Rapid Confirmation and Molecular Identification of Alkaline Protease Producing Aspergillus awamori through Submerged Fermentation

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Abstract

Recent years have witnessed a phenomenal increase in the use of enzymes as industrial catalysts. Proteases constitute a very large and complex group of enzymes, widely utilized in a host of industries. Industrially important efficient alkaline protease producer was isolated from soil samples collected from different regions of Bangalore. Among the 25 isolates Aspergillus awamori KGSR 12 evolved as an potential protease producer by plate assay method and it showed 1.2 cm diameter, 46.15% hydrolysis. The protease were confirmed by TLC method by Rf value i.e 0.84. The molecular tool used for identification of Aspergillus awamori KGSR 12 and also optimum percentage of hydrolysis (0.5%) were determined.

Keywords
Alkaline protease, Molecular confirmation, Casein and Submerged fermentation.

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Introduction

Alkaline proteases are the proteases which have proteolytic activity at pH range of 8 to 14. Due to its stability at high alkaline conditions, alkaline proteases were found its great interest in physiological, industrial, biotechnological and economical applications (Maurer, 2004; Lin et al., 2006; Saeki et al., 2007; Shivasharana and Naik, 2012; Gomaa, 2013; Lakshmi et al., 2014). They are used in detergent industry, textile industry, tanning, leather processing, silk gumming, food processing, pharmaceutical industry, silver recovery, resolution of amino acid mixtures, waste treatment or bioremediation, biotransformation, feather processing (Adler-Nissen, 1986; Nout and Rombouts, 1990; Fujiwara et al., 1991; Pasupuleti, 1998, 2005; Rao et al., 1998; Ota et al., 1999; Kristinsson and Rasco, 2000; Nakiboglu et al., 2001; Garcia-Carreno et al., 2002; Gupta et al., 2002; Freddi et al., 2003; Lin et al., 2006; Bhaskar et al., 2007; Ma et al., 2007; Sareen et al., 2008; Jellouli et al., 2009; Pasupuleti and Demain, 2010; Romsomsa et al., 2010; Shankar et al., 2010; Nasri et al., 2011; Johnny and Chinnammal, 2012; Shivasharana and Naik, 2012; Feng et al., 2013; Gomaa, 2013; Jisha
et al., 2013; Kalantzi et al., 2013; Sanatan, et al., 2013; Anju et al., 2014; Lakshmi et al., 2014; Nosenko et al., 2015; Antony and Chinnamal, 2015; Wisuthiphaet and Kongruang, 2015; Pilli and Siddalingeshwara, 2016; Verma et al., 2016).

Alkaline proteases are produced by all microorganisms like bacteria, fungi and actinomycetes which are not only alkalophilic or halophilic habitats but also non-alkalophilic habitats (Horikoshi, 1990; Grant and Jones, 2000; Gomaa, 2013). Though primarily commercial alkaline proteases are produced by bacteria, predominantly Bacillus sp., fungi are also found its place due to its easy, safe and economic enzyme recovery processing (Gupta et al., 2002; Bhaskar et al., 2007; Sareen et al., 2008; Lakshmi et al., 2014).

Thus it is desirable to search for the new alkaline proteases with novel properties produced by filamentous fungi. In this paper we found that fungi isolated from environmentally stresses, non-alkalophilic soil also could produce an alkaline protease which can exhibit vast range of alkalinity which is found to be the major criteria for the above industrial applications.

This paper highlights on the isolation, screening and molecular identification of the Aspergillus awamori and also confirmation of protease production by thin layer chromatography (TLC).

Isolation and screening of protease producing fungi

Aspergillus awamori (Plate-1) were isolated from the soil sample on CzapexDox agar (CZA) medium. CZA medium composition is as follows. Glucose, 30; NaNO₃, 2; K₂HPO₄, 1; MgSO₄, 0.5; KCl, 0.5; FeSO₄, 0.010; Agar, 15 and pH 6.5 (g/L Distilled water), followed serial dilution (Walksman, 1927) and spread plate method (Cappuccino and Sherman, 2002). The isolated strains were screened for their protease production by plate assay.

The screening medium is as follows. Glucose, 2; skim milk, 0.5 or casein, 0.5; KH₂PO₄, 1.52; KCl, 0.52; MgSO₄, 7H₂O, 0.52; CuNO₃, 3H₂O, trace; ZnSO₄, 7H₂O, trace; FeSO₄, trace; agar, 20.0 and pH 5.0 (g/L distilled water). The clear zone was observed around the colony at regular intervals of time and measured the diameter of clear zone and calculated percentage of hydrolysis. Aspergillus awamori were showed (Plate-2) the best zone of clearance and has produced the high percentage of hydrolysis was considered for further studies.

Rapid Confirmation of Protease by Thin layer chromatography (TLC)

The samples were withdrawn periodically at 24 h in aseptic condition. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for TLC.

The protease production of Aspergillus awamori KGSR12 was confirmed (Plate-3)

Materials and Methods

Sample

Soil samples were selected for the isolation of desired fungi. The soil samples were taken from different regions of Bangalore (12°59’ N latitude and 77°35’ E longitude) of Karnataka.
by subjecting the enzyme extract to thin layer chromatography (TLC). Here the separation and identification of amino acid i.e., tyrosine is a hydrolysed product were carried out by thin layer chromatography technique by using silica gel G and saturated phenol with water used as a solvent system. Rf values were calculated.

**Molecular identification of fungal strain**

The fungal strain *Aspergillus awamori* KGSR12 was identified morphologically (Plate-4), microscopically (Plate-5) and molecular level to confirm its identity.

On CZA plate: Growth rate is rapid and texture of colonies varies from downy to powdery and is produced radial fissures in the agar. Surface colony is initially white becoming yellow to green colour with conidial production while reverse is pale coloured and medium turned orange colour gradually.

**Preparation of culture for colony PCR**

The fungal strain was grown on Capek-Dox agar slant at 30°C for 5 days. This freshly prepared culture was ready for the colony PCR for the identification of the strain.

**PCR amplification**

To avoid any laboratory contamination, the PCR was performed on colonies picked directly from the agar slant culture. The 5.8-ITS (internal transcribed spacer) region was amplified by PCR using universal fungal primers ITS 1 F 5’-TCCGTTAGGTG AACCTGCGG-3’ and ITS4 R 5’-TCCTC CGCTTATTTGATATGC-3’ with amplicon of 600bp (White et al., 1990; Lv et al., 2012; Shilpa et al., 2016). PCR reactions were performed in a total volume of 50 µl of 0.2 ml thin walled PCR tube. The composition contained 33 µl nuclease free water, 1 pick of colony from plate, 2.0 µl of Forward Primer (10 µM), 2.0 µl of Reverse Primer (10 µM), 10 µl of 10X Reaction buffer, 2 µl of dNTP Mix (10mM) and 1µl of Taq DNA polymerase (2.5 U/µl). The amplification reactions were carried out in a Master cycler® Thermocycler using a PCR program (Phalip et al., 2004; Lv et al., 2012; Shilpa et al., 2016), as 10 min of initial denaturation at 95°C, 1 min of denaturation at 94°C, 1 min of annealing at 56°C, 1 min of extension at 72°C and 10 min of final extension at 72°C. The following program was repeated from 1 min of denaturation at 94°C to 1 min of extension at 72°C for 35 cycles. The PCR products from ITS gene PCR reactions were purified to remove unincorporated dNTPS and primers before sequencing using PCR purification kit (GENEI GEL ELUTION KIT).

**Sequencing**

Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequence -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 using appropriate software prior to phylogenetic analysis.

**Phylogenetic analysis**

Sequences were compared to the non-redundant NCBI (National Center for Biotechnology Information) database using BLASTN (Nucleotide Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), with the default settings used to find sequences closest to
each other. The Expected value and e values were noted for the most similar sequences. Ten similar neighbors were aligned using CLUSTAL W2. The multiple-alignment file thus obtained was then used to create a Phylogram using the MEGA5 software.

Hydrolysis of casein at different conc. by A. awamori

Skimmed milk agar plates were prepared with different concentrations of skimmed milk (Amul milk powder) as 0.25%, 0.5%, 0.75% and 1.0%. They were incubated at 30°C and observed for the clear zone at regular intervals of time. The percentage of hydrolysis was calculated.

Results and Discussion

Fungal isolates

In the present study, twenty five fungal strains were isolated from the different environmentally stress soil samples. The potential strains were selected based on clear zone around the colony by plate assay. The results from plate assay were presented in plate – 2. Results revealed that 46.15% of the isolates showed hydrolysis on casein agar plates. Therefore, for the convenience, 46.15% of protease producing fungal isolates was grouped on the basis of zone of diameter they exhibited.

According to Siddalingeshwara and Lingappa (2010). According to this segregation (Plate-5), Aspergillus awamori KGSR12 exhibited the higher zone of diameter 1.2 cm and considered as potential strain for protease production among the strains isolated from the soil. So the fungal strain KGSR12 was selected for the further studies (Plate-1, 2).

Confirmation of protease production through TLC

The conformational studies by using Thin Layer Chromatography reported that Rf value of the crude enzyme was 0.84 while the tyrosine standard reported 0.88.

Molecular identification

The ITS1 and ITS4 primers were used to amplify the ribosomal region which includes the non-coding ITS1 and ITS2 and the 5.8 rRNA gene (White et al. 1990; Lee and Taylor, 1992 and Lv et al., 2012). The amplified ITS regions were run on 1.0% Agarose gel electrophoresis showed PCR product of approx. 600bp and represented (Plate-6).

Plate 1 Aspergillus awamori
**Plate.2** Screening of Protease producer by plate assay

**Plate.3** TLC Plate for confirmation of protease

**Plate.4** Microscopic view of *Aspergillus awamori*
Plate.5 Segregation of fungal isolates based on protease production

Plate.6 1.0% Agarose gel electrophoretic PCR product of 600bp

Plate.7 The aligned sequence of ITS region

ITS F
CGAGGTCACCTGGAAAAATGGTTGAAAAACGTGGCAGCGCGCCCGCAATCTACTACAG
AGCATTGACAAAGCCCATACGCTCAAGGATCGGACGCGTGCCGCCTGCCCTT
TCGGGCGCCTCCCGGAGAGGGGAGCGACCCAAGCACACACACACAAGCCCGGCTTGA
GGGAACATGACGCTCGGACAGGCATGCCCCCGGAAATACCAGGGGCACGTGTT
GCGTCCAAGACCTCGAGATGCTACCAATCTGCAATATCCATTTATCGCACTTTTG
GCTGCGTTCTTCTCAGATGCGGAGGCAAGCAATGATTGAGCGGAGCTCC
GTCATTCAATCAAACCTCAGACTCGACGCTCCACTTACAGACATGGTGGTGTCC
GGCCGCGACAGGGCGGCCGGGGCAAGGCACCCCAGGCGCGAGCAAGCGCGG
GCCGCCGAAGCAGCGGTGTTATACAGACGCGATGGAGGCTGTGGCGGGCCAAAGGA
CCCGCCTCGTATGATGACCTCCTCGGAGGCTACCTACGAAGATTACGGAGTACGG
GTCATTGGGCGCCTCCCATGCCTGCCTGATACCATCCGCTTGGCGGGGCGCCG
CGCTAGTCGGCCGCCGGGGGGGCTTTGCCCCCAGCCCCCCTGTCGACACACAAACAGATCTATATAGCTTAAGTGTCACTCGGTATCCCTACCTGATCTGAGGTCAACCTGAAAAATGGTTGGAAAACGTGCGAAGCGCGCCGCCGCAATCTCACAAGAGCTGACGAGTATGCTTTGATGGGACGCATCAATAAATTCAACAGGACAGTGTCTGGTGCGACGTCGACTAATA

**18S R**

AGTCCCTCGCTTTATTTGATATGCTTAAGTGTCACTGTCGACAGCAGACACCAAAACGATCTATATAAGATGTCTTCAATGGTATTTGATGGGACGCATCAATAAATTCAACAGGACAGTGTCTGGTGCGACGTCGACTAATA

**Effect of casein/skimmed milk concentration on hydrolysis**

Plate 8: Phylogenetic tree

Plate 9: Effect of casein/skimmed milk concentration on hydrolysis
The sequence of the ITS (Plate-7) gene from fungal strain KGSR12 and that of matching sequences from 10 nucleotide sequences were aligned by using the Maximum Likelihood method based on the Tamura-Nei model (Shilpa et al., 2016). The tree with the highest log likelihood (-1422.4280) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows.

When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 517 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Plate-8).

**Effect of concentration of casein/skimmed milk in hydrolysis with Aspergillus awamori KGSR 12**

The clear zone was observed on the skimmed milk agar plates on 24 h culture and the zone was increased with the time period. Different concentrations of casein on the plates have showed variation in the zone of clearance. The diameter of hydrolyzed zone of the 48h culture showed 0.6 cm, 1.4 cm, 0.6 cm and 0.3 cm on the casein agar plates with concentrations 0.25%, 0.5%, 0.75% and 1.0% respectively and their percentage of hydrolysis were 25%, 50%, 33.3% and 13.04% successively (Plate-9). The results showed that the concentration of the casein also effects in the initiation of the protease production. But the plate with conc. of 0.5% skimmed milk showed the best result in 48h. This indicates that the initial phases of increasing was promoted the protease production (from 0.25% to 0.5% casein conc.) but the further increase of casein conc. was demoting the protease production (from 0.5% to 0.75% and 1.0%).

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