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Date : 10th July 2020

To,
The Principal,
Maulana Azad College of Arts, Science & Commerce,
Aurangabad.

Subject: - Proposal of research collaboration for the study of Nanoparticles and Enzymology.

Respected Sir,

We are glad to extend our interest in proposing an active research collaboration between Dr. Madhuri Sahasrabudhe, Associate Professor in Microbiology, Maulana Azad College of Arts, Science and Commerce, Aurangabad and Dr. Savita Kate, Department of Biotechnology, Shivchhatrapati College, Aurangabad Maharashtra, India

We look forward to achieve the following aims and objectives through the proposed collaboration in field of Nanoparticles and Enzymology (2020-2022):

1. Explore the literature,
2. Exchange research ideas
3. Extend support for analysis
4. Use of expertise in result interpretation
5. Research publication in SCOPUS and Web of Science indexed journals.

We are expecting a smooth accomplishment of objectives through the proposed collaboration.

Thanking you,

Yours Sincerely,

(Principal)

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Metallo-collagenase production by *Arthrobacter creatinolyticus* KP015744

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Abstract. Amongst 27 isolates from deteriorated leather samples, *Arthrobacter creatinolyticus* KP015744 zzx28 was found to be an efficient collagenase producer. Collagenase production of 13.33 µmoles/min was shown at an optimum temperature at 37℃ after 72h and at pH 7.5 by using 2 ml/dL inoculum in 10 mg/ml collagen peptide type I as a substrate. In presence of Hg^{2+}, EDTA and *β*-mercaptoethanol the collagenase production by the isolate was strongly inhibited however Fe^{2+}, $Ca^{2+}$and DMSO enhanced production of the enzyme. Specific activity was found to be $19.46X10^3$ U/mg and molecular weight 66 kD by SDS PAGE. Isolate also has potential to hydrolyze keratin which is another important protein found in leather. Experimental results propose that collagenase can be effectively used as a tool for collagen and keratin rich solid waste treatment.

Keywords: collagen peptide type I; dialysis; EDTA; feather meal; leather; metalloprotease

1. Introduction

The leather industry generally uses hide and skin as raw materials, which are the by-products of meat and meat products industry. In this respect, the leather industry could have easily been distinguished as an environmentally friendly industry, since it processes waste products from meat production (Ozgunay *et al*. 2007). It has been reported that the chemical composition of skin and hide vary from species to species as well as their locations. Animal skin/ hide are biological matrices significantly loaded with 33% protein and 2% of fat or triglycerides (Zambare *et al*. 2013). According to the data received from the studies of several researchers, approximately 200 kg of leather is manufactured from 1 ton of wet-salted hide. This amount constitutes about 20% of rawhide weight. More than 600 kg of solid waste is generated during the transformation of raw hide into leather. Solid wastes containing protein and fat that constitute more than 60% of rawhide weight are disposed to the environment by leather factories without turning them to good use (Khandelwal and Bhavar 2014). To overcome the hazards caused by this waste, biocatalyst has often been proposed as a viable alternative. Large amounts of solid waste are produced, mainly

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collagen and keratin proteins, so the degradation of the rigid structure of collagen and keratinis necessary.

Destroying of these proteins is usually made by thermal hydrolysis with acid or alkaline solutions but by enzymatic digestion with specific proteases such as collagenases and keratinases is an alternative to conventional chemical ones because they are eco-friendly (Roming *et al*. 2014). At present, enzyme technology is considered to be the most effective way to hydrolyze collagen (Ling *et al*. 2019). Collagenases [E.C.3.4.24.3] are endopeptidases that digest native collagen in the triple helix region. Collagens are the major fibrous component of animal extracellular connective tissue. Unlike animal collagenases that split collagen in its native triple-helical conformation, bacterial collagenase is unique because it can degrade both water-insoluble native collagens and water-soluble denatured ones. Different authors reported collagenase producing strains *Bacillus* (Rochima *et al*. 2016, Khamson *et al.* 2019), *B. subtilis, B. licheniformis, A. fumigatus, Clostridium histolyticum* and *Achromobacter iophagus*. This biocatalyst is gaining more prominence because it is considered to be cost effective and environmentally friendly technologies in solid waste treatment (Roming *et al*. 2014, Baehaki *et al*. 2012, Liu *et al*. 2010).

The present study aims at isolating the effective collagenase producer and optimizing conditions for the production of the enzyme. As the large portion of proteins present in leather is collagen, the isolate having the potential to produce collagenase could be used to degrade leather waste by tanneries for tenderizing meat thus will help in minimizing the environmental pollution load. Collagenase has wide applications in food, nutrition, biotechnological and medical sectors. This study may help in commercial production of the enzyme which will be used in the different sectors.

2. Material and methods

Dimethylsulphoxide [DMSO], Phenylmethylsulphonyl fluoride [PMSF], trichloroacetic acid was obtained from Sigma Chemical Co. USA. Collagen peptide type I (TC343-10G), Nutrient broth (M002-100G), metal ions and organic solvents were obtained from Hi media Ltd., Mumbai. All other reagents used were of analytical grade.

2.1 Collection of deteriorated leather samples

Deteriorated leather samples were collected by IS: 5868-1969 methods (1969). The samples from the leather factory (Kedar leather Products, Aurangabad, M.S) were collected in sterile polythene bags. Various types of naturally deteriorated finished leather articles like footwears, belts, leather cases, bags or purses and other articles were also collected from different places of Maharashtra.

2.2 Isolation and identification of collagenase producer

Initial isolation of bacterial leather deteriorate was carried out on nutrient agar. A well isolated colony of the isolates was further used to detect the ability to hydrolyze collagen (Collagen peptide type I, TC343-Hi-Media), on collagen agar plates (containing 10 g/L collagen and 20 g/L agar Hi-Media). A drop of mercuric chloride precipitation reagent (15 gm $HgCl_2$ in 20 ml Conc. HCl volume make up to 100 ml distilled water) was added to each colony and incubated at 37℃ of 48

h. Collagenase producers were selected with a larger transparent zone around the bacterial colony (Gautam and Azmi1 2017). At the same time casein, keratin and gelatin hydrolysis were also detected on feather meal (10g/L), gelatin agar (10 g/L) and milk agar plates respectively (100 ml/L), incubated at 37℃for 24h (Mozotto 2011). The most efficient collagenase bacterial isolate was identified as described in the Bergey's manual of systematic bacteriology (1984) and further subjected to genetic analysis by 16s rDNA sequencing (Om gene bio, Pune). The sequences obtained were deposited to Gen Bank for accession number.

2.3 Collagenase production by bacterial leather isolate

Collagenase production was carried out in the medium containing collagen (10 g/L), $CaCl_2$ 0.05 g/L, NaH_2PO_4 (0.5 g/L), K_2HPO_4 (0.5 g/L) & glucose (20 g/L) in triplicate set of flasks. Each flask was inoculated with OD_{600} 1.0 inoculum of selected leather isolate and incubated at 37℃ for 48h in an orbital shaker at 180 rpm followed by centrifugation 4000 rpm for 10min at 4℃. The supernatant was used as a crude collagenase (Tran and Nagano 2002) and subjected to quantitative estimation. Collagenase production with respect to growth phase was studied.

2.4 Assay of Collagenolytic activity

Collagenolytic activity was quantitated as per the method described by Liu *et al*. (2010) with little modification. 50 µl enzyme filtrate was mixed with 250 µl collagen type I substrate (5 mg/ml) in Tris HCl buffer-7.5pH (50 mM) and incubated for 10 minutes at 37℃. Trichloroacetic acid 0.2 M (Merk) was added and incubated further for 10 minutes at 37℃ followed by centrifugation at 4000 rpm for 10 minutes in cooling condition. The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, boiled for 15 min, cooled at room temperature for 5min and the mixture was diluted with 5 mL of 50% (by volume) ethanol, then absorbance was measured at 575 nm. One unit (U) of enzyme activity equals one micromole of hydrolysed amino acid equivalents released from collagen under specified conditions. Hydrolysed amino acids were measured by comparing with the standard graph of L-leucine (Tran and Nagano 2002). Amount of collagenase produced was expressed in µmoles/min.

2.5 Optimization of cultural condition for collagenase production

The effect of inoculum size, inoculum age, substrate concentration, pH, temperature, metals ions and chemicals on collagenase production as mentioned by (Wanderley *et al*. 2015, Lima *et al*. 2015, Daboor *et al*. 2012, Baehaki *et al*. 2012, Liu *et al*. 2010, Wu *et al*. 2010) with some modification was studied. The aliquots of the samples were subjected to centrifugation and were used for the assay. Amount of collagenase enzyme was recorded every day till a decline was observed in the enzyme activity. All the tests were run in triplicates. Abiotic control (without bacteria) was always included.

2.5.1 Effect of inoculum size and inoculum age

To gain maximum collagenase production, the effect of inoculum size was studied by adding $O.D_{600}$1.0 *Arthrobacter creatinolyticus* KP015744 culture density of different concentrations such as 1, 2, 4, 6, 8 and 10 ml/dL (Jogdand *et al*. 2018) in the production medium and then incubated at 37℃ for 24h. To study the effect of inoculum age, the production medium was inoculated with the

2 ml/dLinoculum of the *Arthrobacter creatinolyticus* KP015744 of different ages (8, 12, 24, 48, 72, 96 and 120 h) and incubated at 37℃ to determine the optimum age of inoculum for the maximum production of the enzyme. Amount of collagenase enzyme was noted every day till a decline was observed in the enzyme activity as compared to abiotic control. Control tubes were kept using the same set of medium without inoculum.

2.5.2 Effect of substrate concentration, temperature and pH

The outcome of substrate concentration (collagen type I) was analyzed by adding varying concentrations of collagen peptide typeI - 5,10,15,20,25 mg/ml in the production medium and consequences of pH on collagenase production was determined by inoculating the selected isolated bacterium in the media with different pH viz. 5, 6, 6.5, 7, 7.5, 8 and 8.5, the enzyme assay was carried out after 24h at 37℃. Temperature plays an important role in the production of collagenase. To study the influence of temperature, collagenase production was studied by the incubating the culture media at various temperatures 10, 37, 40 and 45℃. The aliquots of the samples were subjected to centrifugation and were used for the assay. Amount of collagenase enzyme was found out every day till a decline was observed in the enzyme activity as compared to abiotic control.

2.5.3 Effect of metal ions and chemicals

To study the influence of metal ions and chemicals on collagenase production by selected isolate, various metal ions, organic solvents and inhibitors such as 1mM $MnCl_2$, $ZnCl_2$, $HgCl_2$, $CuSO_4$, $MgCl_2$, sodium sulphite, cysteine, PMSF (serine protease inhibitor), EDTA (metaloprotease inhibitor), lead acetate, $FeCl_3$, $CoCl_2$, $CaCl_2$ and 1 ml/dL concentration of β-mercaptoethanol, glycerol, DMSO were added in the media. The effect was measured by assaying activity every day for a period of three days. Collagenase activity measured in the absence of an inhibitor or metal ions was taken as 100% relative activity. The aliquots of the samples were subjected to centrifugation and were used for the assay.

2.6 Partial purification and molecular weight determination by SDS-PAGE

Bacterial cells were removed from the medium by centrifugation under cooling (4℃) at 10,000 rpm for 20 minutes. Cell free supernatant was subjected to precipitation by adding solid ammonium sulphate (30%, 50% and 70%) fractionation at 4℃ for 24 h. The precipitate obtained was dissolved in Tris HCl buffer (pH 7.5) dialyzed overnight against the same buffer and then concentrated on sucrose (Jaouadi*et al*. 2013, Jayaraman 2011). For molecular weight determination, partially purified collagenase along with known molecular weight markers (Hi-Media range 14.3-97.4kD) were subjected to electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with 12% polyacrylamide gels and the protein bands were stained with Coomassie blue R-250. The molecular weight was determined by comparing the relative mobility (Rf) value of the standard protein with that of the enzyme protein, as described by Laemmli (1970) and Sambrook *et al*. (2001).

2.7 Utilization of biocatalyst in collagen rich solid waste treatment

An ability of the biocatalyst from selected leather isolate to degrade the solid leather waste was analyzed in minimal media ($KH_2PO_4$0.4 g/L, $K_2HPO_4$1.4 g/L, NaCl.5 g/L and $MgSO_4$0.01 g/L)

containing leather solid waste samples as a substrate was inoculated with the selected culture and the media with preservative were kept as control. All the tubes were incubated at 37℃ for 24h in an orbital shaker at 160 rpm. The extent of collagen rich leather solid waste digestion by the biocatalyst was noted after 24 h for 15 days. The experiment was carried out in triplicates. The aliquots were centrifuged at 4℃ and 8000 rpm for 10 min and the supernatant filtrate was used to evaluate leather deterioration in terms of the colour assay where leucine was used as a standard amino acid. The intensity of colour developed was measured at 575 nm (Moore 1968). Graph of time versus amount of leucine released was plotted which gives an idea about the extent of degradation of collagen. One proteolytic unit was defined as the amount of enzyme that released 1µmole of leucine as a standard amino acid released under specified condition.

2.8 Statistical analysis

The data was analyzed by one way ANOVA (analysis of variance). Readings were considered significant at $P \leq 0.05$.

3. Result and discussion

3.1 Isolation and identification of collagenase producer

In primary screening, 91 isolates were obtained with varied size, shape, colour and morphology on nutrient agar plates after 24h incubation at 37℃ from fifty deteriorated leather samples. Out of 91, twenty seven bacteria showed collagenolytic activity on 1% collagen agar plate after 48 h of incubation (Fig. 1). The most promising isolate was used for further study. By biochemical and morphological and 16s r RNA sequencing the selected strain was found to be *Arthrobacter creatinolyticus*. Accession number was obtained after sequence deposition to GenBank with *Arthrobacter creatinolyticus* KP015744 (zzx28).Qi Wu *et al* (2010) reported collagenolytic activity by *Bacillus pumilus Col-J*, while *Bacillus subtilis* (Tran and Nagano 2002), *Bacillus* sp. MO-1 (Okamoto *et al.* 2001), isolated from soil or sewage samples were collected from leather house, market, and slaughterhouse. In our present work, we reported for first time *Arthrobacter creatinolyticus* KP015744 (zzx28) collagenolytic activity as per literature cited. Present isolate also hydrolyzed gelatin, casein and keratin.

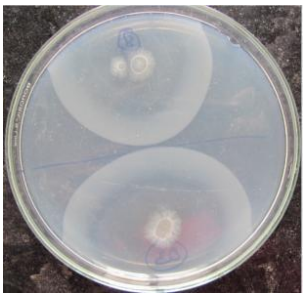

Fig. 1 Zone of clearance due to Collagenase activity

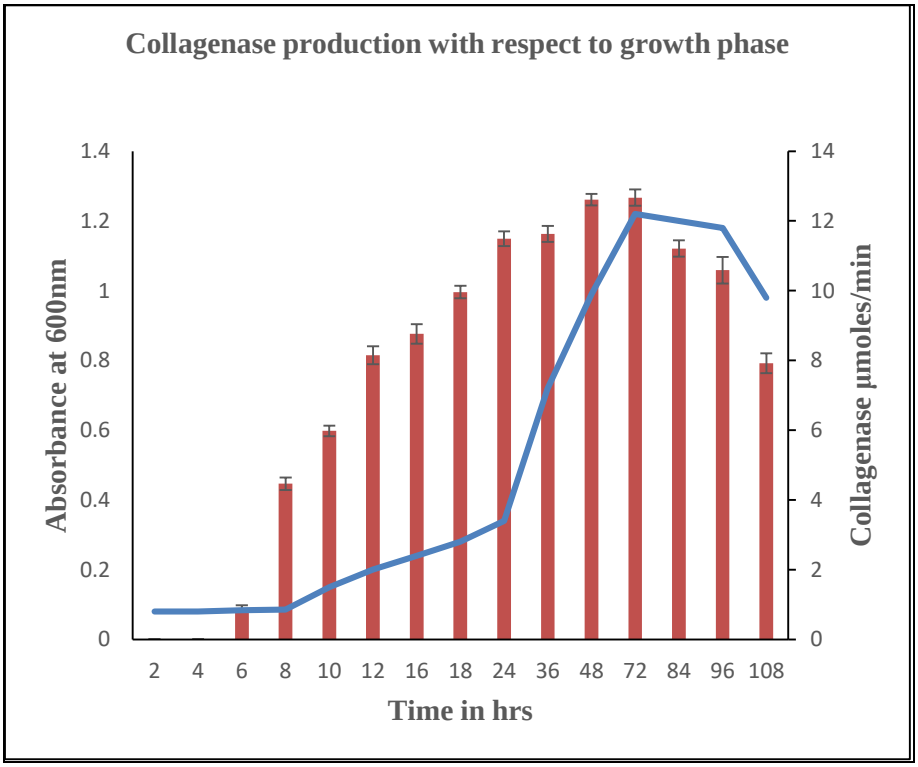

Fig. 2 Collagenase production with respect to growth phase

3.2 Collagenase assay

It was observed that collagenase production started from 6 hr, followed by proportionate increase in collagenase production up to 72 hr in an exponential phase. After 72 hrs, there was a decrease in collagenase production as shown in Fig. 2. *Arthrobacter creatinolyticus* KP015744 (zzx28) produced 11.440µmoles/min of collagenase by using collagen peptide type I as a substrate after 3 days of incubation. *Bacillus amyloliquefaciens* **(**Roming*et al*. 2014) showed collagenase activity 23.7 U/ml after 10 days using sheep fur as a substrate. *Nocardiopsis dassonvillei* NRC2 (Azza *et al*. 2013) shown maximum collagenase activity 240 U/ml after 6 days of incubation using chitin waste as sole carbon source. The present isolate was found to be most efficient as compared to the cited reports.

3.3 Optimization of cultural conditions for collagenase production

3.3.1 Effect of Inoculum size on collagenase production

The *Arthrobacter creatinolyticus* KP015744 (zzx28) under study showed collagenase production by using the different volume of inoculums ranging from 1-10 ml/dL inoculum, The culture density of $O.D_{600}$ 1.0 was used throughout the study.OD_{600} 1.0 had 3.1 X 10^7 CFU/ml. The same cell concentration was used throughout the study. All experiments were carried out under the same set of condition. Control (Uninoculated) was kept at all times. After 24 h initially, 6-10

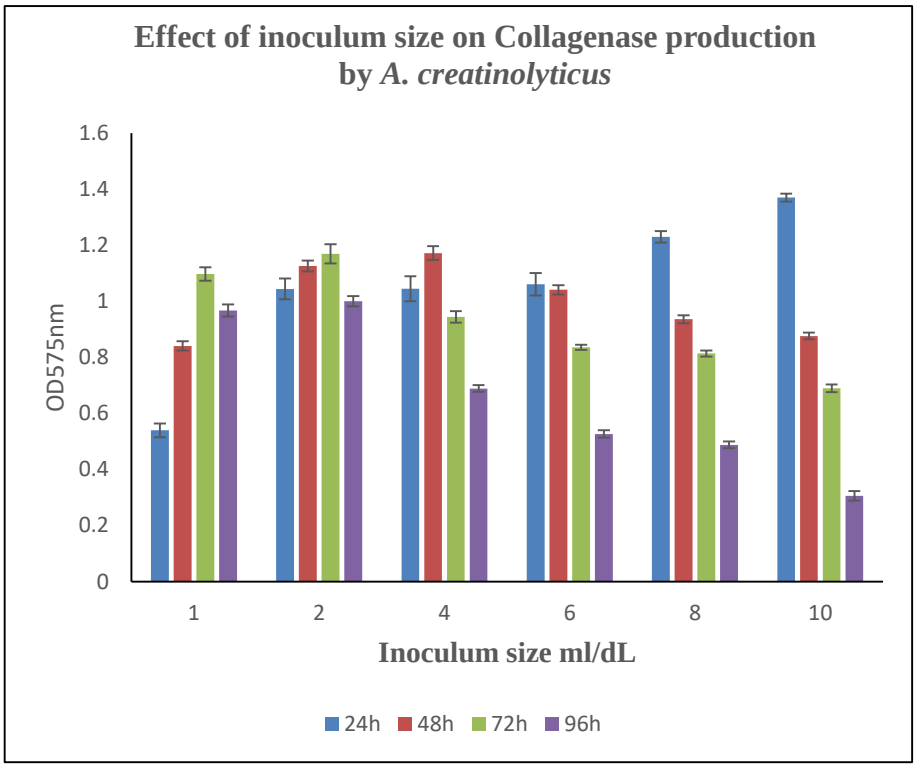

Fig. 3 Effect of Inoculum size on collagenase production by *A. creatinolyticus*

ml/dL inoculum showed highest collagenase production as compared to 2-4 ml/dL inoculum and 1 ml/dL inoculum showed lowest in the same period, but as incubation period increased, 2 ml/dL inoculum showed maximum collagenase production and 10 ml/dL showed lowest after 96h (Fig. 3). The isolate showed 13.16 µmoles/min collagenase production after 72h using 2 ml/dL inoculum. Liu *et al*. (2010) in their research reported that maximum collagenase production by *Bacillus cereus* MBL13 was found in 40g/L of inoculum total activity.

3.3.2 Effect of inoculum age on collagenase production

Arthrobacter creatinolyticus KP015744 (zzx28) began collagenase production from 8 hours. Initially the collagenase production was very less, as inoculum age increased collagenase production was found to be increased. It was observed that inoculum age of 72 h exhibited maximum collagenase activity (12.852 µmoles/min) where the isolate was in late log phase (Fig. 4), above this, the collagenase enzyme production found to be decreased. Liu *et al*. (2010) in their research *B. cereus* MBL13 collagenase production was detected in the late logarithmic phase after 22 h of incubation, and it reached its optimum production. This situation is very similar to the *B. cereus* cultures described by Adiguzel *et al*. (2009). The production of an enzyme normally occurs in the late logarithmic phase of growth, when the cell density is high.

A proportionate increase in the amount of collagenase produced was found to be associated with growth phases. In the lag phase, activity was found to be very less 4.464µmoles/min and maximum activity was found in late log phase 12.848 µmoles/min and amount of collagenase in

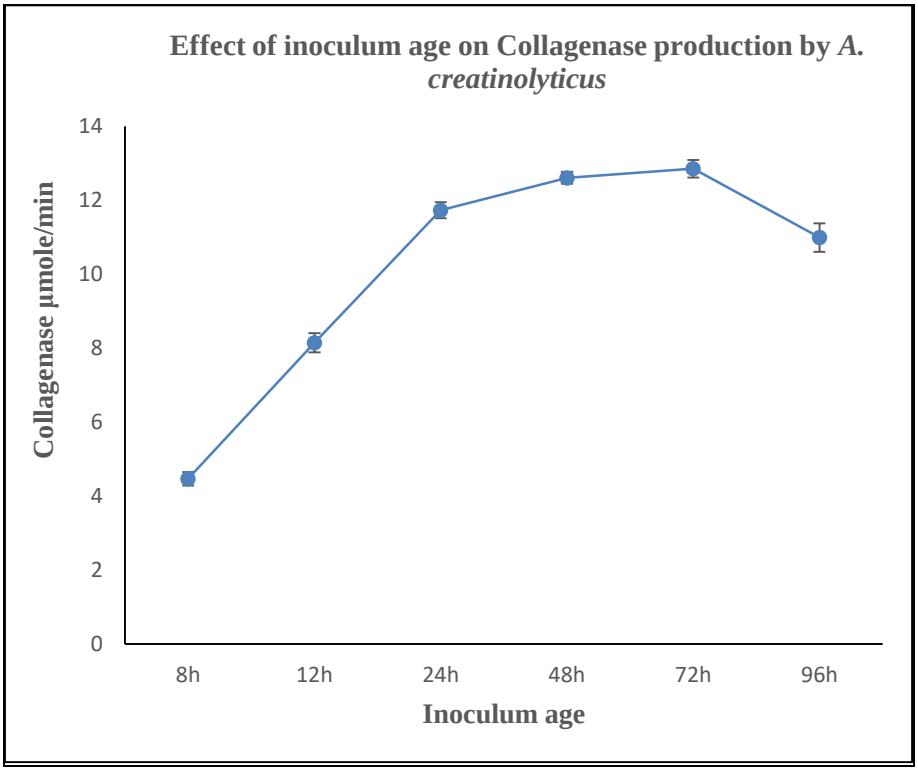

Fig. 4 Effect of Inoculum age on collagenase production by *A. creatinolyticus*

stationary phase was more or less same. The same culture conditions were maintained throughout the study. The selected culture in late log phase with an $O.D_{600}$ 1.0 was used throughout the study.

3.3.3 Effect of substrate concentration on collagenase production

Collagenase production depends on substrate concentration, in this work the isolate showed optimum collagenase 12.852 µmoles/min at 10 mg/ml of collagen type I after 72h (Fig. 5). The *Bacillus* sp. DPUA1728 produced maximum collagenase (86.27 U/mL) at 1.5% substrate concentration as reported by Lima (2015) and the highest collagenolytic enzyme activity was 79.38 UmL^{-1} substrate concentration at 1% (W/V) by *Bacillus stearothermophilus* **(**Arruda *et al*. 2014). Effect of substrate concentration was studied to find the minimum amount of substrate giving maximum collagenase production. This may be of help in the commercial production of collagenase for finding out cost effective substrate concentration.

3.3.4 Effect of pH on collagenase production

The selected leather isolate showed collagenase production in a wide range of pH 5 to 8.5. Highest collagenase production was found at pH 7.5 after 72h. Above this pH, the collagenase production was found to be decreased, because the metabolic activities of microbes respond to pH change (Fig. 6). Baehaki *et al*. (2012) investigated in their work that, the enzyme exhibited the greatest activity in the pH range of 6.0 to 8.0, with an optimum pH 7.0. *Bacillus cereus* showed maximum activity of collagenase within pH range of 7-7.5 (Liu *et al*. 2010). The results in the

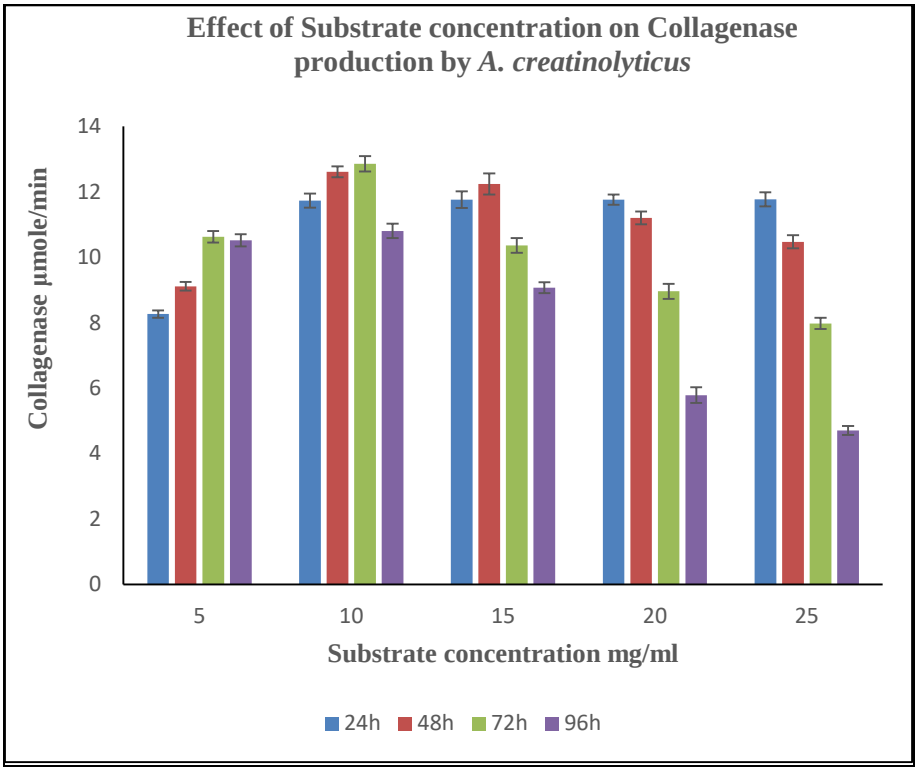

Fig. 5 Effect of substrate concentration on collagenase production by *A. creatinolyticus*

present study are in agreement with cited reports, which showed optimum collagenase production at pH 7.0 to 7.5, but lower than pH 9.0 to 9.5 of *Thermoactinomyces* sp.21E (Petrova *et al*. 2006), *Bacillus* sp. MO-1(Okamoto *et al*. 2001) and pH 9.0 of *B. subtilis* FS-2 (Hiroko and Kim 1999).

3.3.5 Effect of temperature on collagenase production

The effect of temperature on collagenase production was studied by incubating the culture media at various temperatures 10, 37, 40 and 45℃ *Arthrobacter creatinolyticus* KP01574 (zzx28) expressed maximum collagenase production was at 37℃ after 72h followed by this the second best temperature for collagenase production was found to be 40℃ (Fig. 7). On the other hand, the minimum amount of collagenase production was observed at temperature 10℃ and 45℃. Azza *et al*. (2014) noted *N.dassonvillei* NRC2aza maximum collagenase activity was at 55℃ which was higher than the present study. NW4327 collagenase showed higher activity observed at 30℃ compared to that at 20℃ or 40℃ or warmer (Mukherjee *et al*. 2009).

3.3.6 Effect of on metal ions and chemicals on collagenase production

The collagenase activity was strongly inhibited by specific metalloprotease inhibitor such as EDTA (Fig. 8) but not by PMSF. The enzyme, therefore, seemed to be a kind of metalloprotease. Calcium ions and ferric chloride stimulated collagenase production with relative activity of 110%-115% while cobalt, lead, inhibited 31.65% activity. Complete inhibition was observed by *β*-mercaptoethanol and mercury. Recent studies indicated that beside EDTA, *β*-mercaptoethanol and

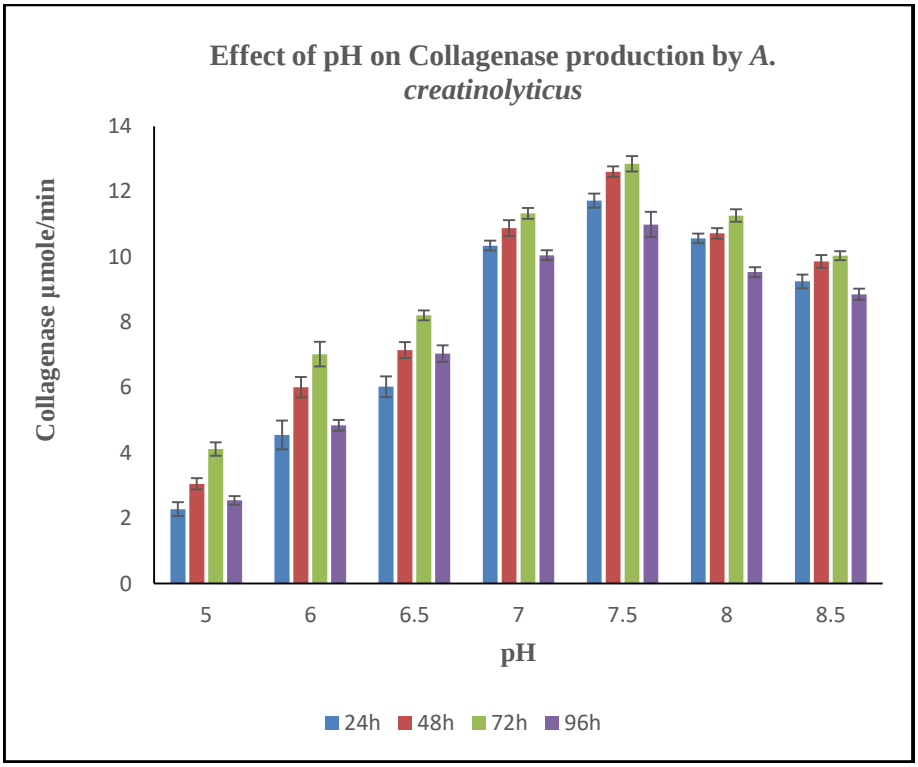

Fig. 6 Effect of pH on collagenase production by *A. creatinolyticus*

mercury were well known inhibitors for collagenases. Cobalt ions may inhibit this enzyme because they can bind to histidine located in the catalytic sites instead of zinc ions (Macartney and Tschesche 1981). Metal ions such as Li^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Ba^{2+} increased and stabilized the collagenase activity, and especially the Ca^{2+} ion displayed the strongest activation with 127% while the collagenase activity was drastically inhibited by EDTA, EGTA, and β-mercaptoethanol. Mn^{2+} and Pb^{2+} ions showed obvious inhibition of the enzyme by 37.8% and 55.2% (Wu *et al.* 2010). Magnesium ions and $CaCl_2$ stimulated collagenase production with relative activity 104%-115%. Some collagenases were partially inhibited or stimulated by Zn, Cu metal ions and cysteine chloride, sodium sulphite. Same results were shown by *Porphyromonas gingivalis*, EDTA also inhibited collagen degradation, but there was no effect on the activity when the enzyme was incubated with PMSF. On the other hand, Ca^{2+} stimulated the activity of the purified protein (Ioannides *et al*. 2004).

3.4 Partial purification and Molecular weight determination

Partially purified collagenase showed increased specific activity from $15.07X10^{3}$ U/mg to $18.45X10^{3}$ U/mg (122.42%) and then to $19.46X10^{3}$ U/mg (129.13%) and decreased total activity 12405.6 U/ml to 10399.3 U/ml and 9780.0 U/ml after fractionation with ammonium sulfate and desalting, respectively. The high protein content was observed before purification was due to the presence of other proteins which resulted in the lower specific activity. As the purification

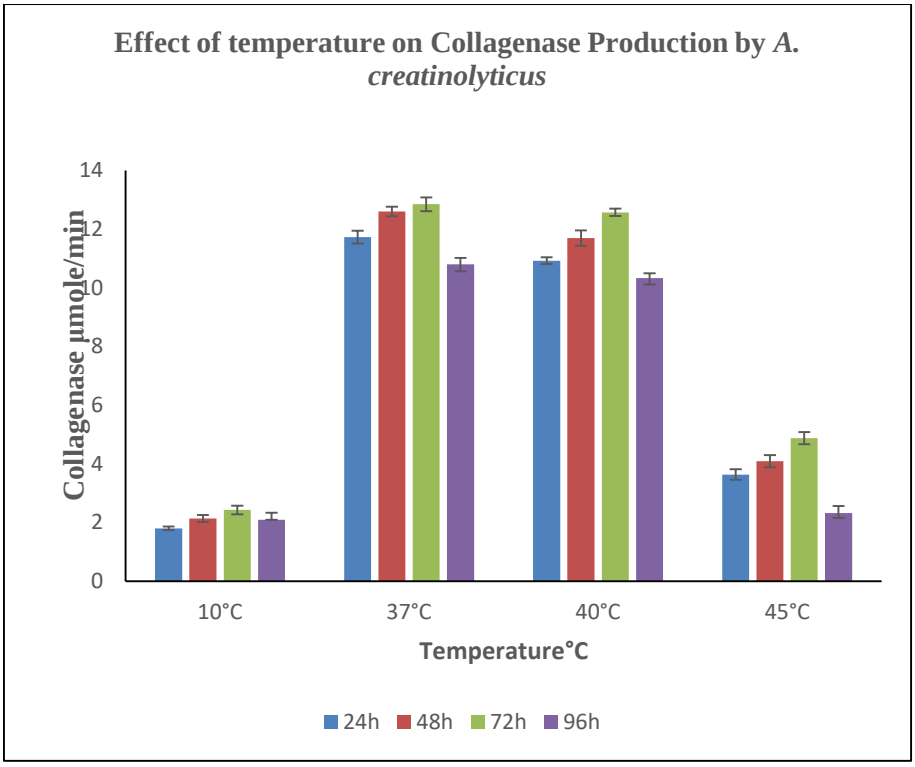

Fig. 7 Effect of Temperature on collagenase production by *A. creatinolyticus*

proceeds, the intervening proteins were eluted, hence the purified enzyme showed increasing specific activity and decreased total activity. Similar results were noted by Daboor *et al.* (2012) and Sayed *et al.* (2012), Daboor *et al*. (2012) reported the total activity of the crude enzyme from fish collagenase was 27.10 ± 4.80 U/mL which decreased to 21.85 ± 3.04 U/mL (80.86%) and then to 18.49 ± 1.10 U/mL (68.23%) after fractionation with ammonium sulfate and desalting, respectively. The crude collagenase was precipitated by $(NH4)_2SO_4$ up to 80%, the specific activity was found to be increased to 0.61U/mg as compared to 0.55U/mg crude enzyme (Sayed *et al*. 2012).

The molecular weight of the partially purified collagenase from SDS–PAGE was found to be 66.55kD, 62.05kD (Fig. 9) as evaluated by matching the electrophoretic mobility of the collagenase with the electrophoretic mobilities of standard marker proteins (Hi-Media range 14.3-97.4kD). *B. licheniformis* F11 by Baehaki et al., (2012), *B. subtilis* FS2120KD, Liu *et al*. (2010) reported molecular weight of collagenase produced by *Bacillus cereus* MBL13 Strain to be 50 kD. The molecular mass of the purified collagenolytic enzyme was much smaller than the molecular mass of the collagenases isolated from *C. histolyticum, C. perfringens, Acinetobacter* sp. and *Vibrio alginolyticus* (molecular masses of 120, 66-125, 102 and 82 kD) reported by Bicsak and Harper (1985), Matsushita *et al*. (1994), LeCorre *et al*. (1985), Hare *et al*. (1983), respectively. Also, the molecular mass of the protease was lower than the molecular mass of collagenolytic proteases purified from *Bacillus cereus* by Makinen and Makinen (1987) and Lund and Granum (1999), which had molecular masses of 87 and 105 kD, respectively. Literature survey showed that

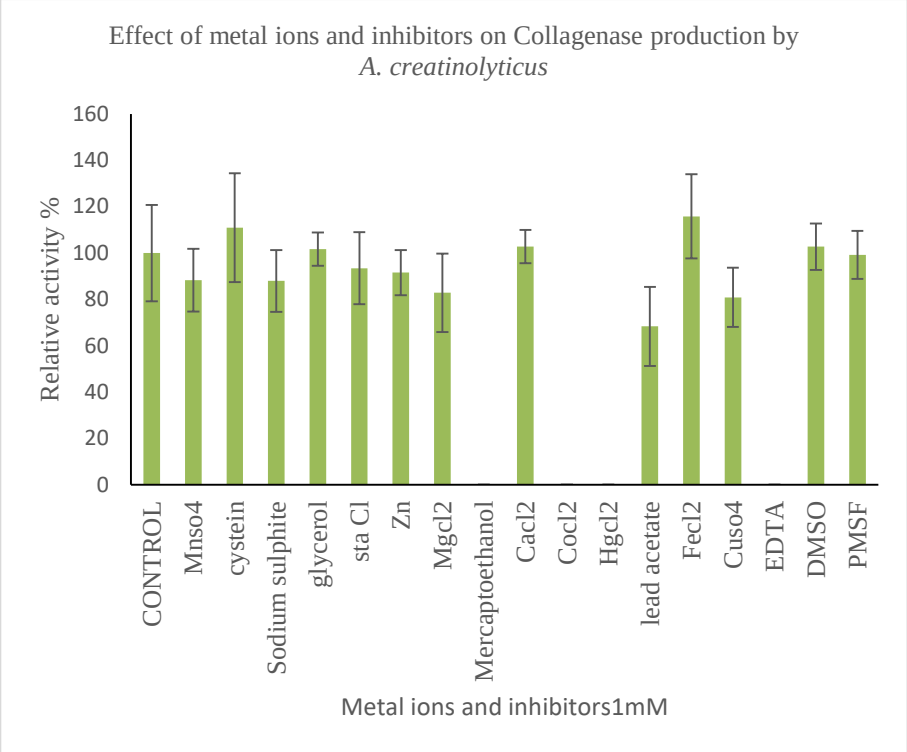

Fig. 8 Effect of Metal ions and inhibitors on collagenase production by *A. creatinolyticus*

Fig. 9 PAGE for collagenase of bacterial isolates

the maximum bacterial collagenases were made up of two subunits and in the present study partially purified collagenases was used that may be the reason for obtaining two bands in electrophoretic separation.

3.5 Utilization of biocatalyst in collagen rich solid waste treatment

Leather solid waste samples were digested by the biocatalyst in minimal media as compared to control. After an interval of 24h the supernatant filtrate was analyzed for leather deterioration by

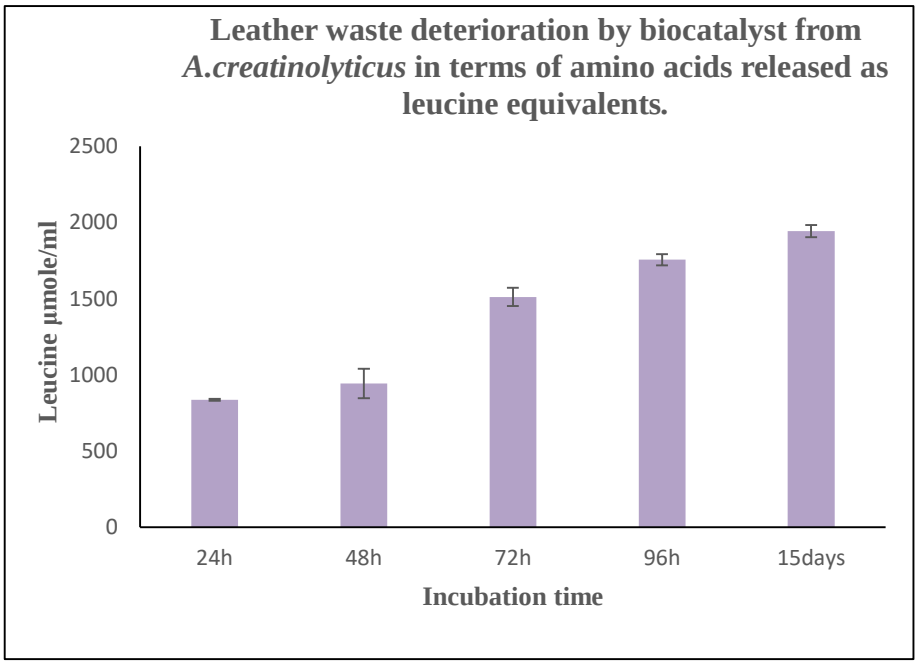

Fig. 10 Leather waste deterioration by biocatalyst from *A. creatinolyticus* in terms of amino acid released as leucine equivalent

the release of amino acids at 575 nm. After 15 days of incubation, the collagenase production by the selected isolate was found to be 32.935±2.768 µmoles/min. Graph of incubation time versus amino acids released in terms of leucine was plotted. Increase in incubation time showed proportionate increase in released amino acids measured in terms of leucine equivalents (Fig. 10). One proteolytic unit was defined as the amount of enzyme that released 1µmole of leucine substrate released per hour. The graph hence, biocatalyst from *Arthrobacter creatinolyticus* was utilized in collagen rich solid waste treatment.

4. Conclusions

The isolate *Arthrobacter creatinolyticus* KP01574 (zzx28) hydrolyzed collagen, gelatin, casein and keratin. Optimum collagenase production was found to be 12.852 µmoles/min in 72h at 2 ml/dL inoculum size using 10 mg/ml of collagen type I as a substrate at 37℃. Optimum pH for collagenase production was found to be 7.5. The collagenase activity was stimulated by calcium ions and ferric chloride with relative activity of 110% - 115% while cobalt, lead, inhibited the activity. Complete inhibition by *β*-mercaptoethanol and mercury and strongly inhibited by specific metalloprotease inhibitors such as EDTA but not by PMSF, the enzyme, therefore, seemed to be a kind of metalloprotease. The molecular weight of the partially purified collagenase was 66.55kD, 62.05kD by electrophoretic mobility as compared to standard marker proteins. Biocatalyst from *Arthrobacter creatinolyticus* KP01574 (zzx28) digested collagen rich leather solid waste;hence this isolate may potentially be used to treat tannery waste with some additional inputs and thus will reduce environmental hazards.

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