



ISOLATION, PRODUCTION AND CHARACTERIZATION OF EXTRACELLULAR PECTINASE FROM *Aspergillus niger* K3

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ABSTRACT

A pectinase producing fungus was isolated from compost soil. Morphological characteristics, microscopic observations and molecular characterization based on internal transcribed spacer (ITS) sequences led to its identification as *Aspergillus niger* (GenBank Accession Number: JX556221) and it was designated as *Aspergillus niger* strain K3. In the present study, the culture condition of the media was found to have a marked effect on pectinase production. The optimum pH and temperature for maximum pectinase activity was 8.0 (70U/ml) and 45⁰C (90U/ml), respectively. The best carbon sources that induce pectinase production was found to be fructose (40U/ml) followed by maltose (38U/ml). Among the nitrogen sources, ammonium nitrate (80U/ml) produced maximum pectinase followed by peptone (72U/ml).

KEYWORDS: Pectinase, pectin esterase, pectin, *Aspergillus niger*, ITS region.



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INTRODUCTION

Pectin is a polymeric material having carbohydrate group esterified with methanol. It is an important component of plant cell wall. It is present in highest concentration in the middle lamella, where it acts as a cementing substance between adjacent cells. Pectinase are enzymes which degrade pectic substances and are of great importance to the food industry. These enzymes are used to facilitate extraction, filtration and clarification and to increase yields in the production of fruit juices and beverages¹. Pectinase is extensively used in food processing industry, souring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extractions, tea and coffee fermentation, bleaching of paper, in the alcoholic beverage². These enzymes are classified according to the criteria whether pectinase acts by transelimination or hydrolysis and they are pectin esterase (PE), depolymerizing enzymes and propectinase². The ability to synthesise pectinases is very common in many microorganisms viz., bacteria, yeast, and fungi^{3, 4}. Fungi have been considered among the most important industrial microorganisms mainly due to their capacity for secretion of the enzymes in the culture medium^{5, 6, 7, 8}. Among fungi, *Aspergillus niger* is commonly used strain for the industrial production of pectinase, because it is regarded as a safe microorganism for its application in the food processing industry^{4, 9}. Selection of the microbial source for enzyme production depends on several features, such as the type of culture (solid-state or submerged fermentation), number and type of the produced pectinase, incubation time, agitation, concentration, pH and thermal stability of the enzymes, and genotypic characteristic of the strain⁶. In this context, the objective of the present study was to produce pectinolytic enzymes by a newly isolated strain of *Aspergillus* sp. by submerged fermentation and to partially characterize the enzymes.

MATERIALS AND METHODS

Sampling and isolation of Fungi

The soil was collected from the compost soil from the Padmashree campus, Padmashree Group of Institutions, Bangalore, India. 5 g of soil sample was taken and performed serial dilution. The soil sample which is serially diluted was poured on to potato dextrose agar (PDA) plates with respective dilutions from 10^{-2} – 10^{-7} . This was incubated for one week at 28°C. Individual fungal isolates were further subcultured on the respective medium in order to obtain pure culture. Pure isolates were maintained at 4°C in refrigerator for further studies.

Screening of soil fungal isolates for pectinolytic activity

A total of 6 isolates (K1, K2, K3, K4, K5 and K6) from soil were assayed for polygalacturonase (PG) activity using pectin containing agar medium (1 g pectin, 0.3 g Diammonium orthophosphate; 0.2 g; KH_2PO_4 ; 0.3 g K_2HPO_4 , 0.01 g MgSO_4 and 2.5 g agar (for 100 mL). The initial pH of medium was adjusted to 4.5. Culture plates with pectin-containing agar were inoculated with each isolate and incubated for 3-5 days at room temperature. After incubation, plates were stained with aqueous 0.05% ruthenium red solution for 1h and rinsed with deionised water. Cultures expressing pectinase activity exhibited a clear zone around the margins of the colony. Isolates without a clear zone usually exhibited a ring of intense staining around the colony. The isolate K3 producing highest clear zone was considered for further investigation.

Identification of fungal isolate

The isolated fungal culture was identified as *Aspergillus* sp. based on its morphological and microscopic characteristics and these values matched with values in standard reference book compendium of soil fungi¹⁰. Molecular characterization was also performed as shown below

Fungal DNA extraction

DNA was extracted from the fungus using fungal genomic DNA extraction kit (Bhat

Biotech Pvt. Ltd., Bangalore). DNA extracts were stored at -20°C prior to use.

PCR Amplification

Amplification of the ITS region (ITS1, ITS2, and 5.8 S rRNA Gene) was performed using the Universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions were performed in an Eppendorf Mastercycler gradient (Eppendorf, Germany). Thermal cycling was carried out using an initial denaturation step at 95°C for 5 min, followed by 30 cycles of de-naturation at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 60 s. Cycling was completed by a final elongation step at 72°C for 10 min. The ~600 bp PCR product was purified by gel elution and used for sequencing.

Sequencing

Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequencer -3037xl DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from Applied biosystems. The sequences obtained for plus and minus strands were aligned manually before performing the bioinformatics analysis. Subsequently, the sequence had been submitted to Genbank database.

Bioinformatics analysis

Sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file

was then used to create phylogram using MEGA5 software^[14].

Enzyme production by submerged fermentation

Inoculum preparation

The fungal inoculum was prepared with addition of 10ml of sterile distilled water to the 7th day slant and was shaken well to obtain homogeneous spore suspension. The fungal culture was used to produce pectinase enzyme using liquid medium (g/L) citrus pectin, 10; (NH₄)₂SO₄, 1.4; K₂HPO₄, 6; KH₂PO₄, 2; MgSO₄.7H₂O, 0.1; pH, 6). Fermentation was carried out in 500 mL Erlenmeyer flask containing 250 mL of growth medium with 10% inoculum (10⁶ spores/mL) and incubated at 28°C under shaking conditions (150 rpm) for 5 days. The biomass was separated by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was used to evaluate the pectinase enzyme activity.

Enzyme Assay

The crude Pectinase enzyme produced by isolates was assayed using the standard protocol of Sigma¹¹. The crude enzyme extracts of different isolates were prepared by the method used to estimate total extra cellular protein. Enzyme assay solution was prepared using 5 ml of 0.5% Pectin solution, 5ml of 500mM iodine (200mM Potassium iodide), 1.0 ml of 1M Na₂CO₃ and 20 ml of 2M H₂SO₄. The enzyme assay solution thus prepared was mixed with 0.1 ml crude enzyme extracts (for samples) and with distilled water for Blank. These assay solution were incubated for 15 min at room temperature and then titrated against 100mM sodium thiosulphate solution to obtain a colourless solution with 1% starch solution indicator. The enzyme activity was calculated by using the following formula:

$$(1) (100) (\text{ml of reagent E for blank} - \text{ml of reagent E for test}) (\text{df})$$

Units / ml enzyme =

$$\frac{\quad}{(5) (0.1) (2)}$$

Effect of incubation period, temperature and pH on pectinase production

The effect of incubation period for pectinase production was determined by incubating production medium at different incubation period viz., 24, 48, 72 and 96h. To study the effect of different temperature on pectinase production such as room temperature, 37°C, 45°C, 65°C was maintained during fermentation process. To study the effect of different pH on pectinase activity, pH 4 to pH 9 were maintained in media during fermentation process.

Effect of different carbon and nitrogen sources on pectinase production

To study the effect of different carbon sources on pectinase activity various carbon sources are used in the media viz., glucose, lactose, sucrose, mannitol and starch. To study the effect of different nitrogen sources on pectinase production, organic nitrogen sources such as peptone, malt extract, casein and inorganic sources such as ammonium nitrate, ammonium chloride were used in the media. The experiments were conducted in triplicates and the results presented are the mean values with standard error.

RESULTS AND DISCUSSION

Isolation, screening and identification of strains for pectinase production

Total, 6 fungal isolates were obtained, among them only one strain (Sample A) was screened as a potent degrader of pectin and showed clear lysis zones on pectin agar plates. The fungal isolate was characterized on the basis of morphology, microscopic appearance and biochemical tests. Molecular characterization of this strain was done by DNA isolation (CTAB method) and ITS region analysis (Fig. 1). Further these amplified ITS region sequence of the fungal strain was blasted using online tool. The taxonomical identification was done by the phylogenetic tree construction and the comparison of this fungal strain sequences with other homologous fungal sequences. After morphological, physiological, biochemical and taxonomical identification, the isolate was identified as *Aspergillus* sp. As per the results obtained the strain was identified as *Aspergillus niger* (GenBank Accession Number: JX556221) (Fig. 2). *Aspergillus* sp. is known to produce multiple enzymes of industrial importance¹².

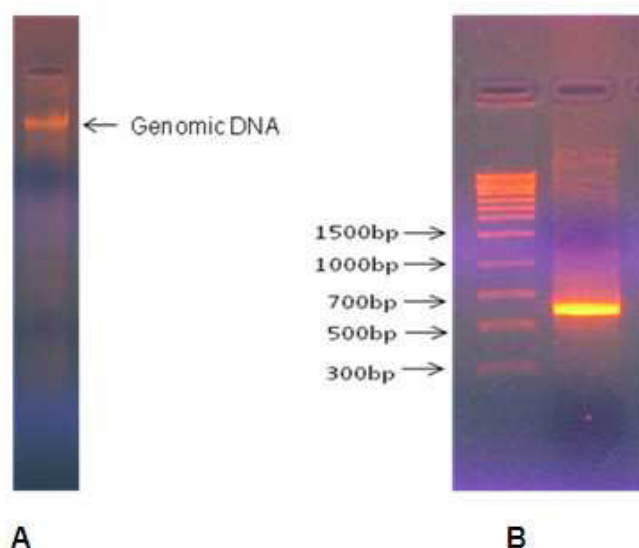


Figure 1

0.5% Agarose gel electrophoresis showing band of fungal genomic DNA (A) and 1% agarose gel electrophoresis showing PCR amplification of the ITS region (ITS1, ITS2, and 5.8 S rRNA Gene) product of ~600bp (B)

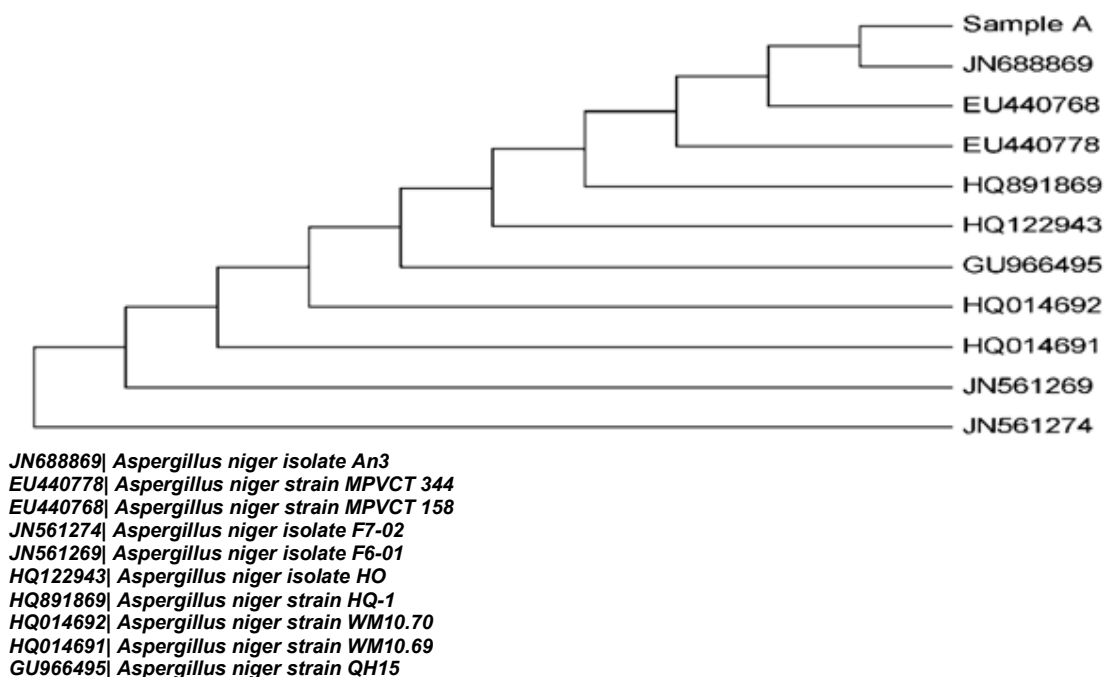


Figure 2
Phylogenetic tree for the fungal isolate (Sample A)

Optimization of cultural conditions

Culture conditions such as incubation period, pH, temperature, source of carbon, nitrogen are known to influence the synthesis and secretion of extracellular enzymes by microorganisms^{13, 14, 15}. Optimization of the culture conditions is hence, necessary in the selection of the fungal source for industrial exploitation of their extracellular enzymes.

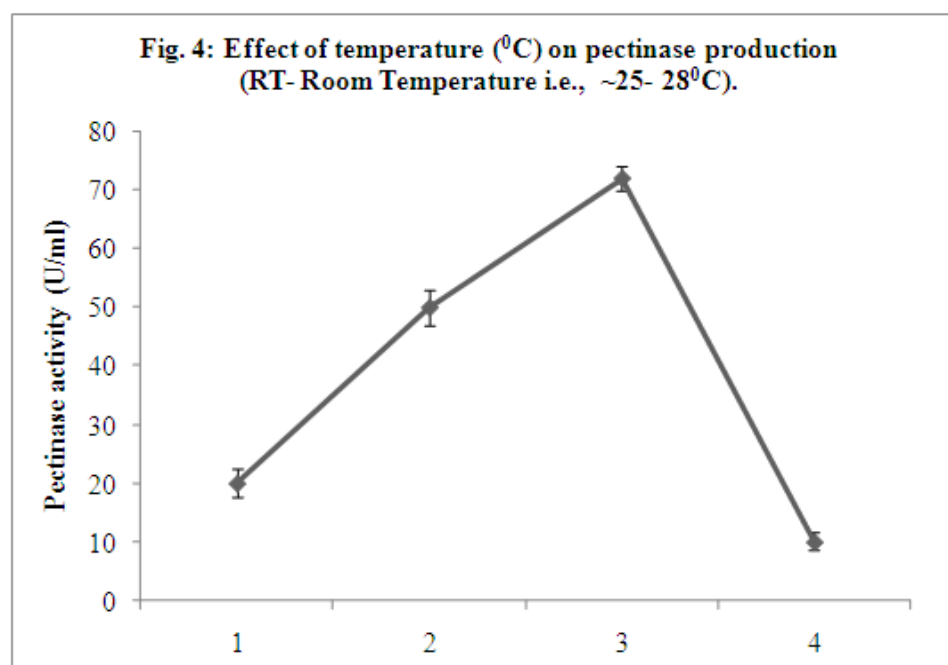
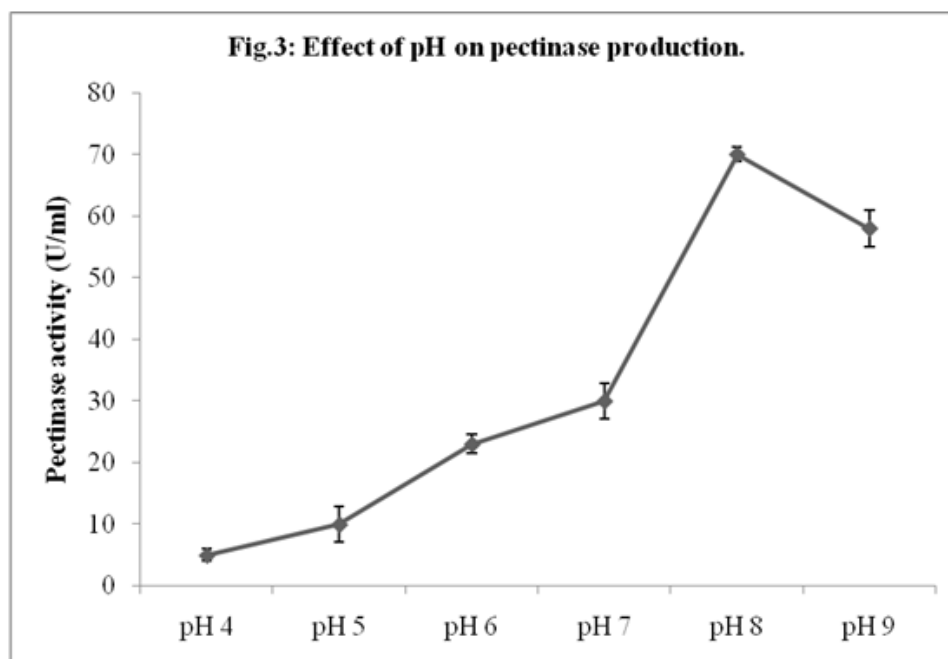
Effect of pH on pectinase production

The enzymatic hydrolysis of pectic substances also depends on several physiochemical factors. In the present study, the pH of the media was found to have a marked effect on pectinase production (Fig. 3). Preliminary studies were carried out to determine the best pH for maximum pectinase production. From the result it can be seen that pH 8 was found to give maximum pectinase activity of 70 U/ml.

The pH optima of the previously reported pectinase have been found to be acidic for *Penicillium canescens* (5.5)¹⁶, neutral for *Penicillium expansum*¹⁷ and basic for *Aspergillus flavus* (8.0)¹⁸ and *Aspergillus terricola* (8.0)¹⁹.

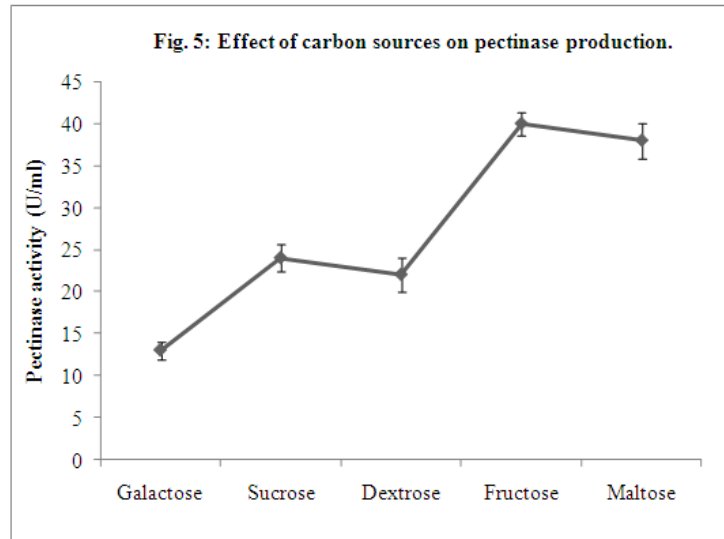
Effect of temperature on pectinase production

Incubation temperature has been found to be a significant controlling factor for enzyme production²⁰. Fig. 4 showed that the temperature has a great influence on the production of pectinase. Maximum enzyme activity 72 U/ml was found at 45⁰ C and lower activity 20U/ml was showed at room temperature (25⁰-28⁰C). Similar results were obtained by Nazneen akhter *et al.*, (2011) in *Aspergillus niger*²¹.



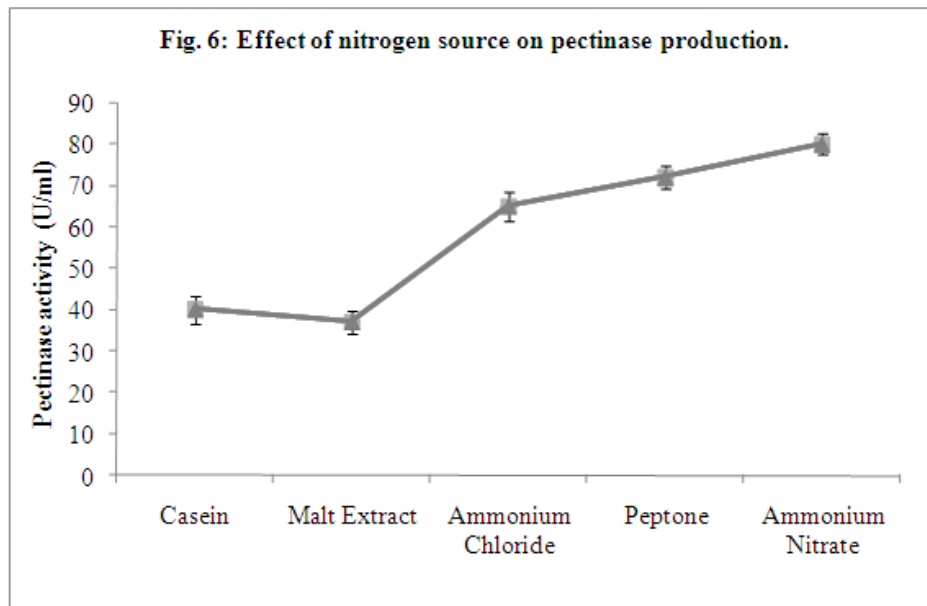
Effect of carbon sources on pectinase production

Type and nature of the carbon source is one of the most important factors for any fermentation process. Carbon source represents the energy source that will be available for the growth of the microorganism. Fig. 5 shows the effect of supplementation of the substrate with mono-, di- and polysaccharides on enzyme production. A maximum enzyme activity of 40 U/ml was obtained with fructose and maltose. Similar results are observed by Rajmane and Korekar (2012) in *Aspergillus* sp.²². In the present study, composition of the medium has showed significant effect on the growth of *A. niger* K3 and production of pectinase enzyme in surface fermentation.



Effect of nitrogen sources on pectinase production

Nitrogen is the basic element of the cellular components. Fig. 6 shows the effect of nitrogen sources on the productivity of pectinase. The maximum pectinase activity of 80 U/ml was obtained with ammonium nitrate. Similarly in *Aspergillus niger*, 84U/ml of pectinase activity was observed when ammonium nitrate was used in the media²¹. Significant results were also observed when peptone and ammonium chloride were supplemented in the production media.



CONCLUSION

The results from the study show that the locally isolated *Aspergillus niger* K3. can be considered as a possible candidate for large scale production of extracellular pectinases. The isolate produces appreciable amount of pectinases at 72 h of incubation. The optimum pH and temperature for maximum enzyme production was found to be pH, 8 and 45⁰ C respectively. The best carbon and nitrogen sources were fructose and ammonium nitrate. Therefore it can be concluded that this isolate can be industrially exploited for the large scale production of pectinase enzyme.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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