Biocatalysis and Agricultural Biotechnology 5 (2016) 58–68

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

# Magnesium dependent proteinaceous protease inhibitor with pesticidal potential from alkali-halotolerant *Streptomyces spp.*: Optimization of production using statistical tools

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ARTICLE INFO

*Article history:*
Received 29 September 2015
Received in revised form
26 November 2015
Accepted 30 November 2015
Available online 2 December 2015

*Keywords:*
*Streptomyces spp.*
Protease inhibitor
PBD
RSM
Soda lake

ABSTRACT

The extensive diversity of microbial proteases in several physiological and industrial applications promoted search for newer protease inhibitors. Of 80 *actinomycetes* isolated from soil–water sediments of Soda lake on starch casein medium (1 M NaCl, pH 9.5) at 30 °C, only 5 have shown significant protease inhibitor (PI) activity (≃20 U) against trypsin. Based on the preliminary phenetic and 16S rRNA gene sequence, the closest related to VL 18, VL 50, VL 61 and VL J2 isolates was *actinomycetes* (99% identity). PI activity of culture broth of short listed strains ranged between 26.90 to 30.72 U. Alternative one variable assortments at a time (OVAT) for PI production by *Streptomyces spp.* VL J2 by optimized medium yielded 55.34 U within 96 h, the highest among the 5 isolates. Further, Plackett–Burman design (PBD) identified starch, $KNO_3$ and $MgSO_4$ for PI production by VL J2 strain as the most significant and they were further optimized by Response Surface Method (RSM) using a central composite design (CCD). Accordingly, 2.49% starch, 0.114% $MgSO_4$ and 0.249% $KNO_3$ were found to yield 72 U of PI by the *Streptomyces sp.* VL J2 in 96 h. The purified PI has molecular mass of ≃35 kD and showed inhibition of fungal mycelial growth and mid gut proteases of insect indicating the scope for its application as a bio control agent. The statistical tools employed in the present study showed 30% increase in PI production over OVAT approach and $MgSO_4$ was found to be a key factor in its production.



## 1. Introduction

Proteolytic enzymes ( > 560) are indispensable for metabolic and regulatory functions as well as crucial for homeostatic control in both prokaryotes and eukaryotes (Drag and Salvesen, 2010; Karthik et al., 2014) and their activity in all life forms is under the control of inhibitors, protease inhibitors (PIs). These molecules of microbial origin are identified as versatile tools in the clinical medicine, agriculture and biotechnology (Sobotic and Kos, 2012). Selective and specific protease inhibition potential of PIs can be promoted to (i) unravel enzyme structures, (ii) treat microbial infections (hepatitis, herpes, AIDS, Aspergillosis) and mortal diseases like arthritis, muscular dystrophy, emphysema, malaria, hypertension, cancer, obesity, cardiovascular, inflammatory, neurodegenerative, cystic fibrosis etc. (Lopez-Otin and Bond, 2008; Karthik et al., 2014), (iii) control herbivorous pests, fungi (Dunse et al., 2010), post-harvest microbial infections (Baird-Parker, 2003), (iv) extend shelf life of protein foods (Bijina et al., 2011b) and (v) stabilize proteases in commercial products (Ganz et al., 2004). Though majority of protease inhibitors reported are of plant origin (Ryan, 1990; Silverman et al., 2001), but microbes remain the preferred source because of (i) rapid growth, (ii) amenability for genetic modifications, (iii) ease of cultivations, (iv) greater versatility in structure and mechanism (Rao et al., 1998), (v) display of unique inhibitory profiles, (v) extensive diversity (Rawlings et al., 2011), (vi) high thermal and broad pH stability and (vii) resistance to proteolytic cleavage. The current fermentative production using microbes was impeded due to lower yield, cost intensive process and difficulties in recovery thereby, preventing its wide use from bench to business. Even large scale production of PI using recombinant techniques is possible, but target PI accumulates and precipitates in host cells as inclusion bodies resulting in low recovery.

Although, much progress has been made in the purification and biochemical characterization of PIs of *Streptomyces spp.*, but no concerted efforts have been attempted yet on the economic production of PIs through optimization of constituents and cultural parameters. The efficient production of PIs involves studies on the reaction parameters either by one variable assortment at a time (OVAT) or statistical tools like Plackett–Burman Design (PBD) or

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http://dx.doi.org/10.1016/j.bcab.2015.11.007
1878-8181/

response surface methodology (RSM). The former approach is tedious, time consuming and unable to determine the interactive effects among input variables, but PBD identifies the significant variables affecting production of PI, predicts the true optimum and allows unbiased estimate of linear effects of all variables (Pal et al., 2009). The statistical approach has been effectively applied to optimize the nutritional and process parameter in submerged and solid state fermentation especially bio-surfactants (Arthala et al., 2015), cleanup of hydrophobic contaminants (Timma et al., 2014) and enzyme inhibitors (Karthik et al., 2013).

PIs from *Streptomyces* of hostile habitats may display stability against harsh conditions prevailing in mid gut of insects (Sobotic and Kos, 2012) and could be potent inhibitors of their proteases (Harsulkar et al., 1999). Hence, screening and isolation of microbes prevalent in harsh habitats may contribute to desirable PIs for industrial/ agricultural purpose. On this premise, the present study was undertaken to screen and isolate potential *Streptomyces* strains from alkaline-saline habitat for the production of PI, optimize medium constituents as well as process parameters to enhance the PI production using statistical tools like PBD, RSM and possible applications towards plant protection.

## 2. Materials and methods

### 2.1. Bacterial strains and cultivation condition

*Actinomycete* strains were isolated from soil sediments of soda lake (natural impact meteorite crater in basaltic rock during Pleistocene Epoch; Lonar, MS, India, Cordinate: 19°58′36″N, 76°30′ 30″ E) and maintained on starch casein agar (pH 9.0 and 0.5 M NaCl) at 4 °C. Each strain of *Actinomycetes* (1% *w*/*v*) was inoculated in 100 mL of same medium, incubated for 6 days on a rotary shaker (120 rpm) at 37 °C, culture broth was centrifuged at 6438*g* (CM 12 plus, Remi, India) for 15 min and the supernatant was used as a source of PI. All the chemicals were procured from Hi- Media, Mumbai and purified trypsin from the Sigma Aldrich (St. Louis, MO, USA).

#### 2.1.1. Enrichment and isolation of Streptomyces spp. for PI activity

Each sample (soil-water sediment, 1 g) collected from different locations of soda lake, Lonar was placed in Erlenmeyer flask separately containing 100 mL pre-sterilized starch casein broth and glycerol asparagine medium (pH 9.5, 0.5 M NaCl) supplemented with cycloheximide (100 μg/mL) and griseofulvin (100 μg/mL) to prevent growth of bacteria and fungi, incubated for 7 days at 37 °C (Balagurunathan and Subramanian, 2001). After two successive enrichment of 7 days each, aliquot of 0.1 mL culture broth was taken on respective agar, incubated at 30 °C for 48 h. After incubation, typical powdery morphotype colonies of *Streptomyces* were purified.

All the 80 *Actinomycete* strains were initially screened for PI activity by radial diffusion method (Vernekar et al., 1999) using skimmed milk agar (SM). Culture supernatant treated with trypsin was added in one of the peripheral wells and sterile distilled water in the other well containing 20 μL of trypsin (1 mg/mL) to serve as a negative control. Each plate was incubated for 24 h at 37 °C. Inhibition of hydrolysis of casein by trypsin was indicated by the absence of clear zone around the well containing the inhibitor.

### 2.2. Protease inhibition activity of culture supernatant

The Kunitz caseinolytic assay with slight modifications was used to determine protease inhibitor activity of isolates (Kunitz, 1947). The reaction mixture (2 mL) contained trypsin (100 μg/mL) in 0.1 M Tris HCl buffer, (pH 8.4), casein (1%) and *Streptomyces* culture extract (0.1 mL) as a source of protease inhibitor and incubated at 37 °C for 20 min. The reaction was terminated by TCA (5%), tubes were kept for 30 min at room temperature and contents filtered through Whatman no.1 filter paper. The absorbance of tyrosine in the filtrate was taken at 275 nm against a standard tyrosine solution (UV 1601, Shimadzu, Japan). Simultaneously, absorbance of the blank and control without inhibitor and substrate were also determined. One unit of protease (trypsin) activity was defined as 1 μM of tyrosine released per minute under experimental conditions. One unit of protease (trypsin) inhibitor (PI) activity is the amount of protein, which inhibits one unit of protease (trypsin) activity. The protein content of culture free broth was determined as per Lowry et al. (1951) using bovine serum albumin (BSA) as standard

### 2.3. Identification of the isolates producing PI

Each strain was screened based on the PI production and efficient strains were identified by preliminary phenetic and 16S rRNA gene sequencing at Royal Life Sciences, Hyderabad, India. The 16S rRNA nucleotide fragment of each of five shortlisted strains was amplified using PCR. The purified PCR products of approximately 1400 bp were sequenced by using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA) and sequencing products were resolved by ABI 3730XL capillary DNA sequencer (Applied Biosystems, USA). The partial sequences of each isolate were analyzed with BLAST and similarity analysis was done using Clustal X.

### 2.4. Purification of protease inhibitor

Purification of PI was carried out by a four step process as described by Bijina et al. (2011a). *Streptomyces spp.* VL J2 was grown in Erlenmeyer flask containing 250 mL of optimized medium comprising (g/L): starch, 24.9; casein, 7.5; $K_2HPO_4$, 0.5; $KNO_3$, 2.49; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 1.14; $FeSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.2 at 37 °C and 120 rpm for 96 h. The cells were removed by centrifugation and the supernatant was used as a source of PI. All purification steps were carried out at 4 °C. The purified PI was checked on 10% native and 12% SDS-PAGE using standard molecular weight markers.

### 2.5. Antifungal activity assay

The laboratory cultures of *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus* and *Fusarium oxysporum* from National Culture Collection of Industrial Microorganisms (NCIM), Pune were used for assessment of antifungal activity of purified PI using agar overlay diffusion method of Menon and Rao (2012).The spores of each fungal strain were harvested from freshly grown culture and suspended in sterile water ($\sim 1.0 \times 10^6$). Freshly prepared spore suspension (1 mL) was evenly spread on Muller Hinton agar (Hi Media, Mumbai) (pH 7.8 ± 0.1). PI concentrations (5, 10, 20, 30 and 40 μg/mL), filter sterilized through 0.22 μm pore size membrane, were inoculated in wells and incubated at 30 °C for 48 h for spore germination and vegetative growth.

### 2.6. In vitro inhibition of C. chinensis gut proteases

The insect pest *C. chinensis* were reared on four different diets and the larvae were collected from each source. Mid gut of larvae were dissected and transferred to a microfuge tube kept on ice at 4 °C containing 10 mM phosphate buffer (pH 7.8). The mid guts were homogenized and centrifuged at 11,183 g (CM 12 plus, Remi, India) for 10 min at 4 °C. The supernatant was used as a source of crude enzyme for PI activity. Also, larval protease and PI activity