*, *acidophilic Bacillus* sp., and *B. stearothermophilus*.²³⁻²⁶

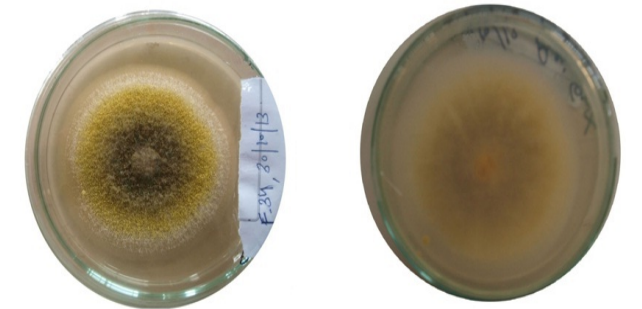


Figure 3: Fungal isolate F3 on YPD agar plate – top & bottom side.

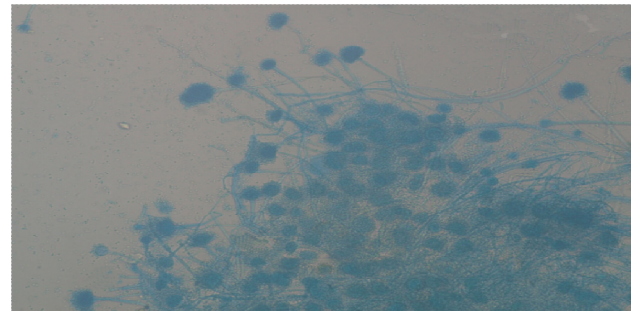


Figure 4: Microscopic view (40x) of fungal isolate F3.

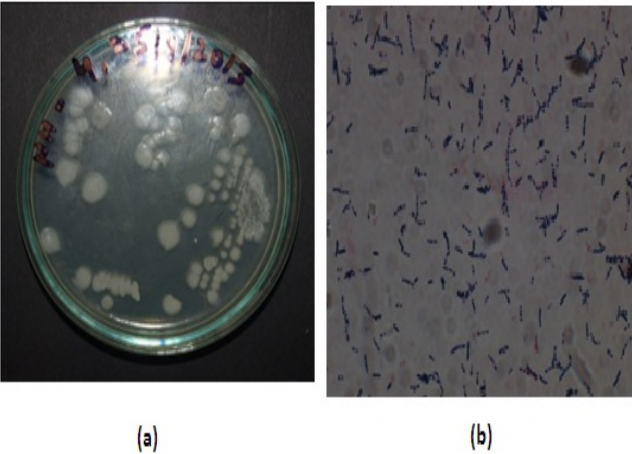


Figure 5: Bacterial isolate NH74 (a) Colonies on nutrient agar (b) Microscopic view (100x).

Table: 1: Biochemical tests of bacterial isolate NH74.

Test	Result	Test	Result
Malonate utilization	-ve	Lysine	+ve
Vogesproskauer	-ve	Ornithine utilization	+ve
Citrate utilization	-ve	Urease	+ve
ONPG	-ve	Phenyl alanine	-ve
Nitrate reduction	+ve	H ₂ S production	+ve
Catalase	+ve	Salicin	-ve
Arginine utilization	+ve	Xylose	-ve
Sucrose	+ve	Inositol	-ve
Mannitol	-ve	Sorbitol	-ve
Glucose	+ve	Rhamnose	+ve
Arabinose	-ve	Adonitol	+ve
Trehalose	+ve	Raffinose	-ve
Indole	-ve	Lactose	-ve

Identification of a bacterial culture using 16S rRNA based Molecular Technique: It has been found that 16 S sequencing is considered as one of the best methods to prepare accurate phylogenetic placement as well as taxonomic classification.²⁷ DNA was isolated from the bacterial isolate and quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed (Figure 6). The PCR

amplicon was purified and further processed for sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using 'BDT v3.1 Cycle sequencing kit' on ABI 3730xl Genetic Analyzer. Consensus sequence of 1287 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genebank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree (Figure 7) was constructed using MEGA 5. Based on the 16SrDNA analysis the bacterial isolate was identified as *Bacillus thuringiensis*.

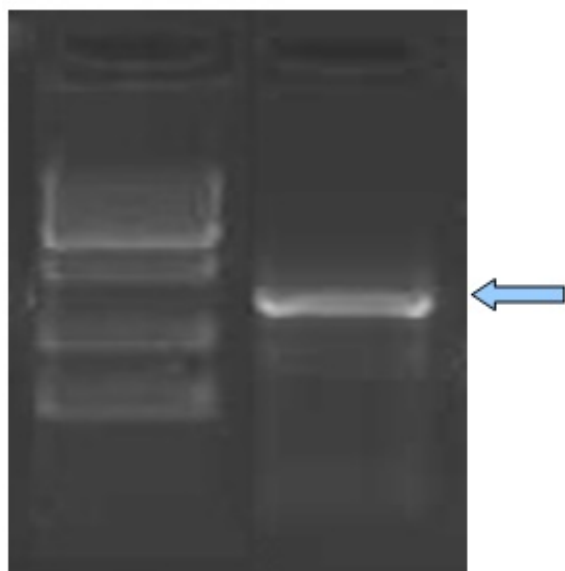


Figure 6: Single 1.5 kb and 16S rDNA amplicon on 1.2% Agarose gel.

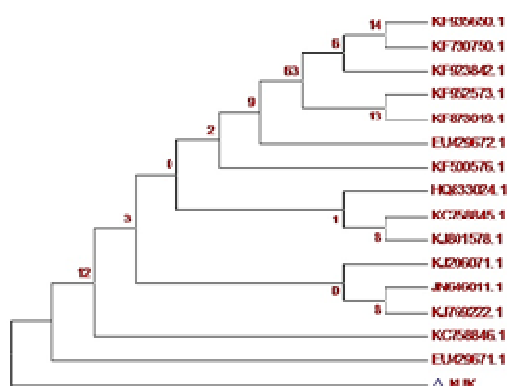


Figure 7: Evolutionary relationships of 11 taxa.

Biocontrol agent: Chitin is an important component of insect cuticle and it provides protection to the insect from external environment. Recently number of

insecticides have been developed which are based on either inhibition of chitin formation or responsible to soften the cuticle.⁷ Chitin deacetylase transforms chitin into chitosan. The bluish patches on cuticle of insect pupae were observed with MBTH staining which indicate the conversion of chitin to chitosan due to the action of CDA. Similarly, blue patches of MBTH stained chitosan have been reported on *H. armigera* pupae cuticle using CDA from *Metarhizium anisopliae* by Pallavi Nahar and her colleagues.²⁸ It shows that chitin deacetylase activity is important in softening the insect cuticle which can further help in initiating the microbial pathogenesis of the insects for their biocontrol.

CONCLUSION: Crop protection always aims to avoid or prevent crop losses or to reduce them to economically acceptable level. However, the losses due to pests have been enormously increased from last few years. Furthermore, a great deal of Insect pests has developed resistance to the insecticides resulting into need of development of more potent and alternative biological control system like enzymes. In present study, we described the isolation and identification of microorganisms which produced one such enzyme i.e. chitin deacetylase and tested the latter for biocontrol of *Helicoverpa*.

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