Original Article

Screening and Isolation of Collagenase Producing Microorganism from Proteins Waste Found in Himalayan Region

Manisha Gautam[1*], Wamik Azmi[1]

Abstract

Multicellular organisms of the human and animal bodies such as skin, bones, tendons, cartilage and blood vessels have collagen as a fibrous element. Collagen is a most representative protein of the leather wastes and collagenase is a proteolytic enzyme. The collagenase is responsible for the degradation of native collagen to small peptide fragments, without affecting the other proteins. Collagenase has widespread applications such as cosmetics, wound healing, diabetic ulcer, arterial ulcers and burns surgery, etc. In the present study, Twenty-seven collagenase-producing microorganisms were identified and isolated from soil/sewage samples of the fish market and slaughterhouse areas. CS-20 sample was found most efficient producer microorganism of collagenase. This sample was identified from Pseudomonas genus and isolated as *Pseudomonas* species. The parameters such as temperature, substrate, pH, incubation period and inoculum percentage were also optimized to screen the hyper producer strain with maximum collagenase activity. CS-20 isolate (Collagenase producing microorganism) was capable of hydrolyzing other protein substrates such as gelatine and azocoll. Thus, CS-20 isolate is high-efficiency strain for the production of collagenase.

Keywords: Microorganisms, Proteolytic Enzyme, Collagenase, *Pseudomonas* Genus

1. Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, India

*** Corresponding Author**
Manisha Gautam
Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, India
E-mail: manisha.gautam21@gmail.com

Submission Date: 4/17/2017
Accepted Date: 8/12/2017

Introduction

Microorganisms are one of the best agents to produce enzymes, and right selection of microbe is a key factor for their large-scale production [1]. In addition, hydrolytic activity and specificity toward the substrate is also one of the primary factors that help to screen healthy strain [2]. Identification, isolation, and characterization of novel strains for the production of industrial important enzymes at economical level are still continuous process [1, 3]. Waste based protein enriched habitats are the most suitable resources of proteolytic agents. Numbers of proteolytic fungi and bacteria have been isolated, but very few of them are commercial exploited. As described in literature, the most extensive works have been reported on the bacterial collagenases because of their broad substrate specificities and abilities to degrade both native and denatured collagens [4]. Collagenases have been isolated and characterized from both microbial cells and animal tissues [5]. Procollagenase is secreted from a variety of cell lines in the culture, including fibroblasts [6]. They are mostly extracted from viscera organ of fish/other animals, but the process of extraction is very tedious and uneconomical [7, 8]. Schenck and his co-workers highlighted that collagenase production by fungi is limited [9]. The collagenases were mostly reported from bacteria such as *Clostridium*, *Pseudomonas*, *Vibrio* and *Streptomyces* sp.. These collagenases can break collagen polypeptides at multiple points [6]. Collagenase avoids the introduction of pathogens derived from the animals into bioprocessing. Highly purified form of collagenase is used for the collagen and biosynthetic studies [10]. Collagenase has widely been used by the molecular biologist in medical industries [11-13]. Collagenase, successfully used for the isolation of cells from bone [14], cartilage, thyroid glands [15], ovarian and uterine tissues [16], skin, endothelial cells [17]. Enzymatic debridement, non-surgical removal of debris from wounds and removal of dandruff are some of the interesting applications of collagenase [18]. Collagenases are also useful in blood clots removal. Proteins enriched waste material from meat, poultry and fish-processing industries is a good economical resource for collagenase production [19, 20]. Moreover, the enzymes activities are not enough to use them for industrial processes. Oftenly, the industrial processes are performed under specific conditions, required for the activity and stability of the enzyme. Therefore, the present study focused to isolate the collagenase-producing microorganism from protein waste discharged soil, which is capable to produce the enzyme of broad substrate specificities with multiple applications for commercial interest.

Materials and Methods

Microorganism and Chemicals

Isolation of Microorganisms

Collagenase producing microorganisms were screened and isolated from the soil/sewage samples collected from the

All rights reserved for official publication of Baqiyatallah university of medical sciences©

local fish market and slaughterhouse area of Bilaspur and Shimla, Himachal Pradesh, India. The soil samples were serially diluted (10^{-8}) by sterile distilled water, and 100 μl suspension was spread evenly on the plate, incubated at 37°C for 24 h [21]. Collagenase activity was tested through a selective medium (pH 7.5). The selective medium contained (per liter): 20 g gelatin, 0.1 g NaCl, 5 g peptone, 0.5 g KH_2PO_4 and 0.2 g $MgSO_4 \cdot 7H_2O$, pH 7.5. After 24 h of incubation at 37 °C, the acidic hydrargyrum solution was used to detect the colonies with glatin hydrolysis activity. The acidic hydrargyrum solution consisted of 15 g $HgCl_2$ and 20 ml dense hydrochloric acid per 100 ml. The bacteria with a bigger ratio of gel hydrolysis collar to colony diameter were inoculated into fluid medium [23]. All the bacterial strains were streaked on to new medium plates before using acidic hydrargyrum solution for the detection of gelatinase activity. The selected positive isolates were grown in a liquid medium [22].

Identification

Morphological characteristics of all the positive isolates were studied and evaluated for gelatin hydrolysis activity. Further, the screening was done on the basis of their ability to digest goatskin and hydrolysis of the substrate azocoll. The bacteria with a bigger ratio of gelatin hydrolysis zone were inoculated in production medium (pH 7.5), contained (%, w/v): glucose 2.0, yeast extract 0.15, tryptone 1.0, $CaC1_2$ 0.005, $NaH_2PO_4 \cdot 2H_2O$ 0.05 and $K_2HPO_4 \cdot 3H_2O$ 0.25 [22]. The medium (50 ml) was supplemented with autoclaved dehaired goatskin pieces (2 g, w/v) of approximately 2x2cm^2 size, and inoculated with 24 h old inoculum of the positive isolates. All the isolates were incubated at 37°C with the agitation rate/speed of 150 rpm. The observation was carried out after 48 h and found the digestion of goatskin. Total 27 microorganisms were isolated, and one hyper producer (CS-20) strain was found which showed highest collagenase activity during screening. This CS-20 was sent to Microbial Type Culture Collection and Gene Bank (MTCC), a national facility of the Institute of Microbial Technology, Chandigarh, for identification. CS-20 sample was *Pseudomonas* species.

Assay of collagenase activity

Collagenase and protease activity of *Pseudomonas* sp. (CS-20) was assayed using different substrates including gelatin, and azocoll.

Gelatin as substrate

Collagenase activity was initially measured using gelatin as substrate, according to the method described by Nagano and Kim, (1999) [23].

Azocoll as substrate

Cultures exhibiting activities on the denatured gelatin were subjected for further assay using azocoll (Insoluble, ground collagen attached with azo-dye) as substrate. This substrate was used because of its sensitivity towards variety of proteolytic enzymes and reproducibility of the assay for its hydrolysis [24].

One ml reaction mixture containing 2 mg of azocoll, 0.5 ml cell free supernatant/ enzyme and 0.1 M Tris-HCl buffer containing 0.05 M $CaCl_2$ (pH 7.5) was incubated at 37°C. After incubation for 30-120 min, the reaction mixtures were placed on ice and undigested azocoll was removed using centrifugation. The absorption of the supernatant was measured at 540 nm (A_{540}).

Enzyme activity

The concentration of hydrolyzed amino acids was determined by a standard curve of L-leucine. One unit (U) of enzyme activity has been defined as the amount of enzyme required for the hydrolysis of 1 μM of substrate per min as per assay conditions. The collagenase from Pseudomonas sp. was found to be an extracellular in nature. Hence, the culture supernatant was used for the enzyme assay.

Optimization of physical parameters for collagenase production by CS-20

The various parameters optimized for the maximum production of collagenase by CS-20 which includes; selection of medium, role of inducer, optimization of medium pH, incubation temperature, inoculum size and age, incubation time and agitation rate.

Results and Discussion

Isolation of the collagenase-producing microorganisms

Isolation of the collagenase producing microorganisms were carried out with selective medium [pH 7.5; %, w/v: gelatin 2.0, agar 1.5, NaCl 0.01, peptone 0.5, KH_2PO_4 0.05 and $MgSO_4 \cdot 7H_2O$ 0.02] from soil and sewage samples. The acidic hydrargyrum solution was used to detect the colonies with gelatin hydrolysis activity after 24 h of incubation at 37°C. Total 27 isolates were isolated.

Screening and selection of the collagenase-producing microorganisms

A large number of samples used for the isolation of collagenase producing microorganisms. The twenty seven positive strains were observed that showed good gelatin hydrolysis zone, and selected to grow in the liquid culture medium [22]. Secondary screenings of these isolates were carried out based on their ability to hydrolyze the substrate azocoll and digestion of goatskin. Out of 27 isolates, only seven bacterial strains produced high collagenase activity with azocoll. These strains were further used to confirm their potential to digest goatskin, by inoculating them with autoclaved piece of goatskin suspended in the production medium (Table 1). The total of 27 microbial isolates were found active but the bacterial isolate CS-20 showed the maximum collagenase activity with gelatin and azocoll substrate (Figure 1). Gram stain was performed with CS-20 isolate, which showed Gram-negative rods. The CS-20 isolate produces colonies with a characteristic greenish pigmentation on bacteriological media (Table 2; Fig. 2).

In mixed cultures, it can be isolated as clear colonies on MacConkey agar (as it does not ferment lactose) which will test positive for oxidase. Confirmatory tests include the production of the blue-green pigment pyocyanin on cetrimide agar. The isolated strain was Gram-negative and identified as *Pseudomonas* sp. at IMTEH Chandigarh. Further, the production and characterization of collagenase was carried out with the CS-20 (*Pseudomonas* sp.). The importance of *Pseudomonas* family in human disease is well known, especially in nosocomial infections as well as the patients being treated with corticosteroids, antineoplastic drugs, radiation and prolonged antimicrobial therapy.

Pseudomonas sp. produces a variety of extracellular substances that may play a role in the disease process particularly the proteolytic enzymes [25-26].

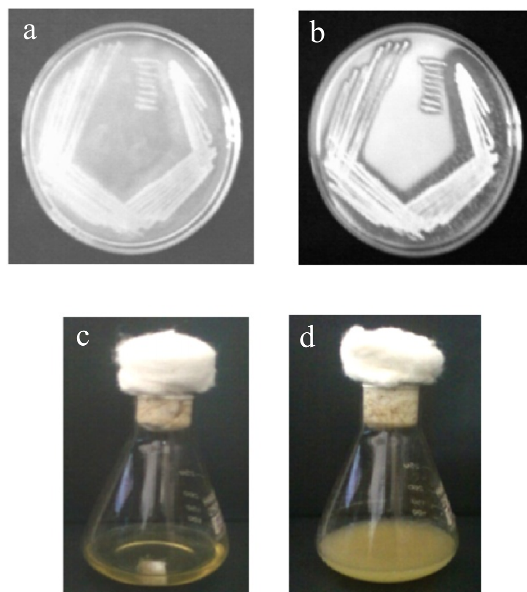

Figure 1. Positive strain with gelatin hydrolysis zone and goatskin digestion ability.
(a) Bacterial strain (b) Gelatin hydrolysis zone observed after 24 h (c) Production medium containing autoclaved piece of goatskin and inoculated with a bacterial isolate having gelatinase/azocoll activity (d) Goatskin digestion observed after 24 h.

Optimization of physical parameters for collagenase production using Pseudomonas sp (CS-20)

Selection of medium for collagenase production

The culture of *Pseudomonas* sp. was grown on fourteen different media and assayed for the growth and collagenase activity and change in the initial pH of the media was also noted (Table 3). The results suggests that the medium M-5 (pH 7.5) containing (%, w/v) sucrose 1.0, yeast extract 2.0, peptone 1.0, $MgSO_4.7H_2O$ 0.04, Na_2HPO_4 0.2 and Na_2CO_3 0.25 was most suitable for the production of collagenase. The maximum collagenase activity obtained with M-5 medium was 0.173 U/ml with 2.21 mg/ml of cell mass. The Pseudomonas sp. showed comparable collagenase activity with media M-10 and M-2 (0.153 U/ml and 0.137 U/ml, respectively). However, the rest of the media exhibited very less amount of collagenase activity and minimum activity was found with M-1, M-6 and M-9 medium. The same medium (M-5) was found most suitable for collagenase production from *Streptomyces* sp. as recorded by Endo *et al*., (1986) [27]. Hence, our results also confirm that medium M-5 was most suitable and selected for the maximum production of collagenase from *Pseudomonas* sp.

Role of inducer in seed and production medium

Earlier reports revealed that collagenase synthesized only in gelatin and collagen induced conditions [28]. Thus, gelatin and collagen was used as inducer to seed and production media. The different combinations of inducers were used at a concentration of 0.3%, (w/v) in seed and production medium (M-5, pH 7.5) and finally, the production of collagenase by *Pseudomonas* sp. was also determined.

Table 1. Summary of the morphological characteristics and collagenase activity of the microbial isolates.

Isolate	Colony colour	Colony shape	Gelatinase Activity	Azocoll assay	Skin digestion (after 24h)
CS-1	White	Rounded	++	+	-
CS-2	Cream	Irregular	++	+	-
CS-3	Cream	Rounded	++	+	-
CS-4	Pale yellow	Rounded	++	+	-
CS-5	Brown	Irregular	++++	+++	Partial
CS-6	Cream	Irregular	++	+	-
CS-7	White	Irregular	++	+	-
CS-8	Cream	Rounded	++	+	-
CS-9	Pale yellow	Irregular	+++	+	-
CS-10	Cream	Rounded	+++	+	-
CS-11	White	Rounded	++++	+++++	Partial
CS-12	Cream	Rounded	++++	+++++	Partial
CS-13	Pale yellow	Irregular	++++	+++++	Partial
CS-14	Cream	Rounded	+++	++	Poor
CS-15	White	Irregular	++	+	-
CS-16	White	Rounded	++	+	-
CS-17	Pale yellow	Rounded	++	+	-
CS-18	Cream	Irregular	++	+	-
CS-19	White	Rounded	++	+	-
CS-20	**Green**	Rounded	++++	+++++	Complete
CS-21	cream	Rounded	++	-	-
CS-22	Brown	Irregular	++	-	-
CS-23	White	Irregular	++	-	-
CS-24	Cream	Rounded	++	+++	-
CS-25	Brown	Irregular	++	+	-
CS-26	White	Irregular	+++	++++	partial
CS-27	Cream	Irregular	+++	+	-

Table 2. Morphological and physiological characteristics of *Pseudomonas* sp.

Morphological Tests		**Remark(s)**
Colony characteristics	Configuration	Round
	Margin	Undulated
	Elevation	Raised
	Surface	Rough
	Density	Opaque
	Pigments	None
	Edge	Entire
	Emulsifiability	Forms uniform turbid suspension
Gram's staining	Shape	Rod
	Gram stain	Gram negative
	Lactose fermentation	-
Spore formation	Endospore	
Fluorescence	Bluish green pigment	+
Broth culture	Culture	Aerobic
	Amount of growth	Moderate
	Surface growth	Present
	Turbidity	Uniform
Physiological tests	Citrate	+
	Oxidase	+
	Methyl red	-
	Catalase	+
	Urea hydrolysis	+
	Gelatin hydrolysis	+
Growth at temperature	**37° C**	+

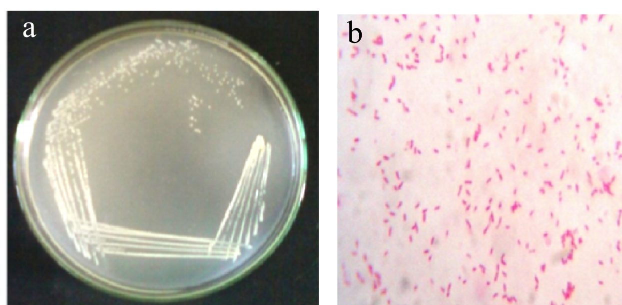

Figure 2. Culture of *Pseudomonas* sp.
(a) The growth of *Pseudomonas* sp. (b) Morphology of *Pseudomonas* sp.

Table 3. Selection of medium for the production of collagenase by *Pseudomonas* sp.

S. No.	**Media used**	**Collagenase activity (U/ml)**	**Cell mass (mg/ml)**	**Final pH**
1.	M-1	0.022	0.41	8.1
2.	M-2	0.137	2.05	7.6
3.	M-3	0.092	2.09	8.4
4.	M-4	0.073	1.76	8.5
5.	**M-5**	**0.173**	**2.21**	**8.6**
6.	M-6	0.042	1.48	8.5
7.	M-7	0.119	1.19	8.3
8.	M-8	0.107	1.97	6.2
9.	M-9	0.055	0.98	7.4
10.	M-10	0.153	1.59	8.6
11.	M-11	0.101	2.50	7.7
12.	M-12	0.111	3.28	7.5
13.	M-13	0.113	2.83	7.6
14.	M-14	0.127	2.75	7.7

The gelatin was found to increase the growth as well as collagenase activity (Table 4). Maximum growth (2.22 mg/ml) and collagenase production (0.208 U/ml) was obtained when 0.3% (w/v), gelatin was used in production media. The final pH of the fermentation broth was found to increase from 7.5 to 8.6. Reid *et al*., (1978) reported that *A. iophagus* requires a peptone inducer for the production of extracellular collagenase while later on Nagano and Kim used the gelatin as inducer in the medium for collagenase production from *Bacillus subtilis* FS-2 [23, 29]. Numbers of reports have already been published that production of bacterial collagenases was greatly enhanced in presence of gelatin, casein and collagen substrates [30, 28]. In contrast, production of extracellular collagenase from *Zygosaccharomyces rouxii* has shown that there was no need to add gelatin [31]. In current study, the addition of collagen in production medium has shown the most significant results, but for cost effective production of collagenase by *Pseudomonas* sp., a most economical gelatin was used as an inducer in production medium.

Table 4. Role of gelatin and collagen the in seed and production medium.

S. No.	Seed medium	Production medium	Collagenase activity (U/ml)	Cell mass (mg/ml)	Final pH
Gelatin					
1	+	-	0.178	2.09	8.60
2	+	+	0.188	2.09	8.57
3	-	-	0.124	1.76	8.97
4	-	+	0.208	2.22	8.60
Collagen					
1	+	+	0.564	1.80	8.67
2	+	-	0.177	1.89	8.66
3	-	+	0.166	2.30	8.62

+ = With inducer; - = Without inducer

Optimization of inducer concentration in the production medium

The varying concentration (0.1 to 0.5%, w/v) of inducer gelatin has been added to the production medium (pH 7.5) containing (%, w/v) glucose 2.0, yeast extract 0.15, tryptone 1.0, $CaC1_2$ 0.005, $NaH_2PO_4{\cdot}2H_2O$ 0.05 and $K_2HPO_4{\cdot}3H_2O$ 0.25. The maximum collagenase activity (0.210 U/ml) was obtained at the 0.3% (w/v) concentration of gelatin in the production medium (Fig. 3).

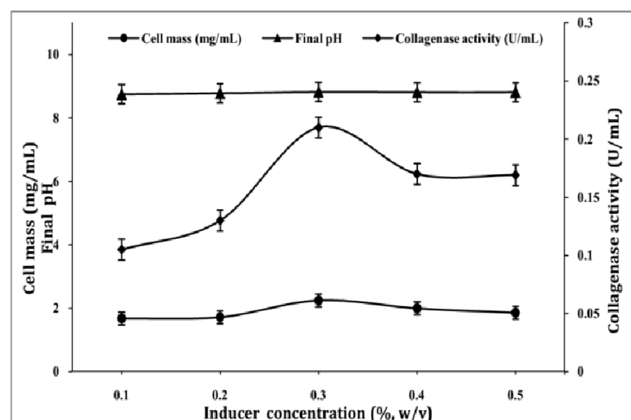

Figure 3. Effect of inducer concentration on collagenase activity in production medium of *Pseudomonas* sp.

However, with further increase in concentration of gelatin, the collagenase production by *Pseudomonas* sp. gradually decreased. The maximum cell mass (2.25 mg/ml) was also obtained with 0.3% (w/v) of gelatin in production medium. Similar concentration (0.3%, w/v) of the gelatin inducer was added in medium for the induction of collagenase production by *B. subtilis* FS-2 [23].

Optimization of medium pH for the production of collagenase

The variation in medium pH greatly affects the uptake of nutrient from the medium which subsequently help in the growth and metabolite production. Therefore, it is very important to optimize the initial pH of the medium. The effect of varying initial pH (4.5-9.0) of the production medium on the growth and collagenase production by *Pseudomonas* sp. was studied (Fig. 4). The present strain of Pseudomonas sp. was found to grow at a wide range of pH 4.5-9.0, but showed maximum collagenase production (0.225U/ml) in culture medium having initial pH 6.5. It was also evident from the results that the collagenase production was greatly affected at highly acidic or alkaline initial pH of the production medium. The maximum cell mass (2.57 mg/ml) was also observed at the same pH 6.5 and on further increasing the initial pH of the production medium, the enzyme activity decreased. Only 0.041 U/ml of collagenase activity was observed at pH 9.0.

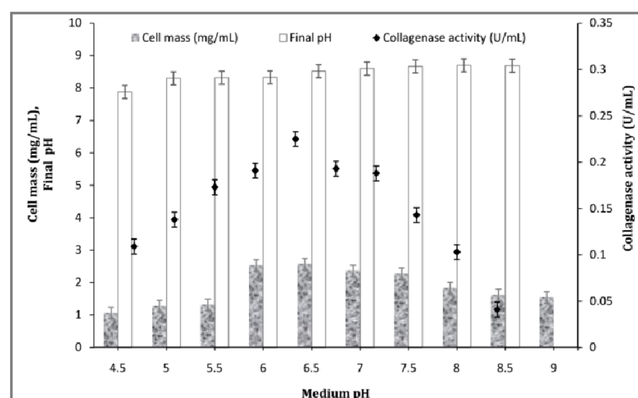

Figure 4. Effect of pH on growth and production of collagenase by *Pseudomonas* sp.

It was reported that most of collagenase producing microorganisms showed optimal growth and enzyme production at neutral range of pH. The maximum growth and collagenase production by *Bacillus* sp. strain MO-1 [32], *Streptomyces exfoliatus* [33] and *Zygosaccharomyces rouxii* [31] was reported at the pH 7.0-7.2. The neutral pH also favoured the growth of bacterial isolate NW4327 which was the primary pathogen of the Great Barrier Reef sponge *Rhopaloeides odorabile* [34]. The pH was optimized as 6.5.

Optimization of incubation temperature

The incubation temperature affects the conversion efficiency of substrates into cell mass and product. The study of collagenase production by *Pseudomonas* sp. was conducted by incubating 50 ml of production medium (pH 6.5) at wide range of incubation temperature (25 to 60°C). The results revealed that the maximum biomass (2.64

mg/ml) was observed at incubation temperature 35 to 37°C, however, the maximum enzyme activity (0.229 U/ml) was observed at incubation temperature of 37°C (Fig. 5).

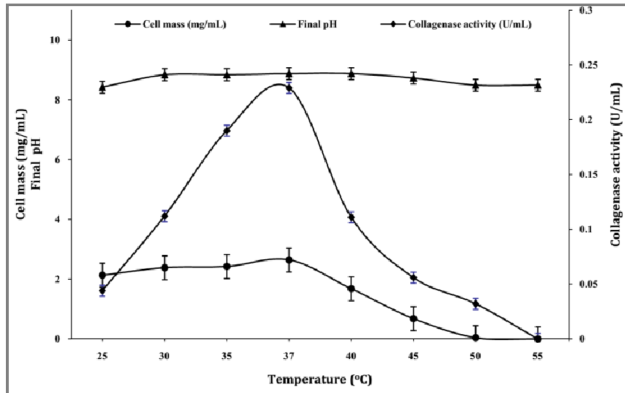

Figure 5. Effect of temperature on growth and production of collagenase by *Pseudomonas* sp.

Moreover, with further increase in incubation temperature, a decrease in growth as well as enzyme production was observed.. The increase in final pH of the production medium was found to be associated with the growth of cells. The same incubation temperature (37°C) was used for collagenase production by *Bacillus licheniformis* F11.4, cultured aerobically in 250 ml flask containing 150 ml of growth medium (%, w/v; NaCl 1.0, triptone 0.5, yeast extract 0.25 and collagen 5 from fish skin) and shaken at 150 rpm [35]. Few other studies have also shown the incubation temperature less than 40°C as optimum for collagenase production [23, 33]. The fungal sources such as *Rhizoctonia solani* have reported the production of extracellular collagenase at low temperature i.e. 30°C [36]. The incubation temperature of 30°C also observed suitable for *Achromobacter iophagus* [29] while thermophilic strain, *A. sendaiensis* produce collagenase at 55°C [37]. The temperature 35 to 37°C was optimum for the production of collagenase by *Pseudomonas* sp.

Optimization of inoculum size

The effect of varying inoculum size (1 to 10%, v/v) on the production of collagenase by *Pseudomonas* sp. was studied. The production medium was inoculated with 24 h old seed and incubated for 24 h at 37°C in a temperature controlled orbital shaker (150 rpm). The results suggest that the varying inoculum size significantly affect the growth of Pseudomonas sp. (Fig. 6). The maximum collagenase activity (0.249 U/ml) with a cell mass of 2.95 mg/ml was observed when 4% (v/v) inoculum was used to inoculate the production medium. The decrease in enzyme production at higher inoculum sizes may be due to competition for nutrients and oxygen in the culture, consequently failing to attain the enzyme production stage.

Optimization of inoculum age

The production medium was inoculated with the 4% (v/v) inoculum of the *Pseudomonas* sp. different ages (6, 9, 12, 15, 18, 21 and 24 h) and incubated at 37°C to determine the optimum age of inoculum for the maximum production of the collagenase by *Pseudomonas* sp. It was observed that inoculum with 21 h old seed exhibited maximum collagenase activity (0.263 U/ml) with 3.08 mg/ml growth after 24 h of incubation (Fig. 7). The cells *Pseudomonas* sp. in the production medium inoculated with 6 h old inoculum and growth was found very poor which results in very low level of collagenase activity (0.114 U/ml). However, with increase in the incubation time beyond 18 h, an increase in cell mass production associated with the increase in collagenase activity was observed. The final pH of the fermentation broth increased in all the cases.

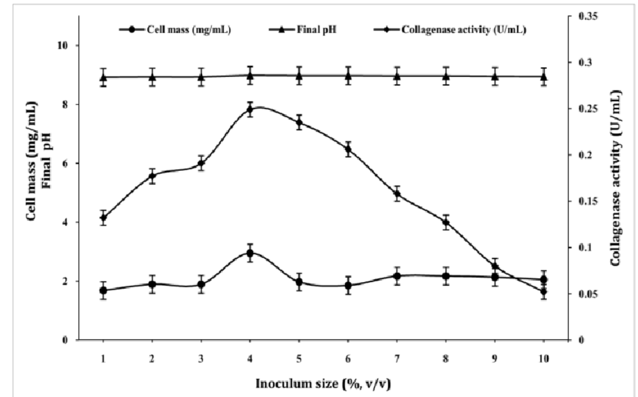

Figure 6. Effect of inoculum size (%, v/v) on growth and production of collagenase by *Pseudomonas* sp.

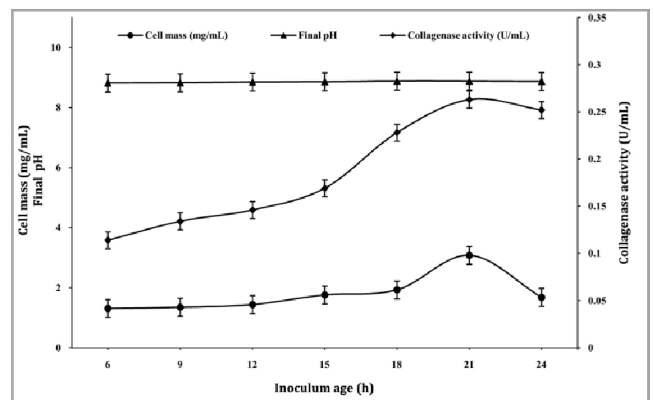

Figure 7. Effect of inoculum age on growth and production of collagenase by *Pseudomonas* sp.

Course of fermentation of Pseudomonas sp. for the production of collagenase

Earlier optimum incubation time for the collagenase production by different strains was reported 24 h, 26 h, 50 h, 108 h, and 5 days [11, 21, 22, 25, 26]. The course of cultivation of Pseudomonas sp. without pH control was carried out in the medium (pH 6.5), containing (%, w/v) glucose 2.0, yeast extract 0.15, tryptone 1.0, $CaCl_2$ 0.005, $NaH_2PO_4 \cdot 2H_2O$ 0.05 and $K_2HPO_4 \cdot 3H_2O$ 0.25. In the initial phase of fermentation, the active cell growth was continued upto 24 h of cultivation and then decline slightly. Upto 34 h of growth, the pH of the fermentation broth increased with very slow pace. The maximum collagenase activity (0.273 U/ml) was observed after 24 h production (Fig. 8).

The optimum incubation time for collagenase production by *Pseudomonas* sp. was observed as 24 h.

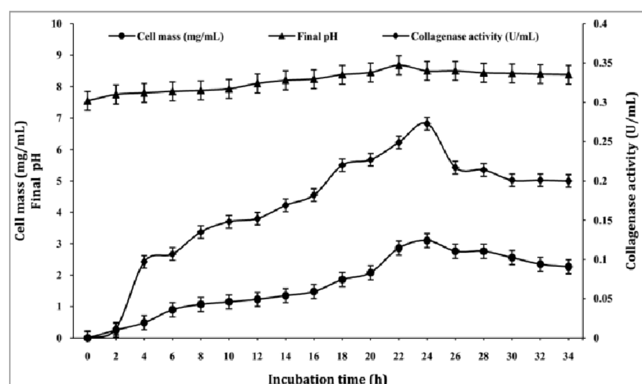

Figure 8. Course of fermentation of *Pseudomonas* sp.

Optimization of agitation rate for the growth and production of collagenase from Pseudomonas sp.

An experiment was conducted to determine the optimum agitation rate for the production of *Pseudomonas* sp. with high collagenase activity. The production medium (pH 6.5) was inoculated with 21 h old inoculum (4%, v/v) and incubated at 37°C in a temperature controlled orbital shaker at varying rotational speed of 50 to 200 rpm for 24 h. It has been observed that with increase in agitation rate up to 150 rpm, the growth of *Pseudomonas* sp. increased continuously (Fig. 9).

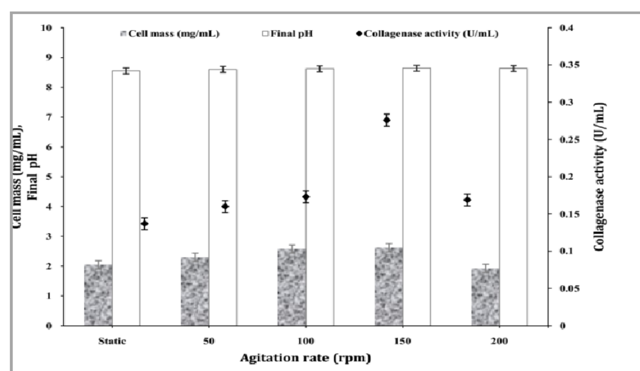

Figure 9. Effect of agitation speed (rpm) on growth and production of collagenase by *Pseudomonas* sp.

The maximum collagenase activity (0.276 U/ml) was obtained at 150 rpm and the further increase in agitation speed lead to decrease in enzyme activity. These results suggest that cells of *Pseudomonas* sp. was not shear sensitive up to some extent, but the considerable increase in the agitation speed might generate enough shears, which adversely affect the collagenase production. The agitation rate 150/175/180 /250 rpm was also used earlier and found suitable for the production of extracellular collagenase [22, 29, 33, 35, 36]

Conclusion

Collagenases have assumed increasing importance every day, having been implicated in angiogenesis, wound repair, inflammation, ageing etc. apart from the various disease conditions described. Collagenase was used to anchor signaling molecules to the collagen containing tissues, presenting a great potential for targeting drug delivery of anti-arthritc and anti cancer reagents [38]. The use of recombinant collagenase preparations also needs to be explored for the development of effective treatment strategies [39]. The use of standard chemotherapeutics together with newer compounds with novel modes of action has emerged as a treatment modality. The developments of therapies are the need of time that abandons the traditional treatments in favour of a more gene specific approach. Further by understanding the roles of collagenase in tumor invasion, researchers may develop drugs that could be effective at various stages of tumor growth and progression. Once the knowledge of molecular mechanisms regulating the expression of the MMPs will completely unfold, this concept might come closer to become a reality [37].
This study was carried out with a Gram-negative bacteria strain identified as *Pseudomonas* sp., producing extracellular collagenase to find out the possibilities offered by this microorganism in the development of a pharmaceutical preparation. The various physicochemical parameters were optimized to maximize the production of collagenase by the *Pseudomonas* sp. Protein waste discharged soil helps to produce the extracellular collagenase enzyme with broad substrate specificities and multiple applications of commercial interest.

Acknowledgements

I would like to thanks Himachal Pradesh University, Shimla for providing me necessary facilities.

References

1. Anbu, P., Gopinath, S., Cihan, A.C., Chaulagain, B.P., Microbial enzymes and their applications in industries and medicine. *Biomed Res Int,* 2013, Vol. 2013, pp. 1-2.
2. Chauhan, B., Gupta, R., Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Process Biochem,* 2004, Vol. 39, pp. 2115-2122.
3. Adrio, J.L., Demain, A.L., Microbial Enzymes: Tools for biotechnological processes. *Biomol*, 2014, Vol. 4, pp. 117-139.
4. Gautam, M., Azmi, W., Purification of extracellular collagenase from *Pseudomonas sp*: remarkable collagenolytic activity. *Adv Biotech Microbiol,* 2017, Vol. 4(2), pp. 1-8.
5. Lima, C.A., Rodrigues, P.M.B., Porto, T.S., Viana, D.A., Lima Filho, J.S., Porto, A.L.F., Carneiro-daCunha, MG., Production of a collagenase from *Candida albicans* URM3622. *Biochem Eng J,* 2009, Vol. 43, pp. 315-320.
6. Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A., Eisen, A.Z., Human fibroblast collagenase: complete primary structure and homology to an oncogene transformation-induced rat protein. *J Biol Chem,* 1986, Vol. 261, pp. 6600-6605.
7. Park, P.J., Lee, S.H., Byun, H.G., Kim, S.H., Kim, S.K., Purification and characterization of a collagenase from the Mackerel, *Scomber japonicas*. *J Biochem Mol Biol,* 2002, Vol. 35, pp. 576-582.
8. Kim, S.K., Park, P.J., Kim, J.B., Shahidi, F., Purification and characterization of a collagenolytic protease from the filefish, *Novoden modestrus*. *J Biochem Mol Biol,* 2002, Vol. 335, pp. 165-171.
9. Schenck, S., Theodore Chase, J.R., Rosenzweig, W.D., Pramer, D., Collagenase production by nematode-trapping fungii. *Appl Environ Microbiol,* 1980, Vol. 40, pp. 567-570.

10. Anandan, D., Marmer, W.N., Dudley, R.L., Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamarii. J Ind Microbiol Biotechnol,* 2007, Vol. 34, pp. 339-347.
11. Beltran, A., Bonnet, M., Quali, A., Collagenase effect on thermal denaturation of intramuscular collagen. *J Food Sci,* 2006, Vol. 56, pp. 1497-1499.
12. Gutierrez-Fernandez, A., Inada, M., Balbin, M., Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J,* 2007, Vol. 21, pp. 2580-2591.
13. Kanth, S.V., Venba, R., Madhan, B., Chandrababu, N.K. and Sadulla, S., Studies on the influence of bacterial collagenase in leather dyeing. *Dyes Pigm*, 2008, Vol. 76, pp. 338-347.
14. Brand, J.S., Hefley, T.J., Collagenase and the isolation of cells from bone. In: Pretlow, T.G. (ed.): Cell separation methods and selected applications. Acad Press, New York, 1984, Vol. 3, pp. 265-283.
15. Patel, V.A., Logan, A., Watkinson, J.C., Uz-Zaman, S., Sheppard, M.C., Ramsden, J.D., Eggo, M.C., Isolation and characterization of human thyroid endothelial cells. *Am J Physiol Endocrinol Metab,* 2003, Vol. 284, pp. 168-176.
16. Marcus, G.J., Connor, L., Domingo, M.T., Tsang, B.K., Downey, B.R., Ainsworth, L., Enzymatic dissociation of ovarian and uterine tissues. *Endocr Res,* 1984, Vol. 10, pp. 151-62.
17. Ray, J.L., Leach, R., Herbert, J.M., Benson, M., Isolation of vascular smooth muscle cells from a single murine aorta. *Methods Cell Sci.,* 2001, Vol. 23, pp. 185-188.
18. Ravanti, L., Kahari, V.M., Matrix metalloproteinases in wound repair. *Int J Mol Med,* 2000, Vol. 6, pp. 391.
19. Anwar, A., Saleemuddin, M., Alkaline-pH acting digestive enzymes of the polyphagous insect pest *Spilosoma obliqua*: stability and potential as detergent additives. *Biotechnol Appl Biochem,* 1997, Vol. 25(1), pp. 43-46.
20. Gousterova, A., Lilova, A., Trenev, M., Spassov, G., Nedkov, P., Thermophilic actinomycetes as producers of Collagenase. *Compt Rend Acad Bulg Sci,* 1998, Vol. 51, pp. 71-76.
21. Wu, C.L., Li, C., Chen, H., Shuliang, L., Purification and characterization of a novel collagenase from *Bacillus pumilus Col-J. Appl Biochem Biotechnol,* 2010, Vol. 160, pp.129-134.
22. Liu, L., Ma, M., Cai, Z., Yang, X., Wang, W., Purification properties of a collagenolytic protease produced by *Bacillus cereus* MBL13 Strain. *Food Technol Biotechnol,* 2010, Vol. 48, pp. 151-160.
23. Nagano, H., Kim, A., Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. *Biosci Biotechnol Biochem,* 1999, Vol. 63, pp. 181-183.
24. Kameyama, S., Akama., Titration of kappa toxin of *Clostridium perfringens* and kappa antitoxin by a modified azocoll method. *Jpn J Med Sci Biol,* 1970, Vol. 23, pp. 31-46.
25. Finland, M., Jones, W.F., Barnes, M.W., Occurrence of serious bacterial infections since the introduction of antibacterial agents. *J Am Med Assoc,* 1959, Vol. 170, pp. 2188-2197.
26. Iococca, V.F., Sibinga, M.S., Barbero, G.J., Respiratory tract bacteriology in cystic fibrosis. *Amer J Dis Child,* 1963, Vol. 106, pp. 315-324.
27. Endo, A., Murakawa, S., Shimizu, H., Shiraishi, Y., Purification and properties of collegenase from a *Streptomyces* Sp. *J Bacteriol,* 1986, Vol. 102, pp. 163-170.
28. Merkel, J.R., Dreisbach, J.H., Purification and characterization of a marine bacterial collagenase. *Biochem J,* 1978, Vol. 17, pp. 2857-2863.
29. Reid, G.C., Robb, F.T., Woods, D.R., Regulation of extracellular collagenase production in *Achromobacter iophagus*. *J Gen Microbiol,* 1978, Vol. 109, pp. 149-154.
30. Mandl, I., Maclennan, J.D., Howes, E.L., Isolation and characterization of proteinase and collagenase from *Clostridium histolyticum. J Clin Invest,* 1953, Vol. 32, pp. 1323-1329.
31. Ok, T., Hashinaga, F., Detection and Production of Extracelluar collagenolytic Enzyme from *Zygosaccharomyces rouxii. J Gen App Microbiol,* 1996, Vol. 42, pp. 517-523.
32. Okamoto, M., Yonejima, Y., Tsujimoto, Y., Suzuki, Y., Watanabe, K., A thermostable collagenolytic protease with a very large molecular mass produced by thermophilic *Bacillus* sp. strain MO-1. *Appl Microbiol Biotechnol,* 2001, Vol. 57, pp. 103-108.
33. Jain, R., Jain, P.C., Production and partial characterization of collagenase of. *Streptomyces exfoliates* CFS 1068 using poultry feather. *Ind J Exp Biol,* 2010, Vol. 48, pp. 174-178.
34. Mukherjee, J., Webster, N., Llewellyn, L., Purification and characterization of a collagenolytic enzyme from a pathogen of the great barrier reef sponge, *Rhopaloeides odorabile*. *PLoS* One, 2009, Vol. 4, pp. e7177.
35. Baehaki, A., Suhartono, M.T., Sukarno, S.D., Sitanggang, A.B., Setyahadi, S., Meinhardt, F., Purification and characterization of collagenase from *Bacillus licheniformis* F11.4. *Afr J Microbiol Res,* 2012, Vol. 6, pp. 2373-2379.
36. Hamdy, H.S., Extracellular collagenase from *Rhizoctonia solani*: Production, purification and characterization. *Iran J Biotechnol,* 2008, Vol. 7, pp. 333-340.
37. Brinckerhoff, C.E., Rutter. J.L., Benbow, U., Interstitial collagenases as markers of tumor progression. *Clin Cancer Res,* 2000, Vol. 6, pp. 4823-4830.
38. Matasushita, O., Koide, T., Kobayashi, R., Nagata, K., Okabe, A., Substrate recognition by collagen binding domain of *Clostridium histolyticum* class I collagenase. *J Biol Chem,* 2001, Vol. 276, pp. 8761-8770.
39. Jin, B., Alter, H.J., Zhang, Z.C., Shih, J.W., Esteban, J.M., Sun, T., Yang, Y.S., Qiu, Q., Liu, X.L., Yao, L., Wang, H.W., Cheng, L.F., Reversibility of experimental rabbit liver cirrhosis by portal collagenase administration. *Labo Invest,* 2005, Vol. 85, pp. 992-1002.