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Scale-up of protease production by *Serratia marcescens* using municipal solid wastes in the bioreactor and its partial purification and characterization

Mohammad Rejaur Rahman, Md. Saddam Hossain, Akhikun Nahar,Md. Faisal Azim, Kamrul Islam, Abul Kalam Azad*

Department of Genetic Engineering & Biotechnology, School of Life Sciences, Shahjalal University of Science & Technology, Sylhet, Bangladesh

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Abstract

A proteolytic bacterial isolate obtained from municipal solid wastes (MSW), was identified as *Serratia marcescens* based on cultural, morphological and biochemical characteristics. In the present study, protease production by *S. marcescens* was maximized using MSW as the sole source of carbon and nitrogen under some optimized physicochemical conditions. The scale-up of protease production with different concentrations of organic MSW was performed. In shake flask fermentation, maximum level of protease was produced with 4 % MSW after 48 h at 30 °C, 120 rpm and pH 8.0. However, in the bioreactor, optimum level of protease was produced with 3 % MSW after 24 h of fermentation at 120 rpm, 30 °C and pH 8.0. In comparison with the shake flask, protease production was scaled-up 2 fold in the bioreactor with reduction in fermentation period. Partial purification by ammonium sulfate fractionation and anion-exchange chromatography resulted in a final 37-fold purified protease with a specific activity of 9411 U/mg protein and a typical yield of 7.1 %. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the estimated molecular mass of the partially purified protease was around 55 kDa. The partially purified protease showed optimum activity at pH 7.0 and 40 °C. The purified protease was stable at pH 5.0 - 9.0 and temperatures up to 45 °C. The enzyme activity was stimulated by Mg^{2+}, K^{+} and Ca^{2+} but was severely inhibited by Zn^{2+} and Hg^{2+}.

* **Corresponding Author:** Abul Kalam Azad ✉ dakazad-btc@sust.edu

Introduction

Proteases, also called peptidases, constitute a large group of hydrolytic enzymes that degrade protein into small peptides and amino acids by cleaving peptide bond (Shankar *et al.*, 2011). As a normal biocatalyst, proteases are the most important industrial enzymes accounting for more than 65% of the total industrial enzyme market (Annamalai *et al.*, 2014). Proteases are obtained from plants, animal and microorganisms, with the majority obtained from bacteria and fungi. A number of bacteria, fungi and yeast have been reported for the protease production (Banerjee and Battacharyya, 1992; Shalinisen and Satyanarayana, 1993). Because of their rapid growth, low production cost and the ease of genetic modification to generate high yielding strains demand for proteases has led to an interest in microbial proteases. Due to their broad substrate specificity, they have a wide range of applications such as in leather processing, detergent formulations, food processing industries, peptide synthesis, pharmaceutical industry as well as analytical tools in basic research (Prakasham *et al.*, 2005; Saran *et al.* 2007; Hou *et al.,* 2017).

The metabolic performance of a microbial culture in the bioreactor strongly depends on complex interactions of the various operating conditions. Up-scaling the fermentation processes from shake flask to bioreactor is challenging due to the difficulty in assessing the factors affecting the scale-up process during the cultivation. It is well known that extracellular protease production by microorganisms in bioreactors is greatly affected by media components, physical factors such as aeration, agitation, dissolved oxygen, temperature; inoculum density and incubation time (Gupta *et al.*, 2002). Therefore, it is essential to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions.

Almost 70–80% of municipal solid waste (MSW) is organic in nature (Alamgir *et al.*, 2007). The organic substitute of MSW comprises mainly of carbohydrates, amino acids, peptides and proteins, volatile acids, fatty acids and their esters. These are easily biodegradable and facilitate the growth of various micro-organisms in order to produce many interesting bioproducts. The large amount of organic MSW should be bio-converted into bio-resources 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 MSW to produce commercially value-added products.

In industries, synthetic medium is used for producing enzymes and the cost of the culture medium corresponds to approximately 60–80% of the total enzymes production cost of enzymes (Kumar *et al.*, 1999).

The organic MSW is reported to support the growth of different microorganisms (Azad *et al.*, 2013), and thus, the enzyme production cost can be substantially reduced by using organic MSW as a raw material. In our previous study, organic MSW was used as nitrogen and carbon sources in fermentation for protease production by the bacterial isolates in shake flask level (Azad *et al.*, 2013). This finding attracted us to study the scale up. Herein, we reported on scale-up of protease production from *Serratia marcescens* by using organic MSW as raw material in the bioreactor.

Material and methods

Source of organisms and culture maintenance

The bacterial culture used in the present study was identified as *S. marcescens* (Azad *et al.*, 2013). The organism was maintained on nutrient agar slants in refrigerator at 4 °C. Subcultures were performed from these slants at 15 days interval.

Production of protease in the bioreactor by using MSW

Cultivation for production of protease in bioreactor was carried out in a 2 L bioreactor (Fermac 360, Electrolab, UK). The bioreactor was equipped with

instrumentation in order to measure and control of agitation, pH, temperature, foam, dissolved oxygen concentration (DO) and exit gases. The inoculation was prepared in shake culture and the inoculum size was one-third of the total fermentation broth in the bioreactor. Basal medium was used to prepare the inoculum. The inoculum preparation was carried out in a shaker at 120 rpm for 20-24 h at 30 °C.

The inoculum was aseptically transferred into the bioreactor through one of the ports on the head plate. In earlier studies, basal media (1.0% glucose, 0.5% peptone, 0.5% yeast extracts, 0.1% K_2HPO_4 and 0.01% $MgSO_4$; pH 7.0) was used for protease production (Azad *et al.*, 2013). To produce protease with proteinous and cellulosic materials of MSW, peptone, yeast extract and glucose of the basal media were replaced by 2, 3 and 4 % each of proteinous and cellulosic wastes. The fermentation was carried out at optimum conditions as stated in earlier studies (Azad *et al.*, 2013). Samples of the culture were withdrawn aseptically with 6 h intervals to investigate the level of protease activity. An aliquot of each sample was used for growth analysis.Cells were separated by centrifugation at 8000 rpm for 15 min at 4 °C and the supernatant was used for the determination of protease activity.

Analysis of growth

Growth was analyzed by total viable cell count. During the time course of protease production, samples taken from the culture were diluted from 10^1 to 10^8 fold. An aliquot of 100 µl from each dilution was spread on nutrient agar medium. After 24 h of incubation at 30 °C, the total viable cell count was performed manually as colony forming unit (CFU). At least three replicas were done for each dilution of a specific sample.

Protease assay and estimation of protein

Protease activity was determined by using azocasein as a substrate according to the method described previously (Azad *et al.*, 2013). Total protein concentration was determined by Bradford protein assay kit (Bio-Rad, USA) using bovine serum albumin as a standard protein.

Optimization of agitation for protease production in bioreactor

The effects of agitation on the growth and production of protease were studied by cultivating the bacteria under different agitation rates and optimized temperature and pH.

Partial purification of protease

Ammonium sulfate fractionation

The crude protease was fractionated with 30, 60 and 90 % of ammonium sulfate saturation. Each of fraction precipitates were recovered by centrifugation at 8000 rpm for 15 min at 4 °C and dissolved in 10 mMtris-HCl buffer in the respective pH. Each ammonium sulfate fraction was dialyzed overnight against the same buffer. The protease activity and protein concentration was measured.

Anion-exchange chromatography

Following dialysis of each ammonium sulfate fraction, samples were applied to anion exchange columns (Econo-Pac, 14 cm length, 20 mL bed volume; Biorad, USA) packed with DEAE-Cellulose. The proteins were slowly mixed and wait for 10 min for equilibrium with resin for strong binding. The unbound materials were washed from the column with the same buffer in which protease solution was prepared.

The bound proteins were eluted step wise with a range of 0 to 0.6 M NaCl in the same buffer at a flow rate of 0.3 ml/min.

The eluted fractions were dialyzed against the same buffer (10 mM) and assayed for the protease activity. The fractions with protease activity were combined and concentrated with ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in same 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.

Partial characterization of purified protease

Effects of temperature on protease activity and stability

The partially purified protease activities at different temperature were observed by keeping enzyme-substrate reaction mixture in water bath at 25, 30, 35, 40, 45, 50 and 55 °C for 30 min and then assay was done as stated above. The thermo stability of protease was studied by treating the enzyme preparation at different temperatures ranging from 25-55 °C for 1 h. The residual protease activities were then assayed to evaluate heat stabilities.

Effects of pH on protease activity and stability

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 to 11.0. Buffers of different pH were used for the preparation of substrate azocasein solution. The pH stability of protease was studied as previously described (Phadatare *et al.*, 1993). 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.

Effects of metal ions on protease activity

The metal ion at a concentration of 10 mM was dissolved in Tris-HCl buffer, pH 7.0. A 100 µL of partially purified protease was treated with 900 µL of 10 mM salt solution for 1 h at room temperature. The residual protease activity was then assayed as described above. The metal salts used in this study were $CaCl_2$, $HgCI_2$, $MgCl_2$, KCl and $ZnCl_2$. The protease activity was then assayed as described above.

Statistical analysis

For statistical analysis, P value of <0.05 was considered to be statistically significant. Data were presented as the means ± standard errors of the means (SEM) of at least three independent experiments.

Results

Production of protease in the bioreactor using MSW

In the bioreactor under controlled conditions of temperature, pH, agitation and aeration the protease production was scaled-up ~2 fold.

The highest protease activity was obtained by *S. marcescens* using 3 % proteinous and cellulosic waste of MSW within only 24 h of cultivation and the activity was 104 U/mL (Fig. 1).

In contrast, the maximum protease activity 52 U/mL was obtained after 48 h of fermentation using 4 % of MSW by *S. marcescens* in shake flask level (data not shown).

Table 1. Partial purification of protease from *S. marcescens*.

Step	Total protein (mg)	Total activity(U)	Specific activity(U/mg)	Purification fold	Yield (%)
Crude	43.500	11250	258	1	100
30% $(NH_4)_2SO_4$ ppt	0.180	280	1555	5	5.3
60% $(NH_4)_2SO_4$ ppt	0.330	1320	4000	15	11.13
90% $(NH_4)_2SO_4$ ppt	0.424	1440	3396	15	10.41
DEAE- Cellulose	0.085	800	9411	37	9.3

However, in concentration of 2 and3% in shake flask level activity was not significant. From the comparative analysis of this study we found highest growth (104 log CFU/ml) of *S. marcescens* with 3 % cellulosic and proteinous materials of MSW, where in case of 2 % and 4 % MSW it was 44 log CFU/ml and 100 log CFU/ml respectively (Fig. 1).

Effects of agitation on protease production by S. marcescens in the bioreactor

The protease activity was found 97, 103 and 85 U/mL at the agitation of 110, 120 and 130 rpm, respectively (Fig. 2). Fermentation was performed with optimized 3 % concentration of cellulosic and proteinous MSW.

Purification of protease

Ammonium sulfate fractionation was performed as the first step of protease purification. Crude protease solution was precipitated with 30, 60 and 90 % saturation of $(NH_4)_2SO_4$. The precipitate was then collected and assayed for specific protease activity after performing dialysis. Fractions collected with 30 – 60 % and 60 – 90 % ammonium sulfate contained the protease activity (Table 1). As the fraction saturated with 90 % $(NH_4)_2SO_4$ saturation contained the highest protease activity, it was further used for purifying by DEAE-Cellulose column. To purify the protease following $(NH_4)_2SO_4$ fractionation, DEAE-cellulose anion exchange chromatography was performed.

Table 2. Effects of metal ions on the activity of the partially purified protease from *S. marcescens*.

Metal ions (10 mM)	Protease activity (%)
None(control)	100
Mg^{2+}	120±2
Ca^{2+}	125±4
Hg^{+}	5±2
Zn^{2+}	10±3
K^{+}	126±3

Dialyzed ammonium sulfate precipitates (60 – 90 %) were then loaded onto a DEAE-cellulose column. Fractions eluted with 0.3 to 0.55 M NaCl revealed the proteolytic activity of every fractions followed by dialysis. Table 1 summarizes the purification of protease of *S. marcescens* resulting in a final 37-fold purified protease with a specific activity of 9411 U/mg and a typical yield of 9.3 %. Following partial purification, the fractions containing protease activity were pooled and were separated on gel.

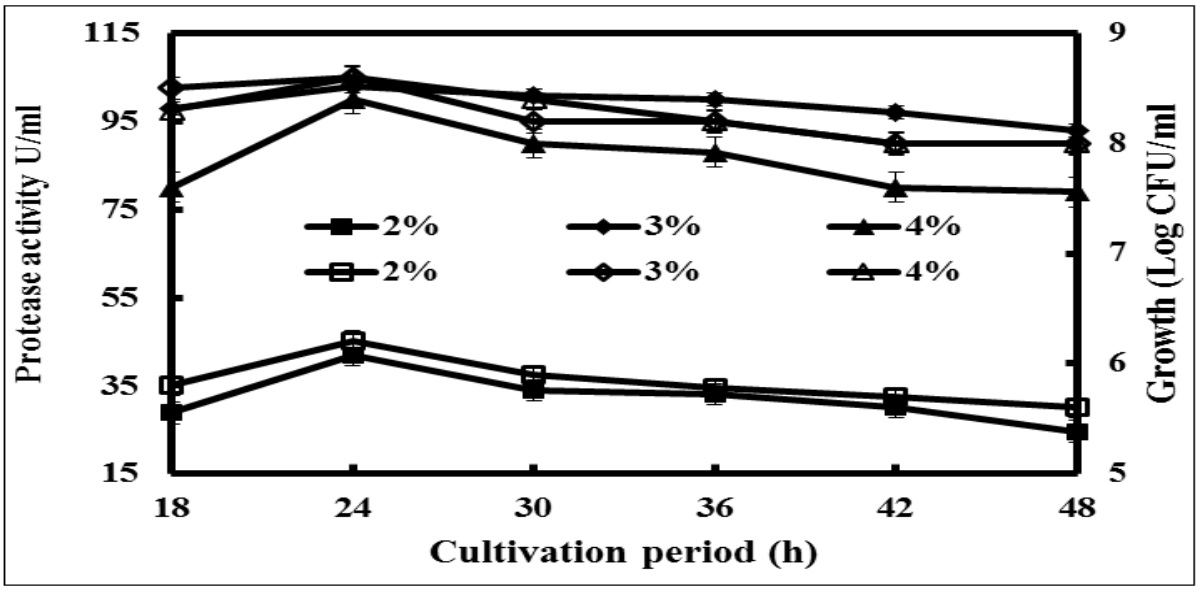

Fig. 1. Time course of protease production and growth in the bioreactor at the concentration of 2, 3 and 4% proteinous and cellulosic organic MSW. The fermentation was carried out at 30 °C, pH 8.0, agitation at 120 rpm and aeration 1vvm. Growth and protease activity was checked every 6 h interval as indicated.

A lot of small peptide fractions were noticed in the crude sample lane compared to 30-60% and 60-90% of precipitate sample. After the final step, the 37-fold purified protease had a specific activity of 9411 U/mg

with a yield of 9.3 %. SDS-PAGE (Fig. 3) showed the presence of one protein of around 55 kDa.

Characterization of partially purified protease

Effects of temperature on protease activity and stability

The optimum temperature for protease activity was found 40 °C(Fig. 4). Partially purified protease of *S. marcescens* was almost stable from 25–45 °C. However, approximately 25 % of protease stability was decreased by the treatment at 55 °C.

Effects of pH on protease activity and stability

The effects of pH on protease activity were investigated using azocasein in 50 mM buffer solutions ranging from pH 4.0 to 11.0. The protease produced by *S. marcescens* was significantly active over a broad pH range from 5.0 to 9.0 having the maximum activity at pH 7.0 (Fig. 5). The partially purified protease from *S. marcescens* lost its activity about 15 and 30 % with the equilibrium of protease in pH 5.0 and 10.0, correspondingly.

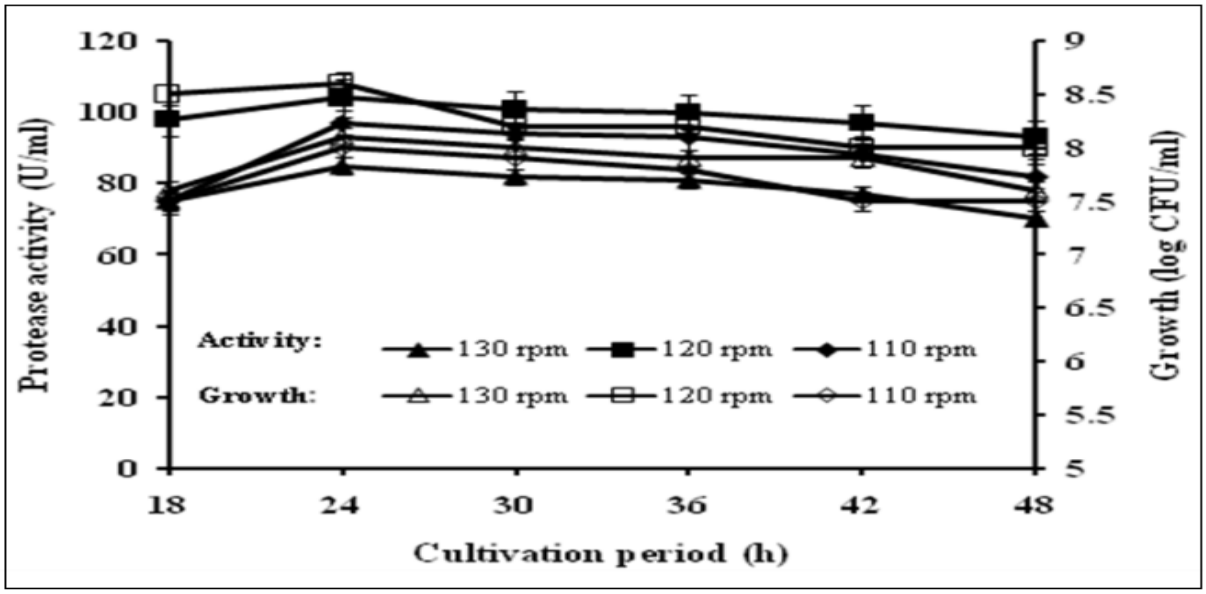

Fig. 2. Effects of agitation on protease production in the bioreactor. The bacterial culture was carried out at 30 °C with shaking at 110, 120 and 130 rpm for 60 h. The highest level of protease was found after 36 h of fermentation.

Effects of metals on protease activity

Mg^{2+}, Ca^{2+} and K^{+} were found to be very strong activators. The activities of the protease in the presence of Mg^{2+}, Ca^{2+} and K^{+} were 20 %, 25 % and 26 % higher than that observed in the control solution. In contrast, Hg^{2+} and Zn^{2+} severely inhibited the activities of protease (Table 2).

Discussion

From the time course study it was found that protease production was almost growth associated and reached its maximum activity after 24 h of incubation. However, maximum protease activity was achieved after 48 h of incubation of *S. marcescens*DEPTK21 (Mukesh *et al.*, 2012). Mohankumar *et al*, (2011) also reported a similar incubation period of 24 h for the production of serratiopeptidase enzyme from *S. marcescens*. There was a steady increase in protease production along with its exponential growth phase and the maximal level was observed at the end of exponential growth phase. It may be suggested that the protein secretion still continued from the cells even after the growth rate was stopped. Deactivation of protease occurred after the highest activity was obtained. Denaturation, degradation and autolysis might be involved in deactivation of protease (Chu *et al.*, 1992).

Not too many studies have been done with the view to produce protease by bacteria using proteinous and

cellulosic materials of MSW and to degrade proteinous waste materials. This study was done to obtain protein degrading protease producing bacteria. With this aim, *S. marcescens* isolate with proteolytic activity was isolated and their fermentation condition at bioreactor level was optimized. The use of MSW materials in producing industrial enzymes will be significantly reduce production costs. Protease production by *S. marcescens* was increased in the MSW media at fermenter level in comparison with commercial media. These results suggested that the bacterial isolate in our experiment have differential capability to utilize proteinous and cellulosic materials of MSW to produce protease.

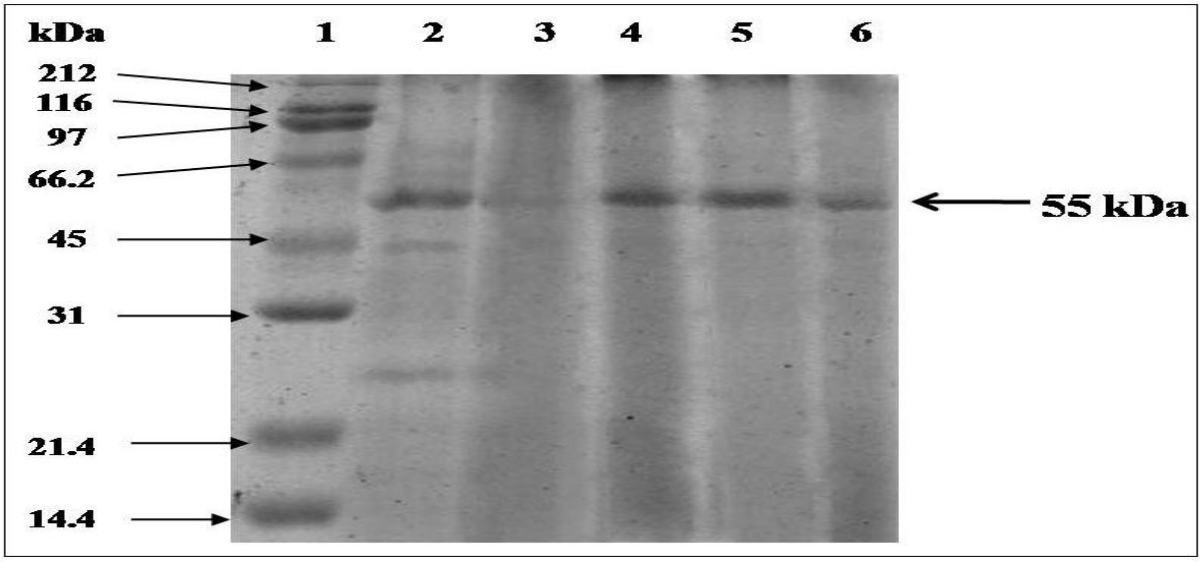

Fig. 3. SDS-PAGE analysis of protease from *S. marcescens* (55 kDa). Molecular masses of the standard proteins are shown in the left (lane 1). An equal concentration of protein 0.8 µg was applied in every lane. Lanes- 2, Crude extract; 3, 30% ppt; 4, 60% ppt; 5, 90% ppt; 6, DEAE-Cellulose pool.

Replacement of glucose, peptone and yeast extract with MSW act as a good alternative for production of protease from bacterial species. When both the carbon and nitrogen sources of the basal media were replaced with 2 %, 3 % and 4 % of cellulosic and proteinous materials of MSW *S. marcescens* took more time around 48 h to produce maximum production. But in case of fermenter *S. marcescens* with these same concentrations of cellulosic and proteinous materials took only 24 h for maximum protease production. The study also shows that the maximum protease was produced with 3 % of cellulosic and proteinous materials of MSW (Fig. 1) which is almost 102 U/ml compared to 43 U/ml with 2 % (Fig. 1) and 98 U/ml with 4 % (Fig. 1) of MSW. It indicates that maximum protease from *S. marcescens* can be produced using 3 % cellulosic and proteinous materials of MSW within 24 h of incubation at controlled environment of bioreactor, which is economically very feasible and beneficial due to its short production time and high production rate.

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Partial purification of protease from *S. marcescens* resulted 37-fold purified protease with a yield of 9.3 % that showed similarity with 119.9 fold serine protease from a study by with its yield 9.9%. (Romero *et al.*, 2001).

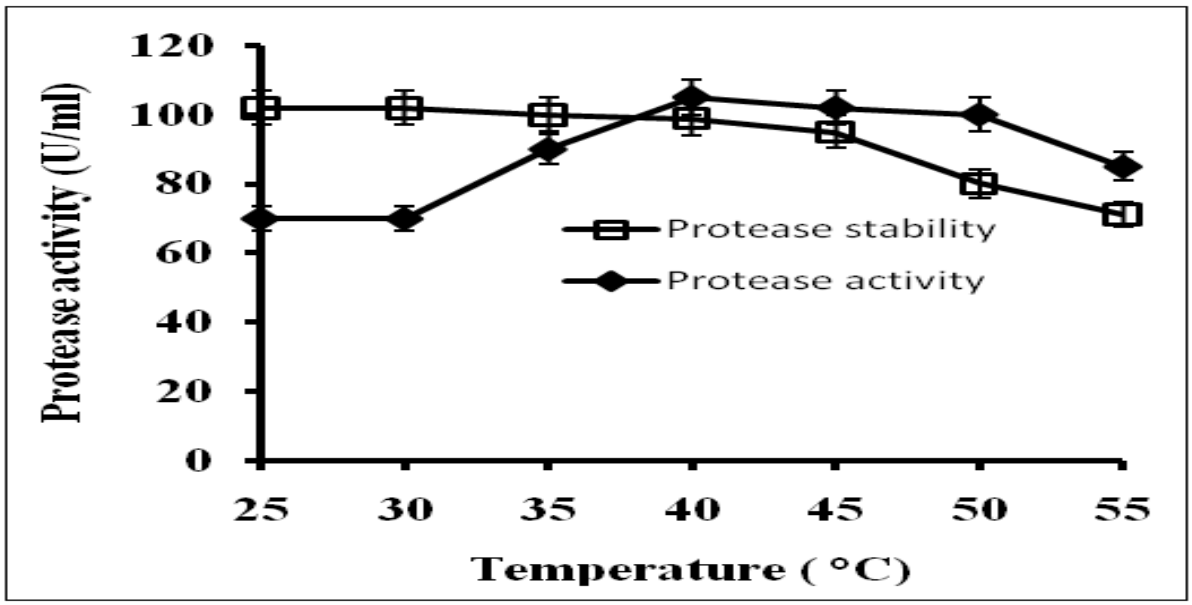

Fig. 4. Effects of temperature on protease activity and stability.

The partially purified protease was estimated to have a molecular mass of around 55 kDa which is quite similar with other reported results like 50 kDa protease produced by *S. marcescens*S3-R1 (Nam *et al.*, 2013), 53 kDa keratinolytic protease from *S. marcescens*P3 (Annapurna *et al.*, 2012), 52 kDa serratiopeptidase from *S. marcescens* (Mohankumar *et al.*, 2011), 56 kDa cold active protease from *S. marcescens* (Tariq *et al.*, 2011).

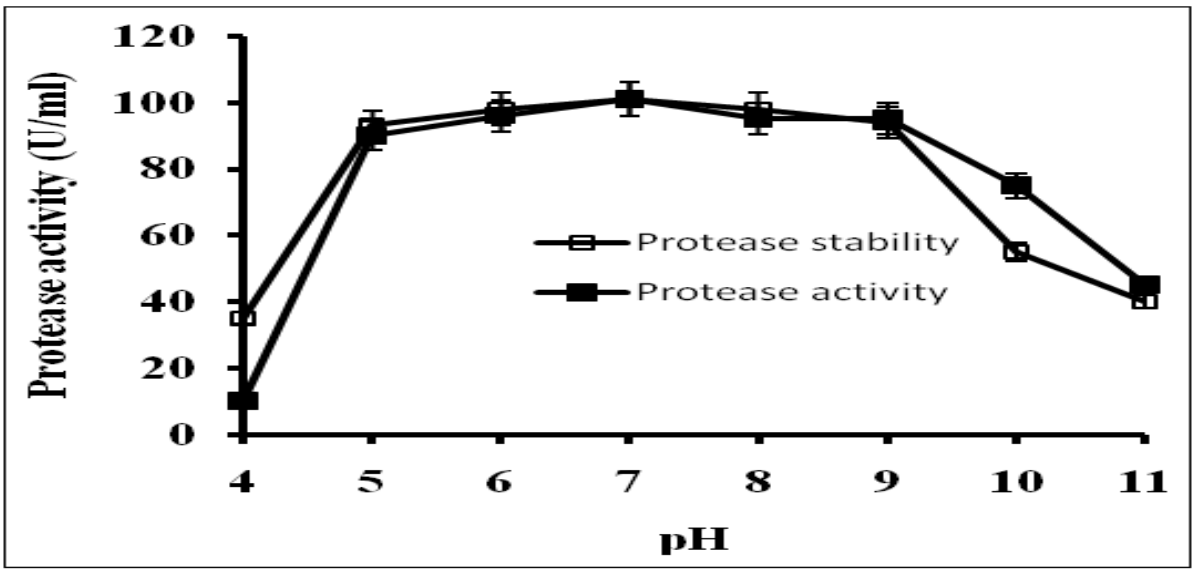

Fig. 5. Effect of pH on protease activity and stability.

Enzymes are protein in nature. So temperature has marked effect on the activities of enzymes. The effects of temperature on the activity of an enzyme are complex and can be considered as two forces acting simultaneously but in opposite directions. As temperature is raised, rate increases, but at the same time, there is a progressive inactivation of enzyme. It is necessary to optimize the physicochemical conditions for maximum activity of an enzyme before its application. Studies were made to determine the optimum temperature of the proteolytic activity of protease secreted by *S. marcescens* and it showed maximum activity at 40 °C. However, significantly

level of activity of protease was retained at the temperature of 50 °C.

The temperature at which denaturation becomes an important factor varies from enzyme to enzyme. Normally it is negligible below 30 °C and starts to become appreciable above 40 °C. The partially purified protease from *S. marcescens* was stable from 25-45 °C for 1 h and lost its activity about 30 % under the treatment at 55 °C.

It is a well-known fact that protein conformation changes at higher temperatures, and hence, causes a decrease in the protease activity (Johnvesly and Naik, 2001). Nevertheless, the protease from halo tolerant *B. subtilis* retains its full activity after 30 min of incubation in the temperature ranging from 37-55°C (Abusham *et al*., 2009).

The present enzymes was less alkaline activity compared to protease produced by *P. aeruginosa* ATCC 27853 (Zivkovic *et al*., 2010) and *B. horikoshii* (Joo *et al*.,2002) with an optimum pH activity of 9.5 and 11.0, respectively. In a study by Nam *et al.* (2013), protease produced by *S. marcescens*S3-R1 was active at pH 7-9. However, pH optima may differ for different substrates (Malathi and Chakraborty, 1991).

As the pH changes, the ionization of groups both the active site and on the substrate can alter, influencing the rate of formation and decomposition of enzyme-substrate complex. Change in pH can cause an irreversible denaturation of enzyme.

Conclusion

The present study reported that protease production was markedly increased when the commercial nitrogen and carbon sources were replaced by proteinous and cellulosic MSW. This indicates that the bacterial isolate *S. marcescens*is capable of utilizing MSW. Partial purification and characterization revealed that an extracellular protease of around 55 kDa was produced by *S. marcescens* which was quite stable at temperatures 25-45 °C and shown stability at ph 6.0-9.0. Theseresults indicate that this protease might find use in industrial applications as well as in MSW management.

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