International Journal of Advanced Research and Development
ISSN: 2455-4030
Impact Factor: RJIF 5.24
www.advancedjournal.com
Volume 2; Issue 5; September 2017; Page No. 60-65

Production and partial purification of collagenase from *Bacillus sp.* isolated from soil sample

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Abstract
Collagenases are endopeptidases which are able to digest both native and denatured collagen. The peptides resulting from the collagen degradation by collgenases have wide range of applications and this bioconversion of collagen in to value added digested peptides have attracted a worldwide attention. Hence, the present work was aimed to isolate new collagenase producing microorganisms, optimize various physico chemical parameters for collagenase production and partial purification of the collagenase enzyme. From different soil samples, 20 bacterial isolates showed digestion of gelatin on gelatin agar medium and out of them only four isolates confirmed collagenase production by azocoll. Among these four isolates, isolate Ad18 showed highest collagenase enzyme activity by ninhydrin assay. Ad18 was then characterized for its morphological and biochemical characters. The effect of different parameters like carbon sources, nitrogen sources, temperature, pH, inoculum size and incubation period was monitored with the selected strain for collagenase production. The maximum enzyme production was obtained when the basal media of pH 7.5 containing gelatin (1.5%, w/v), ammonium ferrous sulphate (1%, w/v) was inoculated with 1 % (v/v) inoculum and incubated at 37°C for 72 h. Further, the enzyme was precipitated out with ammonium sulphate at the saturation of 60-80% and partially purified by using Sephadex G-200.

Keywords: collagenase enzyme, azocoll, *Bacillus sp*., production, partial purification

1. Introduction
Proteases represent one of the largest groups of Industrial enzymes having a wide variety of functions and occupy 60-65% of the worldwide industrial enzyme market [1, 2]. Among the various protease sources, microbial proteases play an important role as they can be produced in large amounts quickly and at very low cost thus extensively utilized in various fields [3, 4]. Among the various proteases collagenases are of great importance as they are the only enzymes that hydrolyse the insoluble fibrous collagen which is a major fibrous element of skin, bones, tendons, cartilage, blood vessels [5]. Collagenases are also produced in mammals by specific cells and are involved in repair and remodeling processes. But the microbial collagenases are of wider application as they cleave the collagen triple helix at multiple sites in comparison to mammalian collagenases which cleave the collagen helix at a single site only [6, 7, 8]. The enzymatic degradation of collagen results various peptides which have incessant industrial applications e.g. an immunotherapeutic agent, a moisturizer for cosmetics, a preservative, seasoning and dietary material. It also contributes to tenderizing dry cured meat products and in generating taste and flavour in meat industry [9].
The importance of collagenase is now a day's extended from industrial area and has found widespread applications in medical field also, such as use in estimation of collagen levels, isolation of specific cell types from attendant connective tissue and pulmonary mast cells from bovine lungs, transplantation of pancreatic islet cells to alleviate diabetic symptoms and they also present a great potential for targeted drug delivery of anti-arthritic and anticancer reagents to anchor signaling molecules to collagen containing tissues [10, 11].
For many years the collagenase of *Clostridium histolyticum* and *Clostridium welchii* were considered as the only enzymes fulfilling many of these conditions thus it is very interesting to isolate collagenase from an aerobic, non-pathogenic collagenolytic microorganism from soil sample. Also, it is well known that the enzyme production in microorganisms is greatly influenced by medium components, especially carbon and nitrogen sources and other physical factors, such as pH, temperature, incubation time and inoculum size. So, the present study deals with the isolation of collagenase producing aerobic bacteria, optimization of collagenase production and partial purification of the enzyme.

2. Materials and methods
2.1 Collection of soil samples
Soil samples were collected from different sources such as human cemetery, burial ground of animals (Dogs, cows, horses, buffalo etc) and meat markets in Chandigarh region by using pre-sterilized petri dishes. The collected samples were dried up and cleared of impurities by sieving and then stored at 4°C until further use.

2.2 Isolation of collagenase producing bacteria
The collected soil samples were serially diluted and spread plated on gelatin agar medium containing (w/v) 1.0% gelatin, 0.5% glucose, 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.02% $CaCl_2.2H_2O$ of pH

7. They were incubated at 37°C for 24 h. The isolates that grew and showed clear zones around colonies after addition of 35% (w/v) Trichloroacetic acid (TCA) were selected for further study [12].

2.3 Preparation of crude extract
100 ml of gelatin medium having pH 7 was inoculated with four positive isolates seperately and incubated at 37°C for 24, 48, 72 and 96 h under shaking condition. After incubation the broth cultures were subjected to centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant so obtained was used to determine the collagenase activity.

2.4 Assay for hydrolysis for azocoll
For determination of collagenase activity of the gelatinase positive isolates the procedure described by Mukherjee *et al.* [13] was followed. Azocoll was prepared in 50 mM Tris HCl with 1mM $CaCl_2$, pH 7.8 and 500 µl of this suspension was incubated with the 500 µl of the test sample at 37°C for 2 h under shaking condition. Absorbance of the suspension containing the azo labeled peptic digestion products was measured at 520nm, with increased absorbance values indicating higher collagenase activity.

2.5 Assay of collagenase activity
The isolates which have shown collagenase activity with azocoll dye were then quantified for enzyme production by ninhydrin method [14].

A reaction mixture of 10 mg of collagen type I and 0.8 ml of 50mM Tris-HCL (pH 7.5) containing 4mM $CaCl_2$ was incubated with 0.2 ml of test sample at 37° C for 1 h. After incubation the reaction was stopped by adding 1 ml of 0.1M acetic acid. Then centrifugation was done at 10,000 rpm for 20 min and 0.2ml of this mixture was incubated at 100°C for 20 min with 0.5ml of ninhydrin solution. Subsequently, the mixture was diluted with 2.5 ml of 50% 1- propanol for an absorbance measurement at 570 nm. One unit of collagenase activity was extressed as one µmol of leucine equivalents released per minute.

2.6 Identification of collagenolytic strain
The Collagenase positive isolate with maximum collagenase production was tested for morphological and biochemical characteristics.

2.6.1 Morphological characterization
The overnight grown bacterial suspension of selected isolate was used for the morphological characterization by Gram staining [15] and other colony characteristics like shape, consistency, margin, opacity, motility and colour using standard protocols.

2.6.2 Biochemical characterization
Different biochemical tests were carried out like catalase, Indole production, urease production, methyl red and voges proskauer, endospore staining and citrate utilization for the biochemical characterization of selected isolate [16].

2.7 Optimization of physico chemical parameters for collagenase production
The experiments in the present study were done by adopting the one variable at a time search technique search. All the experiments were carried out in triplicate and the average values were calculated.

2.7.1 Effect of carbon sources
The effect of different substrates as carbon source like lactose, xylulose, gelatin, mannose, maltose, fructose and sucrose were tested for their effect on enzyme activity. Each substrate was added to a concentration of 1% (w/v) in the basal media containing 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.02% $CaCl_2.2H_2O$ with pH 7. All the flasks containing different substrates were incubated at 37°C for 72 h at 120 rpm. The culture broth was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant served as the crude enzyme source. The collagenase activity was determined by ninhydrin method. The effect of different concentrations (0.5%, 1%, 1.5%, 2%, 2.5% w/v) of selected carbon source was also studied to evaluate its effect on enzyme activity.

2.7.2 Effect of nitrogen sources
The basal media was supplemented with different organic and inorganic nitrogen sources like ammonium sulphate, ammonium ferrous sulphate, yeast extract, peptone, beef extract, urea, ammonium hydrogen carbonate and ammonium nitrate (1% (w/v) concentration) to investigate their effect on enzyme activity. The effect of different concentrations of selected nitrogen source (0.5%, 1%, 1.5%, 2%, 2.5% (w/v)) was also studied on the production of collagenase.

2.7.3 Effect of temperature
Temperature plays vital function in a bioprocess for the production of extracellular enzyme. For the selection of optimum temperature for collagenase production, the bacterial isolate was inoculated in the separate flasks and incubated at different temperatures (28°C, 37°C, 45°C, 55°C) for 72 h.

2.7.4 Effect of pH
pH is another factor affecting the microbial growth as well as enzyme production. Therefore, the experiments were carried out by using different pH (5, 5.5, 6.5, 7, 7.5, 8.5, 9.5) to study their effect on collagenase production.

2.7.5 Effect of inoculum concentration
The effect of different inoculum concentrations (0.5%, 1%, 2%, 3%, 4%, 5% v/v) of selected bacterial isolate were also monitored on collagenase activity after 72 h of incubation.

2.7.6 Effect of incubation time
The effect of incubation time was also observed on collagenase activity by incubating the optimized media at 37°C for different time intervals (24, 48, 72, 96 h).

Statistical analysis
All the experiments were carried out in triplicates to validate the reproducibility of the experiments. The results were analysed using one way analysis of variance with the Holm–Sidak method using SigmaPlot (Systat Software, San Jose, CA) to calculate p values. $p<0.05$ was taken as statistically

significant.

2.8 Extraction and partial purification of Collagenase enzyme

After optimized incubation period the broth was centrifuged at 10,000 rpm for 15 min. The supernatant was collected and precipitated with different saturation rates using ammonium sulphate and kept overnight. The precipitated proteins were collected using centrifugation at 10,000 rpm for 20 min and the broth was collected. The protein solution was dialysed using dialysis membrane against Tris-HCl buffer solution at pH 7.5 for 72 h under cold condition, with the intermediate changes of Tris-HCl to remove the salt. Further, partial purification was carried out by molecular sieve chromatography.

2.8.1 Molecular sieving

Purification of collagenase was done by gel filteration through a Sephadex G-200 (Pharmacia fine chemicals, Uppsala), Column (2cm × 31 cm) equilibrated, eluted with Tris Cl (50mM) buffer pH 7.5. Fractions of 3ml, each were collected and each fraction was read at 280 nm on UV spectrophotometer. The fractions were checked for collagenase activity by ninhydrin assay.

3. Results & Discussion

By primary screening a total number of twenty isolates showed clear zone after the addition of TCA on gelatin agar medium due to the hydrolysis of gelatin (Fig 1). Similar method was also used by Suphatharaprteep *et al.* [12] for the selection of collagenase producing bacteria on the basis of formation of clear zone by using TCA. As, collagenase producing microorganisms can degrade both gelatin and collagen the positive isolates were confirmed particularly for collagenase enzyme production by using azocoll [13] and out of twenty isolates only four isolates namely Ad4, Ad7, Ad11 and Ad18 showed collegenolytic activity.

Further, collagenase enzyme activity was estimated by ninhydrin assay. The maximum collagenase activity of 2.650 IU/ml was obtained with isolate Ad18 after 72 h of incubation. Similar pattern was observed in Ad 7 and 4 wherein an enzyme activity of 1.22 IU/ml and 1.076 IU/ml, respectively was observed after 72 h of incubation. However, in case of Ad 11, enzyme activity was found to be maximum (1.38IU/ml) after 48h of incubation. The same method was also used by some other workers for the estimation of collagenase enzyme production [13, 17].

3.1 Identification of collagenolytic strain

The best isolate was primarily identified on the basis of morphological and biochemical tests. The results of gram staining revealed that the isolated bacterium was Gram positive bacilli with rod shaped cells. The isolate also showed positive results for catalase, oxidase, Indole production, citrate utilization, endospore presence however negative results were observed with urease production and MR-VP test. The results of morphological and biochemical tests are given in Table 1. It was apparent from the results that the isolate was closely related to genus *Bacillus*, which is evident from its morphological and biochemical tests on the basis of Bergey's Manual of Systematic bacteriology [16].

3.2 Optimization of Culture Conditions

The selection of the best medium components and their concentration play an important role in enzyme production also the environmental parameters like temperature, pH, inoculum size and incubation time plays an important role. So, these factors were optimized for the maximum production of collagenase in a stepwise manner.

3.3 Effect of carbon sources

The isolate Ad18 was allowed to grow at 37°C for 72 h in basal media of pH 7 containing 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.02% $CaCl_2.2H_2O$ with combination of different carbon sources like lactose, xylulose, gelatin, mannose, maltose, fructose and sucrose (each with 1% (w/v) concentration) to estimate their effect on collagenase production. The maximum Collagenase activity of 3.911 ± 0.0329IU/ml was found when medium was supplemented with gelatin (Fig 2).

To analyze the effect of different concentrations of gelatin on collagenase production by Ad18, different concentrations of gelatin (0.5%, 1%, 1.5%, 2%, 2.5% (w/v)) were used in the media. The maximum collagenase activity of 3.950 ± 0.0429 IU/ml was observed with 1.5% (w/v) concentration of gelatin.

Similar, observations were also made by Lima *et al.* [18] in the past where maximum collagenase activity was observed with gelatin as carbon source from *Bacillus sp.* in basal medium supplemented with 1.5% of gelatin.

3.4 Effect of nitrogen sources

The presence of external nitrogen source is essential in the media during extracellular enzyme production for efficient consumption of soluble carbohydrates so the effect of different nitrogen sources like ammonium sulphate (AS), ammonium ferrous sulphate (AFS), yeast extract (YS), peptone (P), beef extract (BE), urea (U), ammonium hydrogen carbonate (AHC) and ammonium nitrate (AN), was observed on collagenase production after 72 h of incubation. The maximum activity of 4.331 ± 0.0318 IU/ml, was found with ammonium ferrous sulphate as shown in Fig 3. Kabadjova and Vlahov, [19] also obtained higher collagenase production with sodium nitrate, which is an inorganic nitrogen source. Likewise, the maximum enzyme production by the use of inorganic nitrogen sources was also seen by Guan *et al.* [20] and Sujatha and Dhandayuthapani [21] for the extracellular enzyme production.

The effect of its different concentrations of ammonium ferrous sulphate (0.5%, 1%, 1.5%, 2%, 2.5%, w/v) on collagenase production was determined. The maximum collagenase production of 4.257 ± 0.0259 IU/ml, was observed with 1% (w/v) concentration of ammonium ferrous sulphate.

3.5 Effect of temperature

Temperature plays a crucial role in growth and physiology of microorganisms and its enzyme activity. Hence different temperatures i.e. 25°C, 37°C, 45°C, 55°C were used for the incubation of the media containing Ad18 for 72 h to estimate their effect on enzyme activity. The maximum activity of 3.660 ± 0.0717 IU/ml, was observed at 37°C which was

reduced with further increase in the temperature (Fig 4). As, increase in temperature, above the optimum values, results in thermal denaturation of enzymes which leads to the loss of enzyme activity, hence low enzyme activity was observed above 37°C.

In the same way, maximum collagenase production at 37°C from *Bacillus* KM369985 and *Bacillus cereus* CNA1 was observed by Savita and Arachana [22] and Suphatharaprateep *et al.* [12], respectively.

3.6 Effect of pH

As optimum pH is required to maintain the three dimensional shape of the active site of enzyme and the change in pH results in loss of functional shape of enzyme due to alteration in the ionic bonding of enzyme, therefore, the production media with different pH (5.5, 6.5, 7.5, 8.5, 9.5) was used for growth of Ad18 to investigate its effect on collagenase production. The optimum pH for collagenase production was found to be 7.5 with maximum collagenase activity of 4.052 ± 0.0281 IU/ml (Fig 5). pH 7.5 was also observed optimum for maximum collagenase production by Savita and Arachana [22] and Park *et al.* [17].

3.7 Effect of inoculum concentration

The effect of different inoculum concentration i.e. 0.5%, 1%, 2%, 3%, 4%, 5% (v/v) of Ad18 on collagenase production was evaluated. The optimum inoculum concentration was found to be 1% (v/v) with the enzyme activity of 4.405 ± 0.0172IU/ml (Fig 6). Further, increase in inoculum concentration, leads to decrease in enzyme activity which might be due to the increase in competition for space and nutrients between microorganisms. It also affects the length of stationery phase, in which accumulation of toxic products and secondary metabolites results in loss of the enzyme activity. In a study conducted by Suganthi *et al.* [23] maximum enzyme production from a halotolerant *Bacillus licheniformis* was also obtained with 1% inoculum size.

3.8 Effect of incubation period

The media was incubated for different time intervals i.e. 24, 48, 72, 96 h after inoculating with Ad18 to investigate the effect of incubation time on collagenase production. The optimum time for collagenase production with maximum activity of 3.528 ± 0.0621 IU/ml was observed after 72 h of incubation (Fig 7). The collagenase activity was significantly reduced after 72 h which may be attributed to depletion of nutrients or accumulation of other by products in the media. Similarly, maximum collagenase production from *Bacillus* KM369985 after 72 h of incubation was also obtained by Savita and Arachana [22].

3.9 Extraction and partial purification of Collagenase enzyme

The cell culture of Ad18, grown in optimized medium for 72 h, was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant so obtained was subjected to ammonium sulphate precipitation and the results showed that protein precipitated out at 60-80% saturation, possessed collagenase activity.

3.10 Molecular sieving

The fraction precipitated out at 60-80% saturation with ammonium sulphate was re-dissolved in Tris Cl (50mM, pH 7.5). The dialysis of the precipitated fraction against Tris Cl was carried out extensively at 4°C. Further, purification was done by passing through Sephadex G-200 column. Through column chromatographic pattern, it was seen that the collagenase activity was present in the fractions 5-8 with a peak value in fraction 5, where each fraction was of 3ml quantity **(**Fig 5).

In conclusion, this study provides evidence for the collagenase production from *Bacillus sp.* and also confers that gelatin and ammonium ferrous sulphate can act as best carbon and nitrogen source at the concentration of 1.5% and 1%, respectively. In addition to this maximum collagenase production can be obtained in a media having pH 7.5 at 37°C after the incubation of 72 h from Ad18 with 1% inoculum. Also, the collagenase enzyme can be easily precipitated with ammonium sulphate with 60-80% saturation under 4°C and can be partially purified by sephadex G-200.

4. Table and Figures

Fig 1: Organisms showing clear zones on gelatin agar medium

Table 1: Morphological and biochemical characterstics of Ad18

S.No.	Morphological Characterstics	Observation
1	Shape	Circular
2	Colour	Cream
3	Elevation	Low convex
4	Consistency	Sticky
5	Margin	Irregular
6	Opacity	Opaque
7	Gram Character	+ve rods
8	Motility	Motile

Biochemical Characterstics		
9	Catalase	+ve
10	Oxidase	+ve
11	Indole Production	+ve
12	Citrate Utilization	+ve
13	Methyl Red	-ve
14	Voges proskauer	-ve
15	Endospore	+ve
16	Urease	-ve

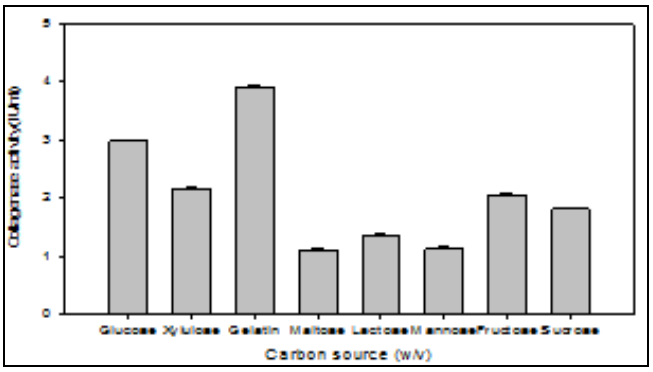

Fig 2: Effect of different carbon sources on collagenase production. Values are expressed as mean ± SD. ANOVA results – p value P = <0.001; F-value 2910.199, overall significance level = 0.05

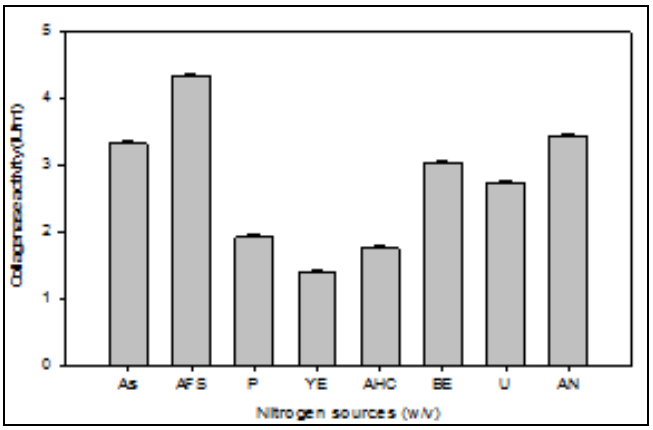

Fig 3: Effect of different nitrogen sources on collagenase production. Values are expressed as mean ± SD. ANOVA results – p-value P = <0.001; F-value 2182.075, overall significance level = 0.05

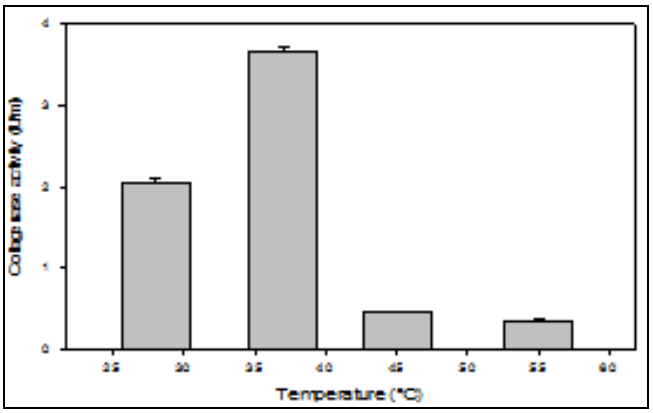

Fig 4: Effect of different temperature on collagenase production. Values are expressed as mean ± SD. ANOVA results – p-value P = <0.001; F-value 3269.819, overall significance level = 0.05

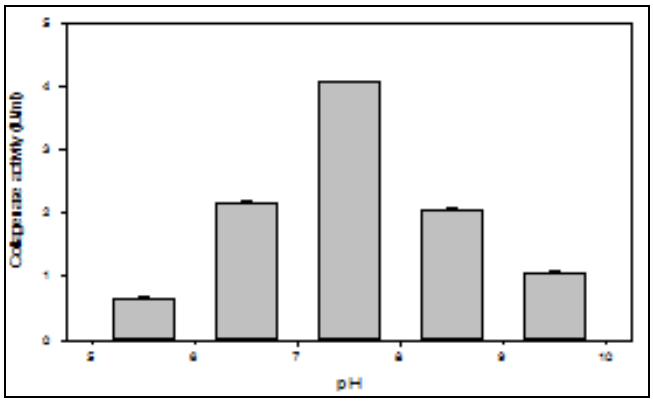

Fig 5: Effect of different of different pH on collagenase production. Values are expressed as mean ± SD. ANOVA results – p-value P = <0.001; F-value 4531.922, overall significance level = 0.05

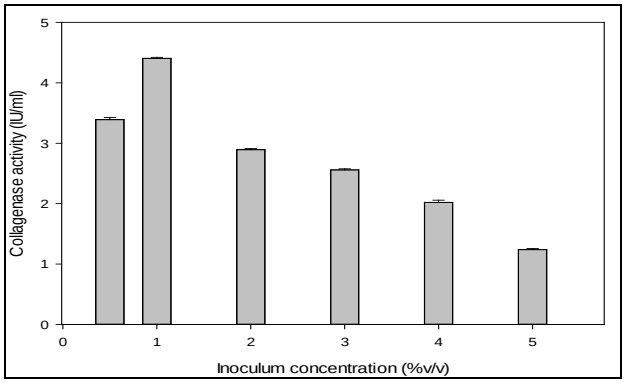

Fig 6: Effect of different inoculum concentration on collagenase production.Values are expressed as mean ± SD. Level of significance represented as p<0.05. ANOVA results – p-value P = <0.001; F-value 5430.518, overall significance level = 0.05

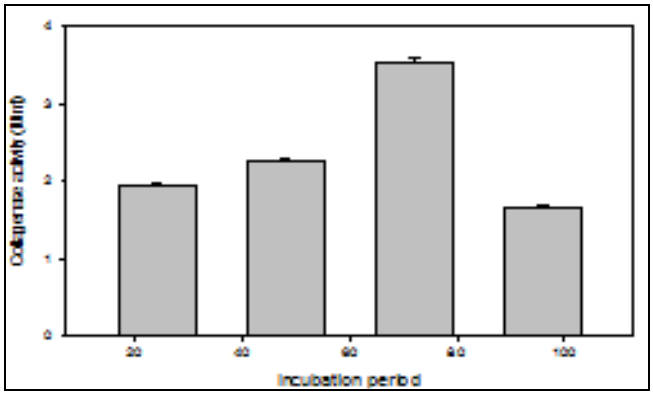

Fig 7: Effect of different incubation period on collagenase production.Values are expressed as mean ± SD. ANOVA results – p-value P = <0.001; F-value 1380.239, overall significance level = 0.05

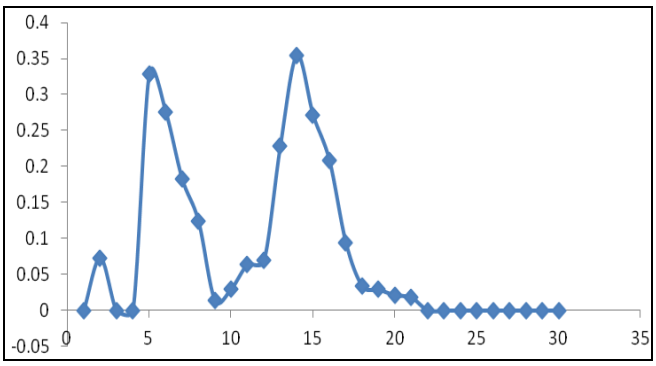

Fig 8: Elution pattern of Collagenase after gel filtration through Sephadex G-200 column showing the presence of collagenase activity in fractions 5-8 with a peak value in fraction 5

Conclusion

In conclusion, this study provides evidence for the collagenase production from *Bacillus sp.* and also confers that gelatin and ammonium ferrous sulphate can act as best carbon and nitrogen source at the concentration of 1.5% and 1%, respectively. In addition to this maximum collagenase production can be obtained in a media having pH 7.5 at 37°C after the incubation of 72 h from Ad18 with 1% inoculum. Also, the collagenase enzyme can be easily precipitated with ammonium sulphate with 60-80% saturation under 4°C and can be partially purified by sephadex G-200.

Acknowledgments

The support of Department of Science and Technology, New Delhi, India (grant no. is DST/INSPIRE/03/2014/000324) and University Grants Commission, New Delhi, India (grant no. F 3- 4-5/ 2016 DRS–I (SAP – II)) are gratefully acknowledged.

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