Production and partial characterization of collagenase from marine *Nocardiopsis dassonvillei* NRC2aza using chitin wastes

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Background
The marine ecosystem has generated considerable interest for the isolation of new microorganisms, especially *Streptomyces* spp. It is considered a cheaper source of precious enzymes such as collagenase.

Objective
This study aimed to produce new collagenase enzymes from the locally isolated marine *Streptomyces* spp. grown on marine wastes for application in industrial fields.

Materials and methods
The marine isolate was identified as *Nocardiopsis dassonvillei* NRC2aza by 16S rDNA sequencing. *N. dassonvillei* NRC2aza was grown on basal medium composed of whole chitin wastes as the sole C and N source for the production of collagenase enzyme. Extraction of the enzyme was performed to study its characteristics.

Results and conclusion
Maximum collagenase activity (240 U/ml) was obtained after 6 days of incubation in shaken liquid cultures when whole chitin wastes (shrimp and crab wastes) were used as the sole nitrogen and carbon source. A *N. dassonvillei* NRC2aza isolate was shown to produce significant amounts of collagenase, reaching 1872 U/g, under solid-state fermentation using a mixture of 10 g chitin waste and 2 g of feather. Successive ammonium sulfate fractionation of *N. dassonvillei* NRC2aza growth extract produced a group of collagenases with different molecular weights. The 80% enzyme fraction was the most active and possessed the highest collagenase activity (1106.66 U/f), reaching about 3.8-fold that of the culture filtrate. The optimum pH and temperature were 8 and 55°C, respectively, and the enzyme was stable at pH range of 6–8. The collagenase exhibited heat stability for 60 min at 50°C. Therefore, collagenases can be applied in food industry as tenderizers of red meat and in fur and hide tanning to ensure uniform dyeing of leather.

Keywords:
azocoll, collagenase, marine *Nocardiopsis dassonvillei* NRC2aza, marine wastes

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Introduction

Collagen is a major fibrous element of skin, bones, tendons, cartilage, blood vessels, and teeth found in all multicellular organisms. Collagen is advertised regularly in the mass media as an ingredient in drugs, drinks, food, cosmetics, and a variety of healthcare products. It has been reported that collagen peptides, the product of collagen degradation, possess several biological activities of industrial and medical interest. Collagenases are collagen-hydrolyzing enzymes that play an important role in connective tissue metabolism [1]. In the animal body they are produced by specific cells and are involved in repair and remodeling processes [2]. The microorganisms producing collagenase are mostly pathogenic in nature and presumably contribute to their pathogenicity by allowing the organisms to penetrate the connective tissue barrier [3–5] or are involved in degradation of collagen under natural conditions [6–8]. Microbial collagenases have wider applications because they cleave collagen helix at multiple sites. Purified collagenases are used in quantification of collagen and in isolation of specific cell types from attendant connective tissue and pulmonary mast cells from bovine tissue [9,10].

In medicine, collagenases are used in experimental transplantation of pancreatic islet cells to alleviate diabetic symptoms [11], enzymatic debridement, nonsurgical removal of debris from wounds, removal of dandruff, etc. [2]. Collagenolytic proteases are classified into two major groups: metallocollagenases and serine collagenases. Among bacterial collagenolytic proteases, metalloproteases are the most frequent, whereas serine proteases and other proteases are rarely seen. All metalloproteases share a zinc-containing HEXXH motif in their active sites. Although collagenase production by bacteria is well documented, little attention has been paid to studying the actinomycetes, known as antibiotic producers. A variety of actinomycetes are actively involved in the processes of degradation of native collagen under natural conditions [12–15]. A higher thermostability

of collagenolytic enzymes would be a significant advantage for their isolation, purification, storage, and different biotechnological applications. There are only a few reports for collagenase forms from thermophilic organisms [16]. It is therefore promising to search for new indigenous thermophilic microorganisms that are able to grow on collagen-rich materials and that produce collagenolytic enzymes of relatively high thermostability. For this purpose, attention has been paid recently to the thermophilic actinomycetes as a possible producer of new thermostable enzymes [17]. They have some advantages in comparison with mesophilic strains, including accelerated accumulation of biomass and desired enzymes, and are also harmless for humans. Considering the importance of collagenase, the present investigation was planned to partially characterize collagenase produced by the thermophilic marine *Streptomyces* strain *Nocardiopsis dassonvillei* NRC2aza.

Materials and methods

Microorganism

A proteolytic marine *Streptomyces* spp. was isolated from the Mediterranean Sea at a depth of about 300 m. The seawater specimen was incubated with pure collagen for about 4 weeks at 37°C. Then the collagenolytic enzyme producer *N. dassonvillei* NRC2aza was isolated using the serial dilution technique and maintained on starch–peptone–yeast slants at 37°C. The maintenance medium was prepared with marine water throughout the study.

Substrate

Chitin wastes composed of a mixture of shrimp and crab waste were obtained from fish markets. The obtained wastes were washed successively with deionized water to remove the sand debris present on the surface. The chitin wastes were then air-dried and ground. The powdered material obtained was used as the substrate for the enzyme production.

Reagents

Azocoll and Folin–Ciocalteu reagents were from Sigma Scientific Services Co. (St. Louis, Missouri, USA). Other reagents were from Merck (Darmstadt, Germany). All other chemicals and bacteriological media were from standard sources.

Taxonomic studies and 16S rDNA sequencing

Isolation of bacterial genomic DNA was performed using Gene Jet genomic DNA purification kit (Fermentas, EU). PCR-restriction fragment length polymorphism analysis was carried out using Maxima Hot Start PCR Master Mix (Fermentas). Thereafter, 4 μl of the PCR mixture was loaded to examine the PCR product on 1% agarose gel against a 1 kb plus ladder (Fermentas); F: AGA GTT TGA TCC TGG CTC AG, R: GGT TAC CTT GTT ACG ACT T. Then PCR clean-up was carried out for the PCR product using Gene Jet PCR purification kit (Fermentas). Finally sequencing to the PCR product on GATC Company with ABI 3730xl DNA sequencer by using forward and reverse primers was made (Sigma Scientific Services Co.).

Culture conditions for enzyme production

Production medium

Liquid starch–peptone–yeast medium was used for inoculum preparation. The basal medium used for the enzyme production consisted of (g/l) 0.7 g of KH2PO4, 1.4 g of K2HPO4, 0.1 g of MgSO4, 0.5 g of NaCl, and 20 g of marine waste (chitin waste). All the constituents were prepared in marine water at a ratio of 1 l. Three-day-old inoculums were prepared using the spore suspension from 7-day-old slants of *N. dassonvillei* NRC2aza and incubated on a shaking incubator at 150 rpm at 37°C. Then 6% of the prepared inoculum was transferred to 250 ml Erlenmeyer flasks, each containing 50 ml of the basal medium, and incubated for 6 days on a rotary shaker at 150 rpm. Packed cell volume of the biomass produced was measured by centrifugation of the content of each flask at 5000 rpm for 10 min.

The protein of the culture filtrate was determined by the method of Lowry *et al*. [18].

Determination of collagenase activity

Collagenase activity was measured using azocoll as a substrate by the following method [19]. One unit of enzyme activity was defined as decomposition of μg azocoll per ml. The reaction mixture containing azocoll (0.0125 g) suspended in 2.5 ml of 0.05 mol/l tris-HCl and 1 mmol/l CaCl2 (pH 7.5) was incubated with 0.1 ml diluted enzyme at 40°C for 30 min. Assays were stopped by immersing tubes in an ice bath. Chilled tubes were then centrifuged and the supernatant was measured at 520 nm. Control tubes, containing azocoll without enzyme, were incubated for the same length of time as the test samples.

Production of collagenase by solid state fermentation (SSF)

This study was performed on the marine *N. dassonvillei* NRC2aza using the different wastes available (chitin

wastes, feather and leather wastes) as the solid substrate. A measure of 10 g of different combinations of the different wastes was prepared in 250 ml Erlenmeyer flasks and mixed with 10 ml of the basal medium and autoclaved at 121°C for 15 min. They were cooled to room temperature and each flask was inoculated with 3 ml of 3-day-old inoculum.

Extraction and enzyme recovery

For isolation of collagenase enzymes produced under solid state fermentation, 10 volumes of distilled water per gram of substrate (based on the initial dry weight of the substrate) were added to the fermented media and the extraction was performed by agitation at room temperature in a rotary shaker for 60 min at 150 rpm. The slurry was then squeezed through sheet cloth and clarified by centrifugation at 5000 rpm at 4°C for 15 min. The clear supernatant was used for collagenase assay and protein content.

Ammonium sulfate fractionation of collagenase

The supernatant of bulk-cultured marine *N. dassonvillei* NRC2aza was centrifuged (10 000*g*, 4°C, and 20 min) to remove cells and debris, and the supernatant was collected. Solid ammonium sulfate was added to the supernatant with a saturation of 50–80% under gentle stirring and then kept at 4°C overnight. The suspension was centrifuged and the precipitate was dissolved in 0.05 mol/l tris buffer (pH 7.5). The solution was then dialyzed against the same buffer and each dialysate was assayed for collagenase activity and protein content.

Results and discussion

A marine *Streptomyces* strain locally isolated from marine habitats was able to grow on chitin wastes as the sole C and N source for collagenase enzyme production. The strain was identified by 16S rDNA sequencing (Sigma Scientific Services Co.).

Phylognetic analysis of 16S rDNA

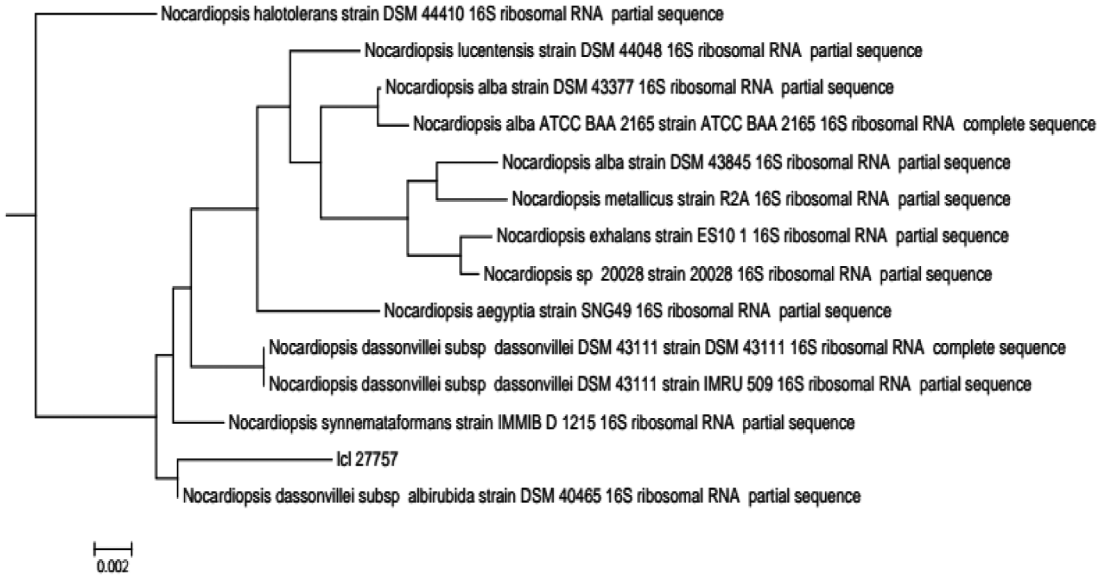
Phylognetic analysis based on 16S rDNA sequencing exhibited a high level of homology (95%) with *N. synnmemataformans* strain IMMIB D 1215, as shown in Figure 1.

N. dassonvillei NRC2aza secretes a large number of hydrolytic enzymes, both inducibly and constitutively on different wastes. This observation was made by Jain and Jain [20], who reported that collagenase enzyme was produced constitutively on poultry feather. However, it was observed that a high level of collagenase was produced by the marine strain in a medium containing chitin waste as the sole N and C source. However, in other *Streptomyces* spp., collagenase production is associated with its induction by the specific substrate [21,22]. *N. dassonvillei* NRC2aza also produced highly active collagenase enzymes in SSF using other wastes like feather and leather, which is a new trend that can be applied in the field of industry (Fig. 2).

Collagenase fractionation

N. dassonvillei NRC2aza collagenase enzymes were easily purified from the culture filtrate by ammonium

Figure 1



Phylogenic tree based on 16S rDNA sequence of *Nocardiopsis dassonvillei* NRC2aza within the genus Nocardiopsis.

sulfate fractionation (with concentrations ranging from 50 to 80%). From the results illustrated in Table 1 it can be seen that three partially pure collagenase fractions (60, 70, and 80%) were produced. This means that we have more than one enzyme. The 80% fraction was the most highly active enzyme (1106.66 U/f) with a 3.8-fold purification and a yield of 7.62% from the crude extract. This indicated that the chitin waste was highly conducive for the production of a type of collagenase that has importance in industrial and medical applications. This is nearly similar to another work with *Bacillus pumilus* COI-J collagenase, in which ammonium sulfate was used to precipitate the enzyme with a 3.39-fold purification and 66.4% yield [23]. Collagenase from *Streptomyces exfoliates* was purified by ammonium sulfate precipitation with 3.29-fold purification and 67.21% yield [20].

Optimum temperature and thermal stability

The effect of temperature on *N. dassonvillei* NRC2aza collagenase activity was determined at different temperatures ranging from 35 to 60°C, followed by assaying the collagenase activity against the control (substrate without enzyme). As shown in Figure 3, the maximum collagenase activity was observed at 55°C. The increase in temperature above 55°C drastically reduced the collagenase activity. The maximum temperature obtained was higher than the maximum temperature reported for *Cytophaga* spp. L43-1, which is between 30 and 42°C [24], and lower than the maximum temperature of *Bacillus* spp. MO-1 and *Thermoactinomyces* spp. 21E, which is between 70 and 75°C [25,26]. From the temperature profile illustrated in Figure 4 it can be seen that collagenase enzyme maintained 100% of its hydrolytic activity for 1 h at 40 and 50°C. At 60°C the relative collagenase activity was only 40.5%.

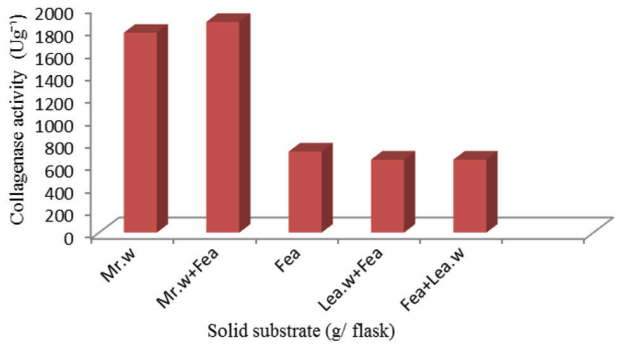
Effect of pH on collagenase activity and stability

The effect of pH on enzyme activity was estimated in three different buffer systems, 50 mmol/l sodium acetate (pH 5.5), 50 mmol/l sodium phosphate (pH 6–7), and 50 mmol/l tris-HCl (pH 8–9). As illustrated in Figure 5 the optimum pH of the collagenolytic enzyme was estimated to be 8, which was the same as for the collagenolytic enzyme of *Treponemadenticola* spp. ATCC 35405-A [27]. The marine *N. dassonvillei* NRC2aza collagenase enzyme maintained 100% activity within the pH range 6–8 (Fig. 6).

The relative activity of the partial pure collagenolytic enzyme, which was highly stable in the pH range of 7–8, was 64.53 and 39.67% after 1 h at pH 8 and 9, respectively.

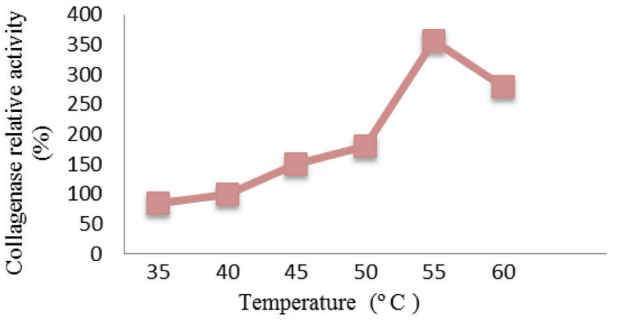
Therefore, production of *N. dassonvillei* NRC2aza collagenase enzyme on cheap N and C sources such

Figure 2



Production of collagenase from marine *Nocardiopsis dassonvillei* NRC2aza using solid-state fermentation. Marine wastes (Mr.w), 10 g; Mr.w, 8 g+feather (Fea), 2 g; Fea, 10 g; leather wastes (Lea.w), 8 g+Fea, 2 g; Fea, 8 g+Lea.w, 2 g.

Figure 3



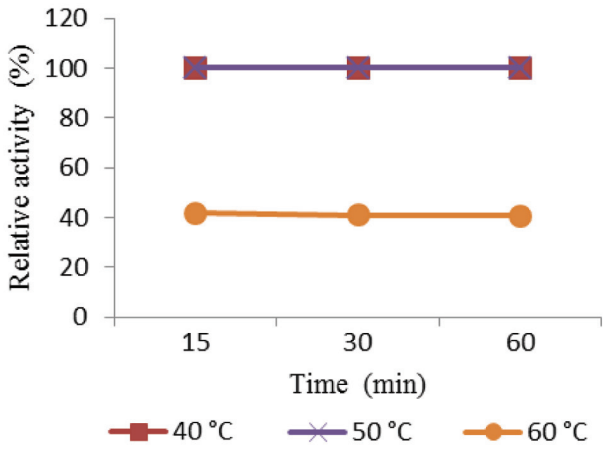
Effect of different temperatures on the partial pure (80% ammonium sulfate fraction) collagenase activity of the marine *Nocardiopsis dassonvillei* NRC2aza.

Table 1 Fractional precipitation of marine Nocardiopsis dassonvillei NRC2aza collagenase enzymes by ammonium sulfate

Ammonium sulfate concentration (%)	Protein content of fraction (mg/f)	Recovered protein (%)	Total activity (U/f)	Recovered activity (%)	Specific activity (U/mg)	Fold purification
50	59.9	5.23	2006.65	1.23	33.5	0.24
60	20.97	1.83	1173.32	0.72	55.95	0.39
70	12.43	1.09	966.66	0.60	77.76	0.55
80	2.04	0.18	1106.66	0.68	542.48	3.81

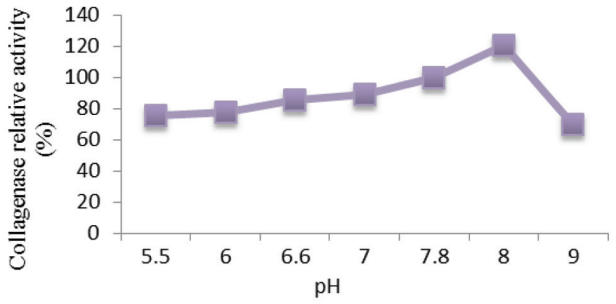
Temperature = 40°C, pH = 7.

Figure 4



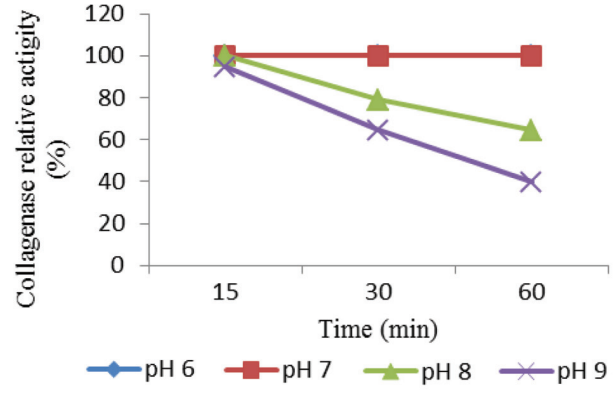
Temperature profile of Nocardiopsis dassonvillei NRC2aza collagenase activity.

Figure 5



Effect of pH on the activity of Nocardiopsis dassonvillei NRC2aza partial pure collagenase enzyme.

Figure 6



Effect of pH on Nocardiopsis dassonvillei NRC2aza collagenase enzyme stability.

as chitin waste and poultry waste is significant as it will reduce the cost of production of the enzyme and may enable efficient hydrolysis of marine and poultry waste from the point of view of waste management.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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