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Research Paper

Production of collagenase by *Bacillus* KM369985 isolated from leather sample

***Kate Savita[1], Pethe Arachana[2]**
[1]Department of Biotechnology, Shiv Chhatrapati College, Aurangabad-431001, (M.S.), INDIA
[2]Department of Microbiology, Shivaji College of Arts, Commerce and Science, Akola-444001, (M.S.) INDIA

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Abstract

Collagen is a major fibrous element of skin, bones, tendons, cartilage, blood vessels and teeth found in all multicellular organisms and which is also the most representative protein of leather wastes. Collagenases are endopeptidase that can hydrolyze both native and denatured collagens. Collagen peptides, the product of collagen degradation, possess various biological activities of industrial and medical interest such as ingredient in drugs, drinks, food, cosmetics etc. In present investigation, twenty six collagenase producing bacteria were isolated from fifty leather samples, one of most efficient collagenase producers was isolated, identified and was found to be belonging to genus Bacillus. 16s r RNA sequencing was done and the sequence was deposited to GenBank with accession number Bacillus KM 369985. The isolate was able to produce 600U/ml in 72h. The optimum temperature, substrate and pH were 37°C, 1.5% and7.5 respectively. Effect of incubation period and inoculum percentage was also studied. The collagenase production was strongly inhibited by Hg^{+2}, EDTA and β-mercaptoethanol however Fe^{2+}, $Zn^{2+}$and DMSO enhanced its production. Isolate was capable of hydrolyzing other protein substrates such casein, gelatin, and keratin. Hence this isolate can be efficiently used to treat tannery waste and recycling of these organic materials.

Keywords: collagenase, tannery waste, Bacillus KM369985, inhibitor, 16s RNA, collagen, keratin.

Introduction

The animal skin is composed of proteins, the main protein in skin is collagen, which also is a main component in bone, cartilage and teeth. Also commonly occurring in skin are the proteins keratin and elastin, though in the process of making leather it is sought to remove as much of any other protein than collagen as possible[1]. Therefore, leather is composed almost solely of collagen. Collagen is a major fibrous element of skin, bones, tendons, cartilage, blood vessels, and teeth found in all multicellular organisms. The repetitive sequence of collagen (Gly-XY) n, where X and Y are commonly Proline and Hydroxyproline, can be proteolytically attacked only by enzymes with a sharply defined specificity profile. Collagenases are endopeptidase that digests native collagen in the triple helix region. 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. It can attack almost all collagen types, and is able to make multiple cleavages within triple helical regions[2].

Type I collagen is the most abundant component of the organic matrix of bone tissue, composing 85-90% of all bone protein. Collagen peptides, the product of collagen degradation, possess various biological activities of industrial and medical interest such as ingredient in drugs, drinks, food, and cosmetics etc[3]. Leather industries as well as meat industries are important generators of insoluble and hard to-degrade animal proteins, which are converted in waste with high potential of environmental pollution. Microorganisms can be used in order to degrade these wastes because they

synthesized extracellular enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, registered an increasing use for industrial applications in fur and leather industry because they are nontoxic and eco-friendly[4]. The objective of the present investigation has been focused on collagenase production by *Bacillus* isolated indigenously from deterioted leather samples.

Materials and Methods

Materials

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

Isolation and identification of collagenolytic bacteria

Bacteria were isolated from deterioted leather samples, collected from different districts of Maharashtra. Primary isolation was carried out on nutrient agar. Among the different organisms isolated, a well isolated colony was used for screening for their ability to hydrolyzed collagen, on collagen agar plates (containing 1% collagen and 2% agar).The plates were incubated at 37°C for 48 h. After incubation to each individual colony, a drop of mercuric chloride precipitation reagent was added. Bacteria showing collagenolytic activitywere screened with larger transparent circle around the bacterial colony. Among these isolated strains, the most efficient isolate was identified by morphological, biochemical characteristics as described in the Bergey's manual of systematic bacteriology and by 16s rDNA sequencing[3].

Collagenase production:

For collagenase production, triplicate set of flasks were inoculated by screened strain (2% inoculum) in a fermentation medium of composition glucose 20g/L, collagen10g/L, $CaCl_2$ 0.05g/L, $NaH_2PO_4$0.5g/L, $K_2HPO_4$0.5g/L. The flasks were incubated at 37°C for 48h in an orbital shaker at 180 rpm. Afterward fermentation medium was centrifuged at 4°C and 4000rpm for 10min, the supernatants were collected as a source of crude collagenase[5].

Enzyme and protein assay

Collagenolytic activity was measured according to the Bergmeyer's (1983) method with modification. 0.5% collagen type I used as substrate. 50µl enzyme filtrate was mixed with 250µl substrate in Tris HCl buffer-7.5 pH (50mM) and incubated for 10 minute at 37°C.Trichloroacetic acid 0.2M was added and incubated for 10 minute at 37°C followed by centrifuged at 4000rpm for 10 minute. The supernatant was mixed with Folin-Lowery reagent incubate for 10 minute followed by addition of Folin Ciocalteau reagent and incubation further at 37°C for 20 minute then measured optical density at 578nm[6].One unit (U) of enzyme activity equals to one micromole of L-leucine equivalents released from collagen under specified conditions.Protein concentration was determined by Folin-Lowery method using Bovine serum albumin as a standard[7].

Effect of various physico-chemical factors on collagenase production

To study the influence of pH and temperature on collagenase production was individually tested by taking the production media at different pH ranging from 5 to 8.5 and temperature from 10°C to 50°C. The effect of substrate concentration on the production of collagenase was tested by adding different concentrations of collagen type I as a substrate in the production medium. The fermentation media were assayed every day for collagenase production till a decline was observed in the enzyme activity. For time course analysis of collagenase production, the isolate was grown in the optimized growth medium and the activity was measured every day for a period of four days. The effect of inoculum size (1%-10%) on the collagenase production also studied.

Effect of metal ions and chemicals on enzyme production

The sensitivity of various metal ions, organic solvents and inhibitors was studied by adding 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% concentration of mercaptoethanol, glycerol, DMSO in the fermentation media .The effect of them was measured by assaying activity every day for a period of three days.Collagenase activity measured in the absence of any inhibitor or metal ions was taken as 100% relative activity.

Results and Discussion

Isolation and identification of collagenolytic bacteria

By primary screening forty eight bacteria having different colour and morphology were isolated on nutrient agar plate from fifty deterioted leather sample after incubation 24h at 37°C.Among these twenty six bacteria shown collagenolytic activity on collagen agar plate after 48 h of incubation(Fig1).one of efficient strain was used for auxiliary study. Given strain shown gelatinase, caseinase and keratinase activity. The biochemical and morphological characteristics of given strain revealed that the organism belongs to the *Bacillus* genus. By analysis of 16s r RNA sequencing shown that the isolated strain was *Bacillus megaterium* and then sequence was deposited to GenBank with accession number *Bacillus KM 369985.*

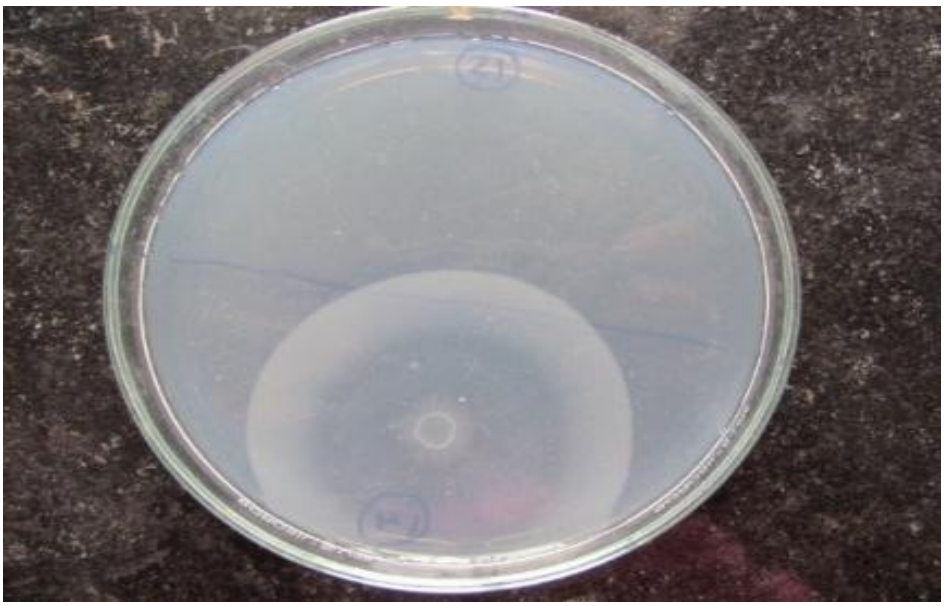

Figure 1: Collagenase activity on collagen agar plate

Effect of various physico-chemical factors on collagenase production

Effect of incubation period

Givenisolate *Bacillus KM 369985*shown collagenase production after 12 h of incubation and maximum collagenase production 600U/ml by using collagen peptide type I as a substrate after 3 days of incubation then reports mentioned uptil. *N. dassonvillei*NRC2[8] shown maximum collagenase activity 240 U/ml after 6 days of incubation using chitin waste as sole carbon source. *Bacillus amyloliquefaciens*[9]showed collagenase activity 23.7U/ml after 10 days using sheep fur as a substrate (Figure 2).

Effect of substrate

Ace Baehaki et al[6], stated that the maximum collagenase production by *Bacillus licheniformis F11.4* using 5% substrate (0.003U/ml) and Lili Liu et al [3], reported 35U/ml collagenase production using 1% substrate by *Bacillus Cereus MBL13,* in this work *Bacillus megaterium KM 369985* was found to give

more or less same amount of collagenase production 613U/ml-628U/ml ±25 atSubstrate concentration ranging from 1-2% for further study 1% concentration was used (Figure 3).

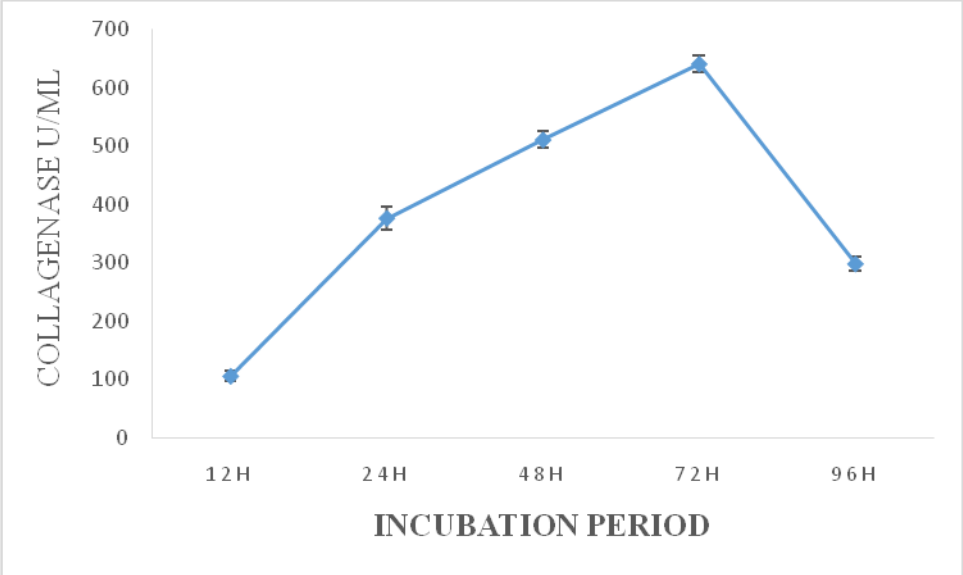

Figure 2: Effect of incubation period on collagenase production

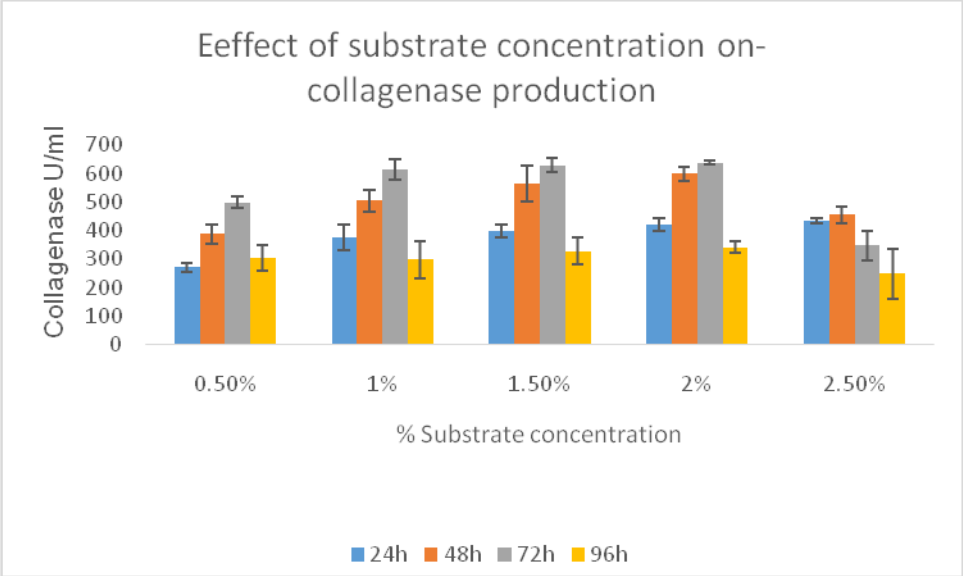

Figure 3: Effect of substrate concentration on collagenase production

Effect of temperature

Optimum temperature for collagenase production of given isolates was recorded as 37°C, the increase intemperature above 40°C drastically reduced collagenase production as shown in fig 4 *Bacillus subtilis* P13 shown maximum collagenase production 1.5U/ml at 40°C[10] and *B. cereus* MBL13[3]also shown maximum production at 40°C.This temperature was a lower temperature than reported (Figure 4).

Effect of pH

The effect of pH on collagenase production wasstudied at pH range of 5 to 8.5, the optimum pH of given isolates was 7.5, *similar collagenase activity was reported at pH 7.5 by Scomber japonicas* [11]. Given isolates *Bacillus megaterium KM 369985* also shows collagenase production on wide range of

pH 5 to 8.5. *B. licheniformis* F11[9]shows maximum activity at pH 7. *Bacilluscereus* shows maximum activity of collagenase has been found within pH range of 7-7.5 [3] (Figure 5).

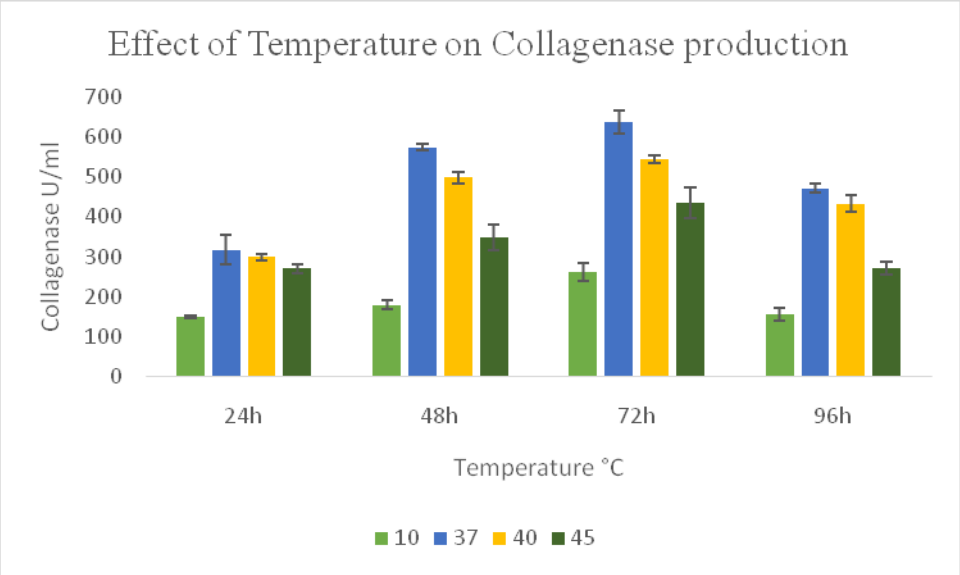

Figure 4: Effect of temperature on collagenase production

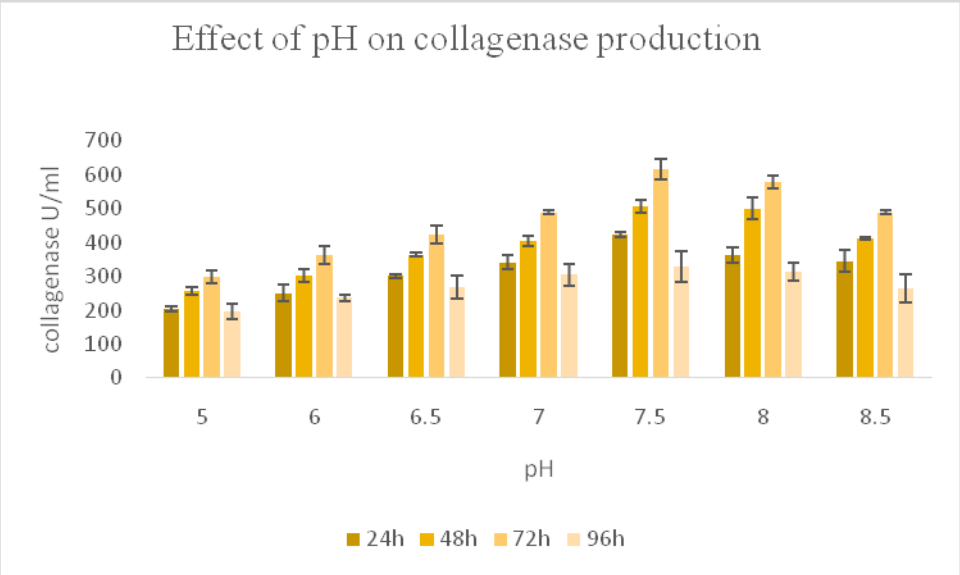

Figure 5: Effect of pH on collagenase production

Effect of various activators and inhibitors

The collagenase production by *Bacillus megaterium KM 369985*was strongly inhibited by Hg^{+2}, EDTA and β-mercaptoethanol however Fe^{2+}, Zn^{2+} Ca^{2+} &DMSO enhanced its production. EDTA, a chelating agent for calcium ions strongly inhibited enzyme activity therefore given *Bacillus megaterium* KM369985 produced collagenase is a member of metaloprotease. Complete inhibition of enzyme production in presence of mercaptoethanol indicating that structure of this enzyme contained disulphide, Similar result was shown by *B. pumilus* col-J[12], by *B. Licheniformis* [9]and by *B. cereus*[13].PMSF and DMSO showed very little or no effect on collagenase production (Figure 6).

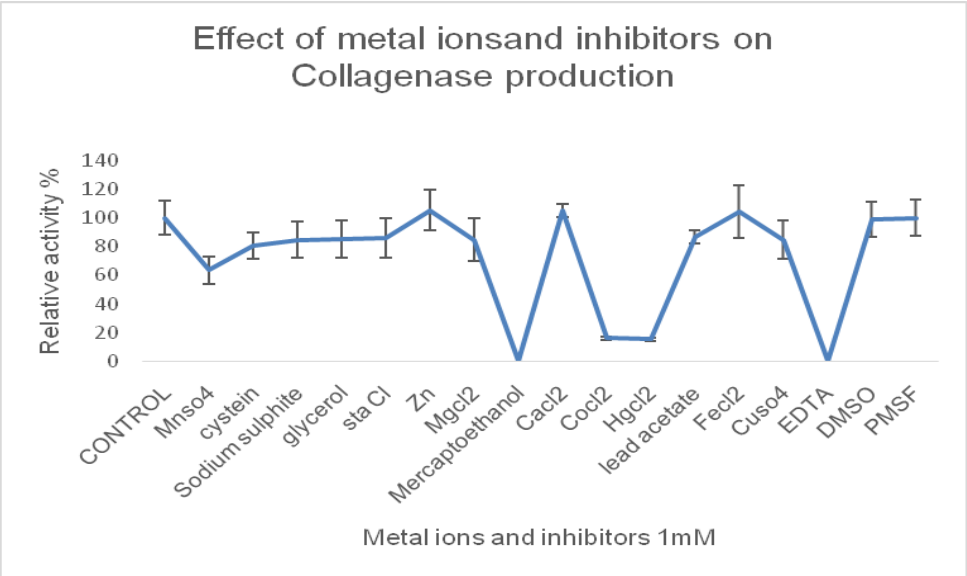

Figure 6: Effect of metal ions and inhibitors on collagenase production

Conclusion

Twenty six collagenase producing bacteria were isolated from fifty leather samples, one of most efficient collagenase producer was isolated, identified and was found to be belonging to genus *Bacillus.*16s r DNA sequencing was done and the sequence was deposited to GenBank with accession number *Bacillus* KM 369985. The isolate was able to produce maximum collagenase 600U/ml in 72h at 2% inoculum size. Substrate concentration ranging from 1-2% was found to give more or less same amount of collagenase production, for further study 1% concentration was used.Optimum pH for collagenase production was found to be 7.5. Optimum temperature was found to be 37°C.The collagenase production was strongly inhibited by Hg^{+2}, EDTA and β-mercaptoethanol however Fe^{2+}, Zn^{2+} Ca^{2+} &DMSO enhanced its production. Isolatewas capable of hydrolyzing other protein substrates such casein, gelatin, and keratin. Hence this isolate can be efficiently used to treat tannery waste and recycling of these organic materials.

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