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Original Article

Partial purification and characterization of serine protease produced through fermentation of organic municipal solid wastes by *Serratia marcescens* A3 and *Pseudomonas putida* A2

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ABSTRACT

Proteolytic bacteria isolated from municipal solid wastes (MSW) were identified as *Serratia marcescens* A3 and *Pseudomonas putida* A2 based on 16S rDNA sequencing. Protease produced through fermentation of organic MSW by these bacteria under some optimized physicochemical parameters was partially purified and characterized. The estimated molecular mass of the partially purified protease from *S. marcescens* and *P. putida* was approximately 25 and 38 kDa, respectively. Protease from both sources showed low K_m 0.3 and 0.5 mg ml^{-1} and high V_{max} 333 and 500 μmole min^{-1} at 40 °C, and thermodynamics analysis suggested formation of ordered enzyme-substrate (E-S) complexes. The activation energy (E_a) and temperature quotient (Q_{10}) of protease from *S. marcescens* and *P. putida* were 16.2 and 19.9 kJ/mol, and 1.4 and 1.3 at temperature range from 20 to 40 °C, respectively. Protease of the both bacterial isolates was serine and cysteine type. The protease retained approximately 97% of activity in the presence of sodium dodecyl sulphate. It was observed that the purified protease of *S. marcescens* could remove blood stains from white cotton cloth and degrade chicken flesh remarkably. Our study revealed that organic MSW can be used as raw materials for bacterial protease production and the protease produced by *S. marcescens* A3 might be potential for applications.

1. Introduction

Protease enzyme catalyzes the hydrolysis of proteins into small peptide fractions and amino acids [24]. It is one of the major groups of enzymes produced and account for 60% of the worldwide sales of the total industrial enzymes [39]. Protease has widespread application fields and mostly used in detergent, leather, textile, food and pharmaceutical industries [4,7,8,25,28]. Bacterial protease is mostly extracellular, easily produced in larger amounts, thermostable, and active at a wider pH range [6]. Because of easy handling, stability and low cultivation cost, bacteria are fascinating sources for protease production [25]. The industrial application of protease highly depends on their stability throughout fermentation, isolation, purification and storage, and it also depends on their activity against solvent, surfactants and oxidants [20,21,34,46,51]. Kinetic study determines the rate of activation and inactivation of enzyme [15], and are indispensable for the evaluation of biotechnological potentiality of any new strain for the development of enzyme-based process in industry [38,40].

Municipal solid wastes (MSW) management in Bangladesh involves collection and dumping of wastes in open field or throwing haphazardly resulting environmental pollution, public health hazards and climate change due to methan gas genration. About 16,015 tons of solid wastes are generated each day from the six divisional cities and other urban areas of Bangladesh, and it is estimated that this amount will rise up to 47,000 tons per day by 2025 [11]. Almost 70–80% of MSW is organic material [3]. The large amount of organic MSW (OMSW) should be bioconverted into bioresources through production of commercially important products as well as renewable biomass energy and thus to mitigate climate change and environmental pollution caused by unmanaged MSW. However, there is no initiative in Bangladesh to utilize the OMSW to produce commercially value added products.

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In industries, enzymes are produced by cultivating microorganisms in synthetic medium and the cost of the culture medium corresponds to approximately 60–80% of the total production cost of enzymes [31]. The OMSW supports the growth of different microorganisms [8], and thus, the enzyme production cost by using OMSW as a raw material could be substantially reduced. Our previous study showed that the OMSW was used as nitrogen and carbon sources in fermentation for protease production by the bacterial isolates in shake flask level [8]. However, the bacterial isolates were not identified based on molecular approaches and crude protease produced in shake flask fermentation was used for partial characterization [8]. In the present study, we have identified the bacterial isolates based on the genetic tool 16S rDNA sequence. Herein, we reported protease production from these bacterial isolates by using OMSW as raw material in the bioreactor, and protease was partially purified and characterized to investigate their potential applications.

2. Materials and methods

2.1. Source of organisms

The bacterial cultures used in the present study were previously isolated from MSW and identified as *Serratia marcescens* and *Pseudomonas* sp. based on morphological, cultural and biochemical characteristics [8]. The organisms were maintained on nutrient agar slants in the refrigerator at 4 °C. Subcultures were performed from these slants at 15 days interval.

2.2. 16S rDNA gene sequencing

The genomic DNA of the two isolates was extracted by using Favorgen Cultured Cell Genomic DNA Extraction Kit in accordance with the manufacture instruction (Favorgen Biotech Corporation, Taiwan). The polymerase chain reaction (PCR) was performed in a thermocycler SimpliAmp TM (Thermo Fisher Scientific Inc; USA). The 16S rDNA was amplified by using a universal forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′), and reverse primers (5′-CGGTTACCTTGTTACGACTT-3′) for *S. marcescens* and (5′-CCG TACATTCMTTTRAGTTT-3′) for *Pseudomonas* sp [17]. Amplification reactions were performed in a total volume of 25 μl containing 1 μl of each 5 μM primer, 2 μl of template DNA (≤250 ng), 12.5 μl of 2x G2 hot start colorless master mix (Promega, Madison, WI, USA) and 8.5 μl of nuclease free water. The thermocycler was programmed for 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 2 min; 1 cycle at 72 °C for 10 min. PCR products were purified from agarose gel by an extraction kit (ATP™ Gel/PCR Extraction Kit, ATP Biotech Inc., Taiwan) and sequenced by a DNA Sequencer (Model 3130, ABI Automated Genetic Analyzer, Hitachi, Japan). The 16S rDNA sequences were analyzed using a free computer program Chromas 2.6.2. The sequence was searched for similarities in the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search program. The sequence was aligned with the similar sequences by using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and a phylogenetic tree was constructed using Molecular Evolution Genetic Analysis (MEGA), version 5.0 [50] as described previously [9].

2.3. Protease production in the bioreactor

Fermentation of OMSW for protease production was carried out in a bioreactor (Fermac 360, Electrolab, UK). The inoculum size of the seed culture was 10% of the total fermentation broth. For seed culture, a fresh isolated bacterial colony was inoculated in a basal media (l.0% glucose, 0.5% peptone, 0.5% yeast extracts, 0.1% K_2HPO_4 and 0.01% $MgSO_4$; pH 7.0) and incubated at 37 °C and 120 rpm for 20 h. Ten ml of this culture was inoculated to 90 ml of MSW media (2–4% of proteinous and cellulosic MSW, 0.1% K_2HPO_4, and 0.01% $MgSO_4$, pH 7.0) and incubated in a shaker incubator at 120 rpm for 20 h. The temperature for cultivation was 30 °C for *S. marcescens* and 37 °C for *P. putida*. Two hundred ml of this seed culture was aseptically transferred into the bioreactor containing 1.8 L MSW media. Fermentation was carried out at pH 8.0, 30 °C for 24–28 h for *S. marcescens* and at pH 7.0 and 37 °C for 36–38 h for *P. putida*. During fermentation, the aeration was 1vvm and the agitation was 120 rpm. After fermentation, cells were separated by centrifugation at 8000 rpm for 15 min at 4 °C, and the supernatant was used as a source of protease.

2.4. Enzyme assay and protein estimation

Protease activity was determined by using azocasein as a substrate according to the method described previously [8]. Total protein concentration was determined by Bradford protein assay kit (1x dye, Bio-Rad, USA) using bovine serum albumin as a standard protein.

2.5. Partial purification of protease

The cultural supernatant was fractionated with 30%, 60% and 90% of ammonium sulphate saturation. The precipitate of each fraction was recovered by centrifugation at 8000 rpm for 10 min at 4 °C. The pellet of each fraction was dissolved in 10 mM Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer overnight at 4 °C. The protease activity and protein content of the dialysed samples were measured. The fraction with protease activity inhibited by phenylmethyl sulphonyl fluride (PMSF) was considered as the source of serine protease and applied to diethylaminoethylene cellulose (DEAE-cellulose) column (Econo-Pac, 14 cm length, 20 ml bed volume; Bio-Rad, USA) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The bound proteins were eluted with NaCl gradients (0.15–0.6 M prepared in 10 mM Tris-HCl buffer (pH7.0)) at a flow rate of 0.3 ml/min using BioLogic Low-Pressure Liquid Chromatography System (BioLogic LP, Bio-Rad, USA). The eluted fractions were dialyzed against the 10 mM Tris-HCl buffer and assayed for the protease activity. The fractions with protease activity were pooled and concentrated with 90% ammonium sulphate saturation. The resultant precipitate was collected by centrifugation, dissolved in 10 mM Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer. The protease activity and protein concentration of each fraction was measured as mentioned above. The molecular mass of proteins was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide resolving gel.

2.6. Effects of pH on protease

The effects of pH on the activity of the partially purified protease were investigated by conducting assay with buffers of different pH in the range of 4.0–11.0. Buffers of different pH (citrate buffer, pH 4.0–6.0; phosphate buffer, pH 7.0–8.0; and Tris-HCl, pH 9.0–11.0) were used for the preparation of 50 mM azocasein solution. The pH stability of protease was studied as described previously [37]. In brief, the partially purified protease was treated for 1 h with different buffers covering the range of pH 4.0–11.0. Residual protease activities were assayed as described above.

2.7. Effects of temperature on protease

The optimum temperature of partially purified protease was determined by incubating reaction mixture of the enzyme at differ-

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