



Systematic functional analysis and application of a cold-active serine protease from a novel *Chryseobacterium* sp.



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ABSTRACT

Psychrotolerant bacteria isolated from natural and artificially cold environments were screened for synthesis of cold-active protease. The strain IMDY showing the highest protease production at 5 °C was selected and phylogenetic analysis revealed that IMDY as novel bacterium with *Chryseobacterium soli*^T as its nearest neighbor. Classical optimization enhanced the protease production from 18 U/mg to 26 U/mg and the enzyme was found to be active at low temperature, activity enhanced by CaCl₂, inhibited by PMSF, stable against NaCl, and its activity retained in the presence of surfactants, organic solvents and detergents. On testing, the meat tenderization, myofibril fragmentation, pH, and TBA values were favorable in IMDY-protease treated meat compared to control. SDS profiling and SEM analysis also showed tenderization in meat samples. Hence, this study proposes to consider the cold-active protease from *Chryseobacterium* sp. IMDY as a pertinent candidate to develop potential applications in food processing industry.

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1. Introduction

Enzymes are biological catalysts with high levels of specificity which has led them emerge as one of the leading areas of biotechnological manufacturing and research. The specificity of the most of the enzymes are not only limited to their substrates but also extends to other factors such as temperature, pH and presence of ions. However, the earth's biosphere includes extreme environments that are inhabitable to evolved living organisms but are flourished with microbial life forms whose biological functions are sustained through extremophilic enzymes or extremozymes (Hough & Danson, 1999). These extremozymes are homogeneous in function to their non-extreme counterparts, but are efficient in catalyzing biological reactions under conditions where non-extreme enzymes are inhibited or denatured. More intriguing is the fact that such extremozymes have evolved to maintain their stability and activity not only to the stress conditions they are exposed to, but are also compatible to multiple stress conditions which makes their studies vital to research and industrial

biotechnology (Gomes & Steiner, 2004). Among biological enzymes, proteases or proteinases catalyze the cleavage of peptide bonds in proteins and in few instances also possess autolytic activity. These enzymes are found to have a large multiplicity in their applications, such as constituent in detergents, industrial processing of food, leather and pharmaceutical products thereby accounting for almost 60% of the worldwide enzymes market (Rao, Tanksale, Ghatge, & Deshpande, 1998).

The colossal use and requirement of proteases in industries have also focused research on proteases with novel properties which can result in significant advancements in product preparation, storage and employment. One such factor can be taken as efficient performance of proteases at sub-optimally low temperatures which is a critical trait in terms of saving energy as a number of domestic or industrial processes utilizing proteases could be performed at room temperatures. In temperate countries for example, detergents that contain proteases are reduced in efficiency as faucet water temperature is usually low requiring energy expenditure in terms of heating the water thereby subsequently increasing costs in the laundry industry (Gupta, Beg, & Lorenz, 2002). There have been several reports on identification of extracellular cold-active serine-proteases in psychrophilic as well as psychrotrophic bacterial species such as *Pseudoalteromonas*, *Colwellia*, *Flavobacterium* and *Shewanella* (Kulakova, Galkin, Kurihara, Yoshimura, & Esaki, 1999; Morita et al., 1998; Wang, Hou, Xu, Miao, & Li, 2008;

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Wang et al., 2005). Such cold-active proteases have high catalytic efficiency even under low temperatures due to their structural flexibility and higher turnover number (kcat).

Cold-adapted or cold-active proteases have found use in a wide range of applications, including bioremediation of polluted environments, cleaning/hygiene products, cosmetics, consumer food products, molecular biology, pharmaceutical and textile industries. Further, the list of applications for proteases keeps extending and emerging uses are in cosmetics (to remove glabellar-frown lines), prevention of bacterial infections (through disruption of biofilms), topical wound management (as a debridement agent for removal of necrotic tissues and fibrin clots), oral health management (plaque removal and prevention of periodontal disease), and in viral infections (through reduction of viral infection in case of human rhinovirus 16 and herpes simplex virus) (Craig, Page, & Madison, 2011; Demirjian, Morís-Varas, & Cassidy, 2001; Fornbacke & Clarsund, 2013; Kuddus & Ramteke, 2012). Furthermore, cold-active protease can provide good alternative to mesophilic enzyme in brewing and wine industries, cheese manufacturing, animal feed and fruit juice industry. Cold-active proteases with high catalytic efficiencies at low-temperatures can be employed to improve the taste of refrigerated meat (Wang et al., 2008). Cold-adaptive proteases with high activity and stability are being discovered which when coupled with manufacturing based on recombinant production techniques possess a promising future as a distinct class of biotechnological products with diverse applications.

Very few reports are available on protease from genus *Chryseobacterium*. A protein-deamidating enzyme (protein-glutaminase) that deamidates glutamyl residues in proteins was purified and characterized from *Chryseobacterium proteolyticum* strain 9670^T which isolated from rice field soil in Tsukuba, Japan (Yamaguchi, Jeenes, & Archer, 2001). Another *Chryseobacterium* sp. strain kr6 isolated from poultry industry waste was found to be capable of degrading chicken feathers by producing keratinase (metallo protease) enzyme (Brandelli & Riffel, 2005). Other proteases from *Chryseobacterium* spp. were metallo protease of *Chryseobacterium indologenes* 1x9a (Venter, Osthoff, & Lithauer, 1999), Keratinase of *Chryseobacterium* L99 sp. (Lv et al., 2010) and an endopeptidase of *Chryseobacterium* sp. (Lijnen, Van Hoef, Ugwu, Collen, & Roelants, 2000). In the present work, we report production and characteristics of extracellular cold-active protease from a novel *Chryseobacterium* sp. focusing on examining its activity and several conditions to recommend its industrial candidature.

2. Materials and methods

2.1. Chemicals, screening and identification of extracellular protease activity

Media components were obtained from Hi-Media, India. All chemicals used were of analytical grade. Pepstatin, PMSF, 1,10-Phenanthroline and protein markers were purchased from Sigma-Aldrich, India.

Isolation of psychrotolerant bacteria was carried out by sampling anthropogenic cold environments namely refrigerated food products (locally made ice-creams, vegetables) and cold storage facilities available in the vicinity of Vellore, India and soil samples from Western Himalayan region. Twenty two psychrotolerant strains were obtained by selectively isolating at 5 °C and screened for the production of cold active protease using casein and skim milk agar plates containing 5 g of Casein enzymatic hydrolysate, 2.5 g of yeast extract, 1 g of dextrose, 29 g of skim milk powder, 15 g of agar, and 1000 mL of distilled water. The presence of clear zones around the colonies after 7 days at 5 °C was taken as evidence of proteolysis (Hantsis-Zacharov & Halpern, 2007).

2.2. Primary enzymatic screening

2.2.1. Quantification of proteolytic activity at low temperatures

The medium used for quantification of protease production contained 1% glucose, 0.5% peptone, 0.15% yeast extract, 0.5% sodium chloride, 0.15% beef extract, 0.08% K₂HPO₄, 0.04% KH₂PO₄. The media was inoculated with 1% (v/v) inoculum and incubated at 5 ± 2 °C in a refrigerated incubator for 6 days. The grown cultures were centrifuged at 4 °C for 15 min at 10,621 × g and the supernatants were used for protease assay with tyrosine as a standard. Aliquots of cell-free supernatants (100 μL) were mixed with 400 μL of 1% casein dissolved in 50 mM Tris buffer (pH 8.0) and incubated at 5 °C for 15 min. The reaction was stopped by adding 500 μL of 1.2 M trichloroacetic acid (TCA). A test blank (control) sample was also prepared by adding TCA prior to the enzyme addition. Test and test blank solutions were kept in ice for 30 min and then centrifuged at 6800 × g for 10 min. For every 500 μL of filtrate 1 mL of 0.4 M Na₂CO₃ and 50 μL of 2 N Folin Ciocalteu reagent were added and mixed thoroughly. The absorbance was measured at 660 nm by the UV Visible spectrophotometer. One unit of protease activity was defined as the amount of enzyme to produce 1 μg tyrosine per min Baghel et al. (2005). Total protein content was measured by Lowry method using bovine serum albumin (BSA) as standard with the absorbance set at 660 nm.

2.3. Identification of the bacterium and protease production

2.3.1. Identification and preliminary characterization

The isolate was identified by 16S rRNA gene analysis using universal primers 27F 5'-GAGTTTGATCTGGCTCAG-3' and 1492R 5'-ACGGCTACCTGTACACTT-3'. The amplified PCR product was purified and sequenced at Macrogen (South Korea). Phylogenetic tree was constructed with MEGA v5.04 using neighbor joining method with a bootstrap value of 1000. Cell morphology, motility, Gram-staining, catalase activity, cytochrome oxidase activity, hydrolysis of sugars, growth at different temperatures and pH values, salt tolerance were studied according to Weon et al. (2008).

2.3.2. Optimization of the culture medium-selection of best media components

Chryseobacterium sp. IMDY was selected for study as it synthesized the highest amount of protease among the studied strains during quantitative assay. During the investigation of culture conditions, growth was carried out in a basal medium containing 1% dextrose, 0.1% K₂HPO₄, 0.05% KH₂PO₄, 0.02% CaCl₂ and 0.05% MgSO₄·7H₂O (pH 7), and supplemented with 1% casein as substrate. Culture samples were collected at 24 h intervals and growth was measured. Protease activity of the cell free supernatant was determined in order to assess the amount of protease produced.

Screening for significant nutritional parameters influencing protease production was performed by one-variable-at-a-time approach. Several carbon sources (1% w/v) such as dextrose, fructose, lactose, maltose, sucrose, arabinose and starch were used. Peptone, urea, ammonium bicarbonate, ammonium chloride, potassium nitrate and sodium nitrate were used at 0.5% (w/v) as supplementary nitrogen sources. Protein substrates (1% w/v) such as skim milk, gelatin, egg albumin, bovine serum albumin and casein were used. Casein concentrations used varied from 1% to 6% and protease activity was quantified. Six metal ions (0.02% w/v) including FeCl₃, CaCl₂, KCl, BaCl₂, ZnSO₄ and MgSO₄ were individually evaluated for their performances in protease production. All experiments were carried out at 5 °C for 6 days. The carbon, nitrogen, substrate and metal ion present in the basal medium mentioned above was replaced with the respective compounds. All the analyses were conducted independently in triplicates and the data presented here are the mean values ± standard deviation.

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