Journal of Genetic Engineering and Biotechnology 16 (2018) 29–37

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Journal of Genetic Engineering and Biotechnology

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

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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ARTICLE INFO

Article history:
Received 15 April 2017
Received in revised form 7 August 2017
Accepted 14 October 2017
Available online 18 October 2017

Keywords:
Municipal solid wastes
Purified protease
Activation energy
Kinetics
Blood stains

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.

Peer review under responsibility of National Research Center, Egypt.
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https://doi.org/10.1016/j.jgeb.2017.10.011
1687-157X/

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-

ent temperatures (20–60 °C) as described previously [8]. The thermo stability of purified protease was investigated by pre-incubating the enzyme for 1 h at different temperatures (20–60 °C) and the residual protease activity was then determined under standard assay condition. The activation energy (E_a) of protease was determined by using azocasein as a substrate at several temperatures ranging from 20 to 40 °C. The E_a was calculated from the slope of linear Arrhenius plot (1) of ln [Protease Activity] versus 1/T, where E_a = −slope × R, R (gas constant) = 8.314 J/K/mole, and T is the absolute temperature in Kelvin (K) [36,44].

$$\ln(\text{protease activity}) = \left(\frac{-E_a}{R}\right) \times \frac{1}{T} + \ln A \quad (1)$$

The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to raise in the temperature by 10 °C. The Q_{10} was calculated using the following Eq. (2) of Dixon and Webb [14].

$$Q_{10} = antilog_e\left(\frac{E \times 10}{RT^2}\right) \quad (2)$$

where E = Ea = Activation energy; all the formulas in this study were typed by a standard tool, 'MATLAB Toolboxes' as described by Valipour [51].

2.8. Determination of kinetic parameters

For determining the K_m and V_{max} of the partially purified protease, azocasein was used as a substrate upto a concentration 0.2–2.2 mg/ml under optimum condition. The kinetic parameters were calculated by using Lineweaver-Burk double-reciprocal plot [12]. The reciprocal of substrate concentration (1/[S]) was plotted against the reciprocal of reaction rate ($1/V_o$) by using following Eq. (3).

$$\frac{1}{V_0} = \left(\frac{K_m}{V_{max}}\right) \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

where K_m is the Michaelis–Menten constant, indicates substrate concentration at which the reaction rate is half of the V_{max}. The [S] is the concentration of substrate, V_0 and V_{max} represent the initial and maximum velocity of a reaction, respectively.

2.9. Determination of thermodynamic parameters

The thermodynamic parameters for azocasein hydrolysis were calculated using Eyring's absolute rate Eq. (4) derived from the transition state theory [16].

$$K_{cat} = \frac{k_B T}{h} \times e^{\left(\frac{-\Delta H^{\#}}{RT}\right)} \times e^{\left(\frac{\Delta S^{\#}}{R}\right)} \quad (4)$$

where k_B is the Boltzmann's constant (1.3805×10^{-23} J/K), h is the Planck's constant (6.6256×10^{-34} Js), T is the absolute temperature in Kelvin (K), R is the gas constant (8.314 J/K/mol), $\Delta H^{\#}$ is the change in enthalpy, and $\Delta S^{\#}$ is the change in entropy.

The value of activation Gibbs free energy ($\Delta G^{\#}$) and enthalpy ($\Delta H^{\#}$) were calculated by using the Eqs. (5) and (6), respectively.

$$\Delta G^{\#} = -RT \ln\left(\frac{K_{cat} h}{k_B T}\right) \quad (5)$$

$$\Delta H^{\#} = E_a - RT \quad (6)$$

From Eqs. (5) and (6), the value of activation entropy ($\Delta S^{\#}$) was calculated by using following equation:

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \quad (7)$$

The free energy of substrate binding and transition state formation was calculated using the following derivations:

$$\text{Free energy of substrate binding } (\Delta G^{\#}_{E-S}) = -RT \ln(K_a) \quad (8)$$

where $K_a = 1/K_m$

$$\text{Free energy for transition state formation } (\Delta G^{\#}_{E-T}) = -RT \ln\left(\frac{K_{cat}}{K_m}\right) \quad (9)$$

2.10. Effects of inhibitors on the partially purified protease

To detect the type of protease, the partially purified proteases were treated for 1 h with 1 mM solution of PMSF, 1, 10 phenanthroline, ethylene diamine tetra-acetic acid (EDTA), mercuric chloride, 25 μM solutions of pepstatin and leupeptin. PMSF, pepstatin and 1, 10 phenanthroline were solubilized in 50% (w/v) ethanol in 50 mM Tris-HCl buffer (pH 7.0) and rests of the inhibitors were dissolved in 50 mM Tris-HCl buffer (pH 7.0).

2.11. Effects of metal ions on protease activity

The partially purified protease was treated for 1 h with each of 10 mM metal ion prepared in 50 mM Tris-HCl buffer, pH 7.0. The metals were $CaCl_2$, $FeCl_3$, $HgCl_2$, $MgCl_2$, KCl and $ZnCl_2$. The protease activity was then assayed as described above.

2.12. Effects of surfactants and oxidizing agent on partially purified protease

The effects of different surfactants and oxidizing agent on the activity of the purified protease were studied by treating the enzyme for 2 h at room temperature. One percent of different surfactants and oxidative agents (Tween-20 in v/v, SDS in w/v and H_2O_2 in v/v) were used. The residual protease activity was assayed as described above.

2.13. Removal of blood stains and degradation of chicken flesh by the partially purified protease

To investigate the blood stain removal capability of the partially purified protease, clean white cotton cloth pieces (4 cm^2) were stained with chicken blood for 10 min and then dried for 5 min at 70 °C. The stained cloth pieces were soaked in 2% formaldehyde and washed with distilled water to remove execs blood from the cloth pieces [34,41]. The stained cloth pieces were then treated with 25 ml of purified protease solution; (total protease activity was 1557 U/ml) in the 50 ml conical flasks. One control contained distilled water with white cotton cloth piece and another control contained distilled water with blood stained cotton cloth piece. The conical flasks containing the cloth pieces were incubated at 40 °C and 120 rpm for 1 h. After incubation, the cloth pieces were dried at room temperature for 1 h and observed for evaluating the stains removal capability of the partially purified protease. Degradation of chicken flesh with the protease was investigated as described previously [8].

2.14. Statistical analysis

For statistical analysis, Student's 't' test was used. A p value of <0.05 was considered statistically significant. Data were presented as the Means ± SEM of at least three independent experiments, or as noted in the figure legends.

3. Results and discussion

3.1. Molecular identification of bacterial isolates

In the previous study, bacterial isolates were identified as *Pseudomonas* and *Serratia* based on cultural, morphological and biochemical characteristics [8]. The 16S rDNA gene sequencing was performed for molecular identification of the species of these two isolates. The PCR amplicons of the genomic DNA of *Pseudomonas* and *Serratia* isolates with the primers mentioned earlier was approximately 900 and 1550 base pair, respectively. BLAST similarity search with the DNA sequences of PCR amplicon obtained from *Pseudomonas* (DDBJ, EMBL and Gene Bank accession no. LC177213) and *Serratia* (DDBJ, EMBL and Gene Bank accession no. LC177214) revealed that these isolates were like *Pseudomonas* and *Serratia* spp. Phylogenetic tree constructed with the similar sequences showed that the *Pseudomonas* and *Serratia* isolates were closely related to the strains of *Pseudomonas putida* and *Serratia marcescens*, respectively (Fig. 1). We named them as *Pseudomonas putida* A2 and *Serratia marcescens* A3.

3.2. Protease production in the bioreactor using OMSW

In order to produce protease using OMSW, the carbon and nitrogen (glucose, peptone and yeast extract) sources of the basal media were replaced by 2, 3 and 4% each of cellulosic and proteinous MSW. The highest level of protease was produced with 3% of OMSW (Fig. 2). Interestingly, almost similar level of protease was produced when the carbon and nitrogen sources of the basal media was substituted by 3% of cellulosic and proteinous MSW, indicating that OMSW might be suitable for protease production. Protease production by the both bacterial isolates with OMSW was optimized in shake flask and bioreactor. Optimization is the important strategy to maximize the yields [51–54]. The optimum temperature, pH and agitation for fermentation of OMSW for maximum production of protease by *S. marcescens* at shake flask and bioreactor was 30 °C, 8.0 and 120 rpm, respectively, whereas those for *P. putida* was 37 °C, 7.0 and 120 rpm, correspondingly (data not shown). Optimum fermentation period for protease production by *S. marcescens* and *P. putida* at shake flask was 30 h and 42 h, respectively. However, optimum fermentation period for protease production in the bioreactor by the both isolates was reduced by 6 h compared to at shake flask.

In the bioreactor under controlled conditions of temperature, pH, agitation and aeration, the protease production was scaled-up about 2–2.5 fold compared to at the shake flask (Fig. 2). This result further indicated that the both isolates produced almost the same level of protease with OMSW. Growth kinetics during the time course study showed that the protease production was growth-associated (data not shown).

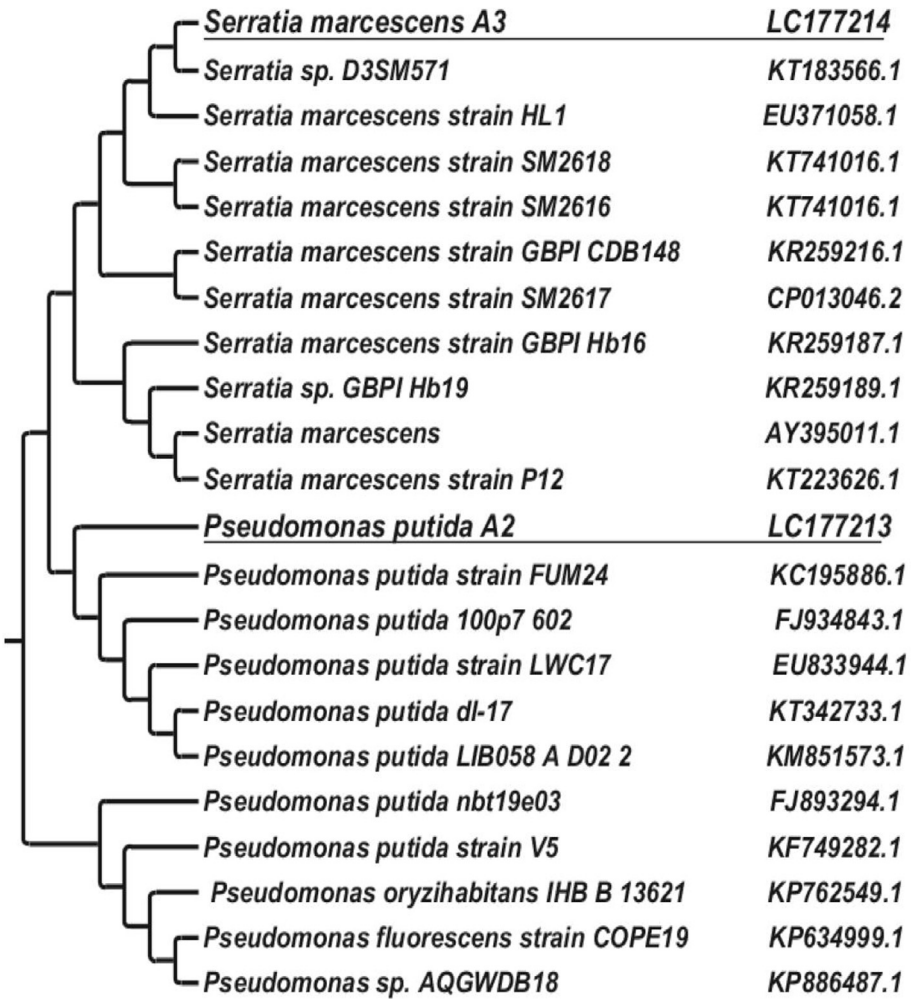

Fig. 1. Phylogenetic tree showing the relationship of *P. putida* A2 and *S. marcescens* A3 with other species of *Pseudomonas* and *Serratia*, respectively. The evolutionary history was inferred using the Neighbor-Joining method. The genetic distance was estimated by the p-distance method. *Serratia marcescens* and *Pseudomonas putida* isolated and identified in this study are underlined. The accession numbers of *Pseudomonas* and *Serratia* species to which the phylogenetic tree has been constructed are shown in the right column.

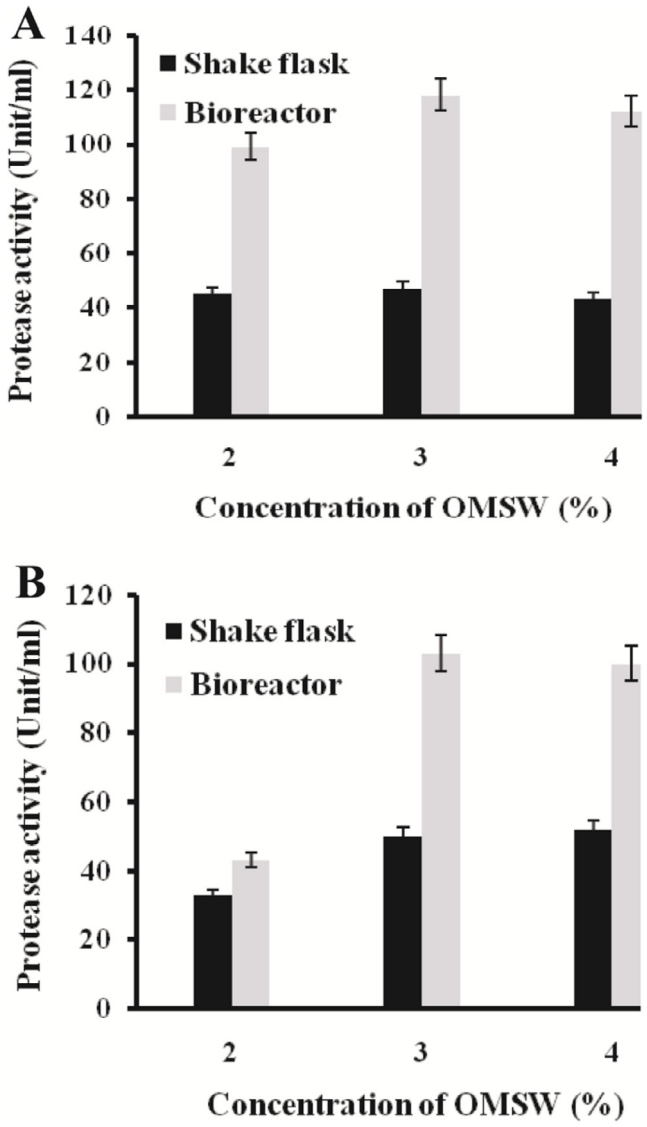

Fig. 2. Production of protease by *S. marcescens* A3 (A) and *P. putida* A2 (B) using OMSW. Black and gray bars indicated the protease level produced during fermentation at shake flask and bioreactor, respectively. The fermentation with *S. marcescens* was carried out under temperature, pH, and agitation at 30 °C, 8.0 and 120 rpm, respectively. These conditions for *P. putida* were correspondingly 37 °C, 7.0 and 120 rpm. The aeration in the bioreactor was maintained at 1 vmm. Results are mean ± SEM of repeated experiments (n = 3).

3.3. Characterization of partially purified protease

3.3.1. Molecular weight of protease

Crude protease from *S. marcescens* and *P. putida* had specific activity 403.9 and 312.5 U/mg protein, respectively. Protease from both bacteria was precipitated by 60% and 90% ammonium sulphate saturation. However, protease from *S. marcescens* was also precipited with 30% $(NH_4)_2SO_4$ saturation. This result indicated that both bacterial isolates might have more than one protease. The 90% saturated protease from the both isolates was further purified by DEAE-cellulose chromatography. Purification of protease obtained from *S. marcescens* resulted in a final 52-fold purified protease with specific activity of 21,081 U/mg proteins and a typical yield of 7.5%. Purification of protease from *P. putida* resulted in a final 62-fold purified protease with specific activity of 19,267 U/mg proteins and a typical yield of 8.6%. The partially purified protease appeared as a prominent single protein band on 12.5% SDS-PAGE as compared to the crude sample. Appearance of a prominent single band in SDS-PAGE indicated that the purified protease of *Serratia* and *Pseudomonas* isolates was a homogenous monomeric protein with molecular mass of 25 and 38 kDa, respectively (Fig. 3). These results are consistent with the reports which showed that the molecular mass of serine proteases was between 18 and 35 kDa [39]. Rao et al. [39] further reported that cysteine protease had molecular mass between 32 and 50 kDa. However, the molecular mass of protease depends on their types and bacterial species. The molecular mass of alkaline protease from *Bacillus* sp is less than 50 kDa [47], whereas that of alkaline protease from halophilic bacteria is between 40 to 130 kDa [2].

3.3.2. Effects of pH on protease activity and stability

The partially purified protease of the both isolates was significantly active over a broad pH range from 5.0 to 9.0 having the maximum activity at pH 7.0 (Fig. 4). However, protease in our study was less active at alkaline condition compared to protease produced by *P. aeruginosa* ATCC 27853 [26] and *B. horikoshii* [27]. In a study of Nam et al [35] protease produced by *Serratia marcescens* S3-R1 was active at pH 7–9 [33,49]. However, pH optima may differ based on different substrates. The purified protease from the isolates was almost stable at pH 6.0–9.0. However, about 40% activity was lost when the enzyme solution was treated at pH 10.0.

3.3.3. Effects of temperature on protease activity and stability

The partially purified protease from *S. marcescens* and *P. putida* was active within a wide range of temperatures with the optimum at 40 °C (Fig. 5A & B). However, almost similar protease activity was observed at 40–50 °C. The purified protease was almost stable up to 45 °C (Fig. 5C). Nevertheless, stability of protease from both bacteria sharply reduced when the enzyme was heated beyond 45 °C and approximately 75% of protease stability was lost at 60 °C. These results indicated that the protease from the both sources can be applied at a wide range of temperature above the room temperature. The temperature optima and thermostability of protease vary based on the type of bacterial species. It is a well-known fact that protein conformation changes at higher temperatures, hence causes a decrease in the protease activity [29]. Nevertheless, the protease from halo tolerant *B. subtilis* retains its full activity after

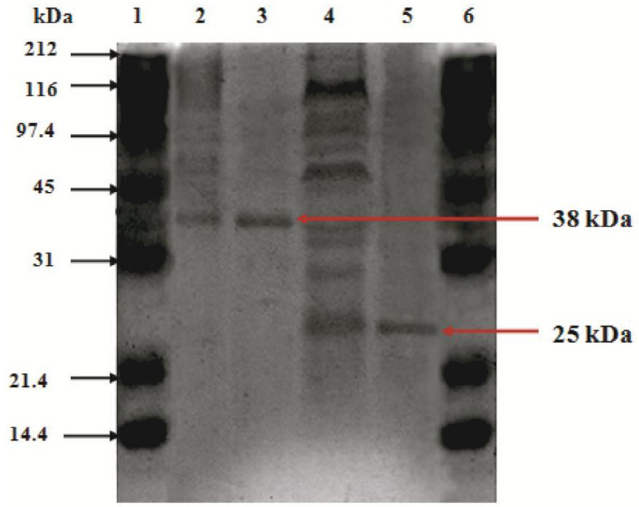

Fig. 3. SDS-PAGE of the partially purified proteases from *S. marcescens* and *P. putida*. Molecular masses of the standard proteins are shown in lanes 1 and 6. An equal amount of protein 3.4 μg was applied in every lane. Lanes 2 and 3 indicated the crude and purified protease of *P. putida*, respectively, and lanes 4 and 5 indicated the crude and purified protease of *S. marcescens*, respectively.

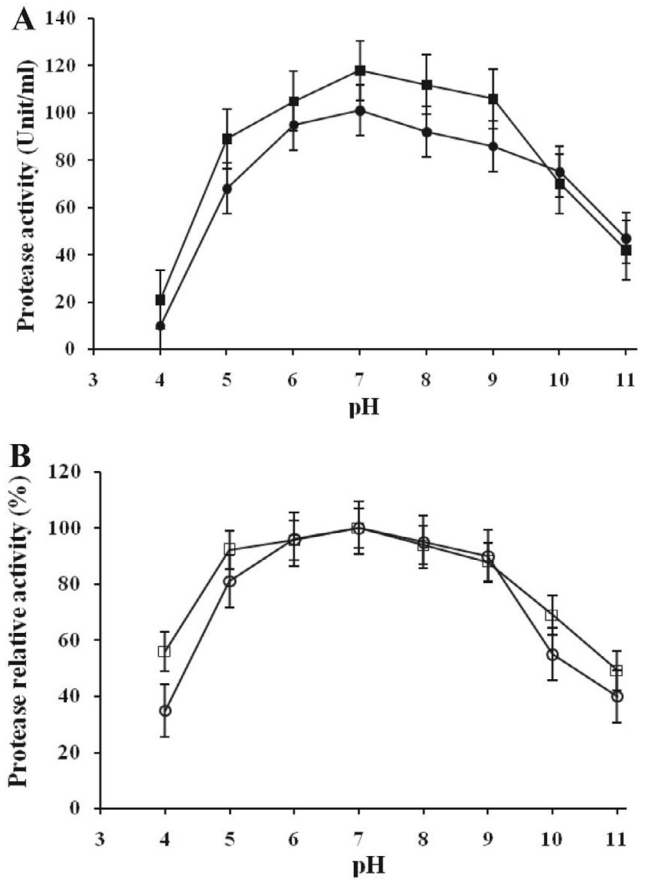

Fig. 4. Effects of pH on activity (A) and stability (B) of protease from *S. marcescens* and *P. putida* isolates. Filled and open circles indicate the activity and stability of protease from *S. marcescens,* respectively and filled and open rectangles indicate those of protease from *P. putida*, correspondingly.

30 min of incubation in the temperature ranging from 37 to 55 °C [1].

The enzyme-catalyzed reaction may show more complex temperature dependence because increased temperature may provoke conformational change and even denaturation that lower the effectiveness of the enzyme [43]. It is clearly revealed (Fig. 5A & B) that activity of protease from *S. marcescens* and *P. putida* reduced over 40 °C, suggesting that inactivation of protease started from or above 40 °C. Therefore, the activation energy (E_a) for the azocasein hydrolysis was calculated at temperature 20–40 °C by using Arrhenius plot. The Arrhenius plots (insets of Fig. 5A & B) for the protease from both bacteria showed a linear variation with temperature increase, suggesting that the protease from both sources has a single conformation up to the transition temperature [13]. The E_a of the protease from *S. marcescens* and *P. putida* was 16.2 and 19.9 kJ/mol, respectively. However, Hernandez-Martinez et al. [22] reported comparative higher activation (62 kJ/mol) for a similar neutral serine protease from *Aspergillus fumigates*. The low E_a values measured for the protease from both bacteria suggest that less energy is required to form activated complex of azocasein hydrolysis, thus indicating an effective hydrolytic capacity of the protease from both bacteria [45,48].

To investigate the effects of temperature on the rate of protease reaction, we examined the temperature quotient (Q_{10}) which is a value used to infer whether or not the metabolic reactions being examined are controlled by temperature or by some other factors [14]. The values of Q_{10} for the protease of *S. marcescens* and *P. putida* were approximately 1.2 and 1.3 at 20–40 °C, respectively.

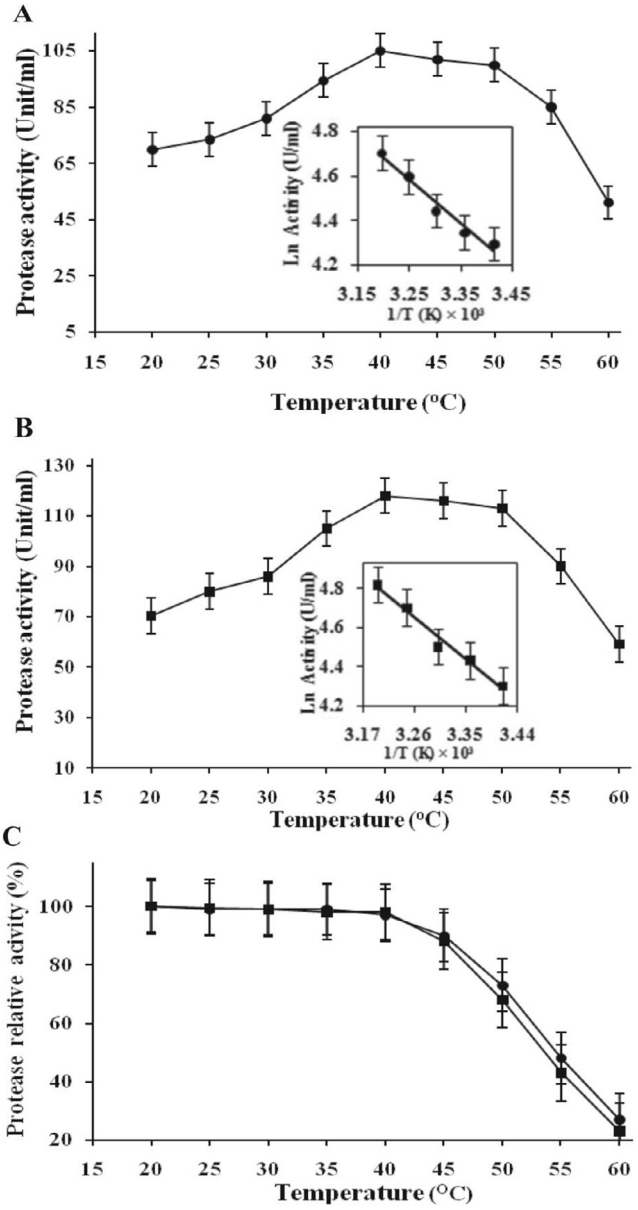

Fig. 5. Effects of temperature on activity of protease from *S. marcescens* (A) and *P. putida* (B). The Arrhenius plot for determining the E_a of protease from *S. marcescens* (A) and *P. putida* (B) are shown in the insets. Effects of temperature on stability of protease from *S. marcescens* (circles) and *P. putida* (rectangles) isolates are shown in C.

In general, enzymatic reactions show Q_{10} values between 1 and 2 and any deviation from this value is indicative of involvement of some factor other than temperature in controlling the rate of reaction. A Q_{10} value of 2 suggests doubling of the rate of reaction with every 10 °C rise in temperature [14].

3.3.4. Kinetic and thermodynamic parameters

The maximum velocity (V_{max}), Michaelis–Menten constant (K_m), turn over number (K_{cat}), and catalytic efficiency (K_{cat}/K_m) are important parameters for most of the enzyme-catalyzed reactions. The values of V_{max}, K_m, K_{cat}, and K_{cat}/K_m measured for the partially purified protease were summarized in Table 1. The K_m value of an enzyme indicates the affinity of the enzyme for its substrate: a low value of this parameter indicates a higher affinity [36]. It was observed that purified protease of *S. marcescens* had high affinity for its substrate compare to the purified protease of

Table 1
Kinetic and thermodynamic parameters for azocasein hydrolysis by partially purified protease of *S. marcescens* and *P. putida* isolates.

Kinetic studies			Thermodynamic studies		
Parameters	*S. marcescens*	*P. putida*	Parameters	*S. marcescens*	*P. putida*
K_m (mg/ml)	0.33	0.5	E_a (kJ/mole)	16.2	19.9
V_{max} (μmole/min)	333.3	500	$\Delta H^{\#}$ (kJ/mole)	13.6	17.3
K_{cat} (s^{-1})	9.1	18.5	$\Delta G^{\#}$ (kJ/mole)	71	69.2
K_{cat}/K_m (s^{-1}/mg/ml)	27.5	37	$\Delta S^{\#}$ (kJ/mole)	−0.18	−0.17
			$\Delta G^{\#}_{E\text{-}S}$ (kJ/mole)	5.7	7.6
			$\Delta G^{\#}_{E\text{-}T}$ (kJ/mole)	−8.6	−9.4

P. putida with K_m value 0.3 and 0.5 mg/ml, respectively. The V_{max} is the amount of enzyme involve in the enzymatic reaction. The V_{max} values for the protease from both bacteria were 333 and 500 μmole/min, respectively. The K_{cat} value for the protease of *P. putida* was higher than the protease of *S. marcescens*, which can be attributed to the high number of conformationally active enzyme molecules. The K_{cat}/K_m values for the protease of both bacteria were 37 and 27.5 s^{-1}/mg/ml. The K_{cat}/K_m is the measure of the enzyme's catalytic efficiency and depends on how often the enzyme encounters substrate molecules in active conformation [10]. Results indicated that the protease from both bacteria had high affinity for its substrate and efficient catalytic role in the enzyme-catalyzed reaction compared to literature reported neutral protease from *Streptomycin* sp. A6 [45], acidic protease from *Aspergillus oryzae* [13], and alkaline proteases from *Bacillus clausii* GMBAE 42 [30] and *Pseudomonas aeruginosa* PseA [20].

The thermodynamic parameters for azocasein hydrolysis are shown in Table 1. The negative values of entropy ($\Delta S^{\#}$) for the protease of *S. marcescens* and *P. putida* suggest the formation of a more ordered enzyme-substrate (E-S) complex at the transition state of enzyme-catalyzed reaction [10,23]. The Gibbs free energy ($\Delta G^{\#}$) is the measure of spontaneity of any reaction [42]. Low $\Delta G^{\#}$ values for both bacteria signify that the conversion of a transition state E-S complex into a product was more spontaneous than the native protease. For both bacteria, low values of free energy for substrate binding ($\Delta G^{\#}_{E\text{-}S}$) and transition state formation ($\Delta G^{\#}_{E\text{-}T}$) indicate the high affinity of protease for azocasein hydrolysis [10].

3.3.5. Effects of protease inhibitors

The protease of *S. marcescens* and *P. putida* was classified based on their responses to different protease inhibitors. The protease obtained from *S. marcescens* and *P. putida* was strongly inhibited by PMSF and $HgCl_2$ but not by EDTA (Table 2). The effects of protease inhibitors revealed that the protease from *S. marcescens* and *P. putida* was serine and cysteine type [8].

3.3.6. Effects of metal ions on protease activity

Mg^{2+}, Ca^{2+} and K^+ were very strong activators as they significantly increased the activities of protease from the both isolates. In the presence of Mg^{2+}, Ca^{2+} and K^+ the activity of purified protease from *S. marcescens* was increased 20, 25 and 26%, respectively and that from *P. putida* was increased correspondingly 33, 12 and 5% (Table 3). In contrast, Hg^{2+} and Zn^{2+} severely inhibited the activities of the protease. It was also reported by Annapurna et al. [5] that the activity of alkaline protease from *S. marcescens* was increased due to the presence of $CaCl_2$ and inhibited in the presence of $HgCl_2$.

3.3.7. Effects of surfactant and oxidizing agents on protease activity

For detergent formulation with protease, it is very important to investigate the protease stability in the presence of surfactants and oxidizing agents [18]. Commercial detergent proteases such as Esparase, Alcalase, Carlsberg, Savirage and Subtilisin are stable in the presence of surfactants and oxidizing agents [19]. The activity of purified protease of *S. marcescens* and *P. putida* was enhanced by approximately 30% and 5%, respectively in the presence of tween 20, a non-anionic surfactant. In the presence of SDS, a strong anionic surfactant, the purified protease of *S. marcescens* and *P. putida* could retain its activity 97% and 83%, respectively (Table 4). Li et al. [32] and Gupta et al. [20] reported similar results for protease from *Serratia* sp and *P. aeruginosa* PseA, respectively. These literatures also indicated that protease from *Serratia* sp. was more stable against non-anionic and anionic surfactants compare to protease from *Pseudomonas* sp. According to an earlier study, most of proteases are unstable in the presence of oxidizing agent like H_2O_2

Table 2
Effects of different protease inhibitors on the activity of partially purified protease from *S. marcescens* and *P. putida*.

Inhibitors	Concentration	Relative activity (%) of purified protease from	
		S. marcescens	*P. putida*
Control[a]	None	100	100
PMSF	1 mM	35 ± 1	47 ± 1
1, 10 phenanthroline	1 mM	90 ± 3	81 ± 1
$HgCl_2$	1 mM	10 ± 2	7 ± 1
EDTA	1 mM	93 ± 1	95 ± 3
Leupeptin	25 μM	86 ± 1	92 ± 2
Pepstatin	25 μM	91 ± 4	73 ± 1

[a] The control represents the activity of protease without treatment of any protease inhibitor and was considered as 100%. Results are presented as the Mean (n = 3) ± SEM.

Table 3
Effects of metal ions on the activity of partially purified protease of *S. marcescens* and *P. putida*.

Metal ions (10 mM)	Relative activity (%) of purified protease from	
	S. marcescens	*P. Putida*
Control[a]	100	100
Mg^{2+}	120 ± 2	132 ± 4
Ca^{2+}	125 ± 4	112 ± 4
Hg^{+}	5 ± 2	15 ± 3
Zn^{+}	10 ± 3	15 ± 3
K^{+}	126 ± 1	105 ± 1

[a] Protease activity without treatment by any metal ions was considered as control and its activity was 100%. Results are presented as the Mean (n = 3) ± SEM.

Table 4
Effects of surfactants and oxidizing agent on the partially purified protease of *S. marcescens* and *P. putida*.

Surfactants and oxidizing agent	Concentration (%)	Relative activity (%) of purified protease from	
		S. marcescens	*P. putida*
Control[a]	None	100	100
Tween-20	1	133 ± 2	103 ± 3
SDS	1	97 ± 1	83 ± 3
H_2O_2	1	84 ± 1	66 ± 4

[a] Protease activity without any surfactant was considered as control and its activity was 100%. Results are presented as the Mean (n = 3) ± SEM.

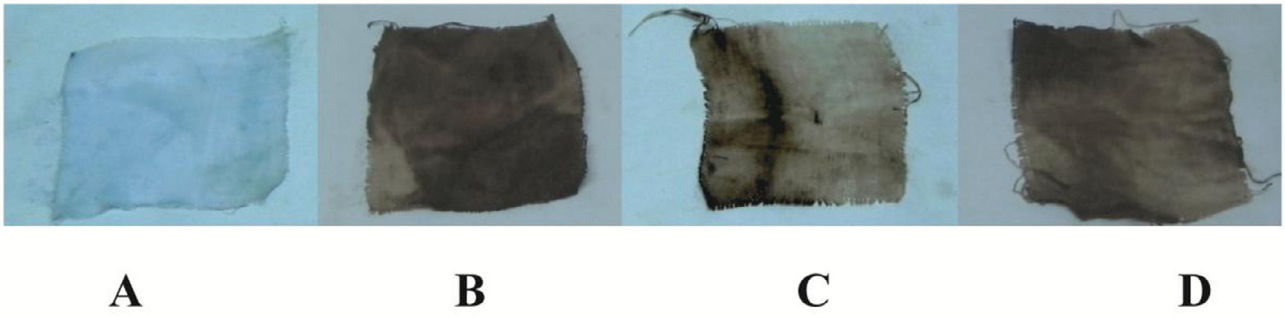

Fig. 6. Removal of blood stains from cotton cloths by partially purified proteases. (A) untreated white cloth, (B) untreated blood stained cloth, (C) blood stained cloth treated with the partially purified protease of *S. marcescens*, and (D) blood stained cloth treated with the partially purified protease of *P. putida*.

[19]. The similar result was obtained in our present study. H_2O_2 inhibited the activity of the purified protease from *S. marcescens* and *P. putida* approximately 20% and 35%, respectively (Table 4). This data indicated that protease obtained from *S. marcescens* retained good stability and activity against surfactants and oxidizing agent.

3.3.8. Evaluation of blood stains removal and raw protein degradation capability of the partially purified protease

Proteases have wide range of applications in the detergent and laundry industries. Protease is primarily used as cleaning additives in the detergent industries [55]. In the blood stain removal experiment, visible inspection revealed that the partially purified protease of *S. marcescens* and *P. putida* removed 80–85% and 30–35% of stains from the cloth piece, respectively (Fig. 6). This result indicated that the purified protease of *S. marcescens* had more capability to remove blood stains from cloth compared to that of *P. putida*. When chicken flesh was treated with the purified protease as described previously [8], about 75% and 60% of dry weight of chicken flesh was lost by protease from *S. marcescens* and *P. putida*, respectively. These results indicated that the purified protease could successfully degraded the proteinous material.

4. Conclusions

Two proteolytic bacterial isolates were identified as *S. marcescens* and *P. putida* based on the 16S rDNA sequence. The OMSW was successfully used as raw material instead of commercial carbon and nitrogen source for production of industrially important bacterial protease. Some physicochemical parameters were optimized for fermentation of OMSW by the both bacterial isolates to produce protease. Proteases produced from the two bacterial isolates by using OMSW as raw materials were partially purified. The partially purified proteases from *S. marcescens* and *P. putida* with molecular mass of 25 and 38 kDa, respectively was serine and cysteine type. The kinetic parameters, the effects of surfactants, removal of blood stain from the white cloth and degradation of chicken flesh collectively revealed that the protease particularly from *S. marcescens* could be applicable as proteolytic agent. However, further study is necessary to determine the carbon nitrogen ratio in the OMSW media to enhance the level of protease production in the bioreactor and for molecular characterization of the protease produced by *S. marcescens* isolate.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was partially supported by grants in aid from The World Academy of Sciences (TWAS) for the Advancement of Sciences in Developing Countries (No. 12-150 RG/BIO/AS_G), the Ministry of Science and Technology, Bangladesh (No. 39.009.006.01.00.042.2012-13/BS-135/518), the Ministry of Education, Bangladesh (No. 37.20.0000.004.033.020.2016.7725) and University Grant Commission of Bangladesh.

The nucleotide sequences data reported in this paper has been deposited in DDBJ and will be found in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number LC177213 and LC177214 for *Pseudomonas* sp. and *Serratia* sp., respectively.

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