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Production of Collagenase from a Novel Non-pathogenic Isolate *Chryseobacterium contaminans* KU665299

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Abstract: Collagenase is a vital therapeutic enzyme that carries out the degradation of collagen, the most abundant protein in vertebrates, into small peptide fragments and has potential applications in medical and meat based food industries. In the present study, isolation and screening of collagenase producing bacteria from soil samples were executed. A novel and non-pathogenic bacterial isolate, identified as *Chryseobacterium contaminans* by 16S- rRNA sequence analysis, with collagenase activity was reported. The sequence was deposited in GenBank with accession number KU665299. This is the pioneer report on the production of collagenase by *Chryseobacterium* sp. Maximum extracellular collagenase production (2.0 U/mL) by obtained when this isolate was grown in medium (pH 6.5) containing (%, w/v) sucrose 1.0, peptone 1.0, gelatin 0.3, yeast extract 0.2 and few salts for buffering action at incubation temperature 30°C and agitation 150 rpm for 24 h of fermentation.

Keywords: Collagen, Collagenase, *Chryseobacterium contaminans*, enzyme, isolation, optimization.

Introduction

Collagenases (E.C.-3.4.24.3) are proteolytic enzymes accountable for the degradation of helical regions of native collagen fibrils into small peptide fragments. The substrate, collagen is an extensive fibrous constituent of extracellular connective tissue such as skin, bones, cartilage, tendons, blood vessels and teeth found in all multicellular organisms [17, 20]. Collagenase is an endopeptidase that is highly specific for both native and denatured collagen. Bacterial collagenases are usually considered to be enzymes that cleave helical regions of fibrillar collagen molecules under physiological conditions [15].

Collagen is the most abundant component of the extracellular matrix (ECM) in vertebrates, which provides not only a flexible scaffold for embedded cells but also regulates crucially important cellular processes including differentiation, cellular growth, survival, migration and much more[37]. Nearly 30 % of the total proteins in mammalian bodies are various types of collagens, which are essential structural components of all connective tissues [33]. Different types of collagens are characterized by substantial complexity and diversity in their structure, the existence of additional non-helical domains, their assembly, functions and splice variants [8, 11]. The polypeptide chains are mostly composed of repeating Gly-X-Y triplets, where X and Y are frequently proline and hydroxyproline, respectively. Each polypeptide chain is composed of triple-helical domains which are flanked by non-helical regions [12].

Microbial collagenases have been mostly obtained from pathogenic microorganisms, particularly *Clostridium histolyticum*, which is the ex-

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tensively used commercial source [6, 39]. Collagenases are known proteases that seemingly contribute to the pathogenicity of bacteria by allowing these pathogens to penetrate the connective tissue barrier [21]. From therapeutic as well as industrial point of view, pathogenicity can restrict and negatively influence the use of microorganism for bioprocess development [21, 39]. Microorganism's *viz*. *Bacillus subtilis*, *B. licheniformis, B. tequilensis, Streptomyces* sp., Thermoactinomyces, screened from the soil, water, earthworm and caviar are generally considered safe to produce collagenase [21]. The collagenase genes have been successfully cloned and expressed in non-pathogenic strains that would simplify their production, purification and also abstain the presence of undesirable toxins resulting to the safe use collagenase in the medical and food industry [7].

Collagenases can be used in the treatment of more specific diseases where excessive collagen deposition is the main problemc[4, 26]. Microbial collagenases have the potential applications in food and nutrition sector such as meat tenderization, collagen peptides, hydrolysates, collagen extraction, by-products utilization and functional foods [31]. Collagenase enzyme appears to be a convenient and a cheap medication for the treatment of burns, wound healing, and some other diseases [1, 22]. Collagenase has numerous applications in therapeutics, leather industry, dye industry, food industry and waste management [7, 40]. The purpose of this study was to isolate collagenase producing non-pathogenic bacteria from soil samples and production of extracellular collagenase.

Materials And Methods

Chemicals

All the chemicals used in the present study were procured from HiMedia Laboratories Pvt. Ltd., Mumbai and were of high purity analytical grade. Collagen and azocoll used were procured from Sigma Aldrich, USA. The media constituents were of bacteriological grade.

Isolation of collagenase producing microorganisms

Collagenase producing microorganisms were isolated from the soil samples collected from local meat markets and slaughterhouse of various places of Himachal Pradesh. The soil samples were serially diluted (upto 10^8 dilutions) in the sterile physiological saline and 100 µL of each dilution was spread on the agar plates containing selective medium [40] (%, w/v): gelatin 2.0; peptone 0.5; KH_2PO4 0.05; NaCl 0.01; $MgSO_4.7H_2O$ 0.02 and agar 2.0 (pH 7.5). The plates were incubated at 37°C for 24-48 h. Morphologically distinct pure colonies of bacteria were subjected to further screening by the acidic hydrargyrum solution (consisting of 15 g $HgCl_2$ and 20 ml concentrated HCl per 100 ml) method.

Screening and selection of collagenase producing microorganisms

Colonies showing a positive zone of hydrolyzation of gelatin (denatured collagen) were selected. Further screening was done on the basis of their ability to digest goat skin and hydrolysis of substrate azocoll [18]. These isolates were grown under submerged conditions in a liquid medium containing (%, w/v): gelatin 2.0; peptone 0.5; KH_2PO4 0.05; NaCl 0.01 and $MgSO_4.7H_2O$ 0.02 (pH 7.5) and the flasks were incubated at 37°C for 24 h in an orbital shaker at 150 rpm. The collagenolytic activity in the supernatant was measured spectrophotometrically. The growth was also monitored by measuring A_{600} fermentation broth (Spectrophotometer Labindia, India).

Collagenase Assay

Enzyme activity was measured by determining the extent of collagen/gelatin (substrate) breakdown using Rosen's modified colorimetric ninhydrin method [35]. Amino acids liberated were expressed as micromoles of L-leucine released in one minute under standard assay conditions. Tris-HCl buffer 250 µl (0.1M, pH 7.5), crude enzyme 50 µl and 300 µl of 0.3 % (w/v) substrate (gelatin prepared in 0.1M Tris-HCl buffer, pH 7.5) were incubated at 37°C for 30 min. The reaction was stopped by addition of 600 µl chilled TCA (50 %, w/v). A set of control was also run. Then, 200 µl of the reaction mixture was withdrawn and amount of L-leucine released was measured by ninhydrin method. One unit (U) of enzyme activ-

ity has been defined as the amount of enzyme required for the release of 1mmole of L-leucine per minute as per assay conditions.

Identification of selected bacterial strain on basis of 16S rRNA sequencing

The selected bacterial strain CGR-6 was sent to IMTECH, Chandigarh for 16S rRNA sequencing. The consensus sequence of 16S rDNA gene obtained was subjected to BLAST with non reductant database of NCBI genbank database. On basis of maximum identity score, first few sequences were selected and aligned using multiple sequence alignment software ClustalW. Distance matrix was generated and a phylogenetic tree was constructed using MEGA 4. The evolutionary history was implied using the Neighbor-Joining method [36].

Selection of medium for production of extracellular collagenase from *C. contaminans*

In order to find out the most suitable medium, 15 reported media (Table 1) were tested for collagenase production by *C. contaminans*. The initial pH for all of the media was adjusted to 7.0. The 24 h old 2 % (v/v) inoculum (seed) was used to inoculate the different production medium and flasks were incubated at 37°C for 24 h in an orbital shaker (150 rpm). The fermentation broth was assayed for collagenase activity.

Role of inducer on the production of collagenase from *C. contaminans*

The effect of inducer (gelatin/collagen) on collagenase production was studied with or without the addition of (0.3 %, w/v) gelatin/collagen in seed and production medium.

Effect of various physiological factors on collagenase production from *C. contaminans*

Various physiological factors such as medium pH (5.0 to 9.0), incubation temperature (25 to 50°C) and agitation rate (100 to 250 rpm) were studied in order to optimize these conditions for production of collagenase.

Time course analysis of collagenase production from *C. contaminans*

For the course of cultivation, the bacterial isolate was grown in selected medium under optimized physiological conditions and enzyme activity was measured at different time intervals ranging from 2 to 34 h. Samples were withdrawn at the time interval of 2 h.

Results and discussion

Isolation, screening, selection and identification of collagenase producing microorganism

Primary screening was done on the basis of gelatin hydrolyzation zone by the acidic hydrargyrum solution. Gelatin is denatured form of collagen. A total number of 46 positive isolates were obtained from different soil samples and 22 bacterial isolates exhibiting a larger zone of hydrolysis were selected for further studies. Secondary screening was done on basis of hydrolysis of substrate azocoll and digestion of goat skin. Seven isolates showed complete digestion of goat skin after 24 h of incubation and high azocoll hydrolysis. The selected isolates were also evaluated for estimation of collagenase activity by ninhydrin method [35] using collagen and gelatin as substrate. Among all these isolates, the most potent CGR-6 bacterial strain (Fig. 1) was selected and observed as Gram- negative, small rods, catalase positive and oxidase positive. It forms round, smooth and deep yellow coloured colonies on agar plates. Based on 16S rRNA sequencing, it was identified as *Chryseobacterium contaminans* and a phylogenetic tree was constructed using the Neighbor-Joining method (Fig. 2). The sequence was deposited in GenBank with accession number KU665299. The bacterium *Chryseobacterium contaminans* was first reported/isolated from rhizosphere contamination on an agar plate in Alabama, USA in 2014 [19]. There has not been any pathogenicity report on *C. contaminans* so far, so this study offers collagenase production from a novel non-pathogenic bacterium.

Selection of medium for production of extracellular collagenase from *C. contaminans*

C. contaminans was grown on 15 different reported media (Table 1). Among all the media tested for production of collagenase from *C. contaminans*, maximum collagenase activity (0.93±0.06 U/mL) was achieved in medium 8

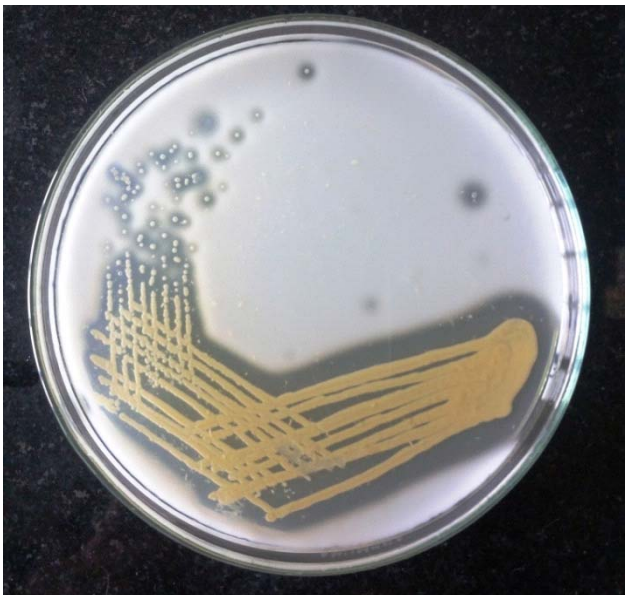

Fig. 1. Bacterial isolate *C. Contaminans* showing gelatin hydrolyzation zone

Table 1. List of media used for production of collagenase from *C. contaminans.*

Medium no.	Media composition (%, w/v)	References
M1	Gelatin 2.0; Peptone 0.5; $MgSO_4.7H_2O$ 0.02; NaCl 0.01; KH_2PO_4 0.05	40
M2	NaCl 1.0; Tryptone 0.5; Yeast Extract 0.25; Gelatin 5.0	2
M3	Dextrose 2.0; Yeast Extract 0.15; Tryptone 1.0; $NaH_2PO_4.2H_2O$ 0.05; $K_2HPO_4.3H_2O$ 0.25; $CaCl_2$ 0.005	25
M4	Dextrose 1.0; Soyapeptone 1.0; NaCl 0.05; $FeSO_4.7H_2O$ 0.01; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.05	17
M5	Gelatin 1.5; Peptone 0.4; Yeast Extract 0.1 Aged sea water 50 %	3
M6	Glucose 2.0; Yeast Extract 0.15; Tryptone 1.0; $NaH_2PO_4.2H_2O$ 0.05; $K_2HPO_4.3H_2O$ 0.25 and $CaCl_2$ 0.005	41
M7	Gelatin 1.5; NaCl 0.085; Yeast extract 0.01; KH_2PO_4 0.5 and $MgSO_4.7H_2O$ 0.001	28
M8	Sucrose 1.0; Peptone 1.0; Gelatin 0.3; Yeast extract 0.2; Na_2HPO_4 0.20; Na_2CO_3 0.25 and $MgSO_4.7H_2O$ 0.04	9
M9	Glucose 1.0; Meat extract 1.0; Polypeptone 1.0 and NaCl 0.5	14
M10	Glucose 1.0; Meat extract 0.2; Polypeptone 0.5; Gelatin 0.2 and $FeSO_4.7H_2O$ 0.001	14
M11	Casein 0.5; KH_2PO_4 0.25 and NaCl 0.1	16
M12	Tryptone 1.0; Yeast Extract 0.5 and NaCl 0.5	30
M13	K_2HPO_4 0.7; KH_2PO_4 0.2; $MgSO_4.7H_2O$ 0.01, Trisodium citrate 0.05; Yeast Extract 0.1; $CaCl_2$ 0.01; Gelatin 0.3; Polypeptone 0.15 and NaCl 1.0	27
M14	NaCl 2.34; K_2HPO_4 1.06; KH_2PO_4 0.46; Trisodium citrate 0.05; $(NH_4)_2SO_4$ 0.1; $MgSO_4.7H_2O$ 0.01; Glucose 0.25	32
M15	Glucose 10; Polypeptone 1.0 and Yeast Extract 0.5	29

(Fig. 3) containing (%, w/v) Sucrose 1.0; Peptone 1.0; Gelatin 0.3; Yeast extract 0.2; Na_2HPO_4 0.20; Na_2CO_3 0.25 and $MgSO_4.7H_2O$ 0.04. Although maximum activity was obtained in medium 8, maximum growth (4.56±0.07 mg/mL) was seen in medium 4. These results proposed medium 8 to be most suitable for collagenase production.

Role of inducer on the production of collagenase from *C. contaminans*

Inducer (gelatin/collagen) plays a critical role in the production of collagenase. Its effect was studied in different combinations of seed and production medium (Table 2). In case of gelatin as inducer, maximum collagenase production

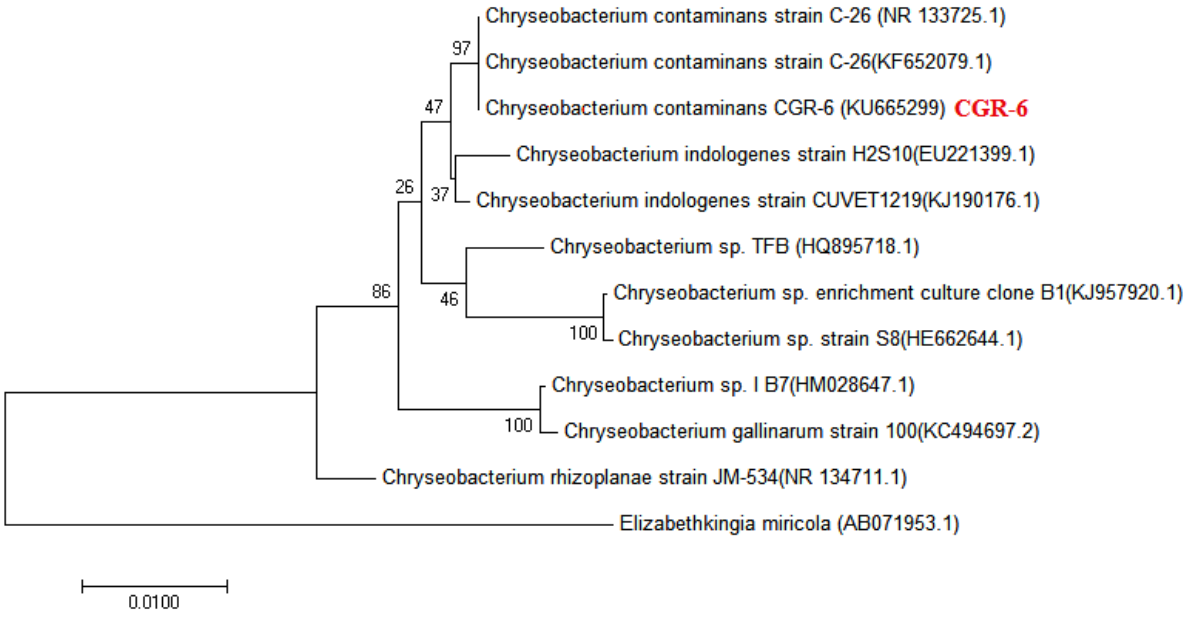

Fig. 2. Phylogenetic tree of bacterial isolate CGR-6. A branch length is correlative to 0.01 changes per amino acid and bootstrap values are designated at branch points

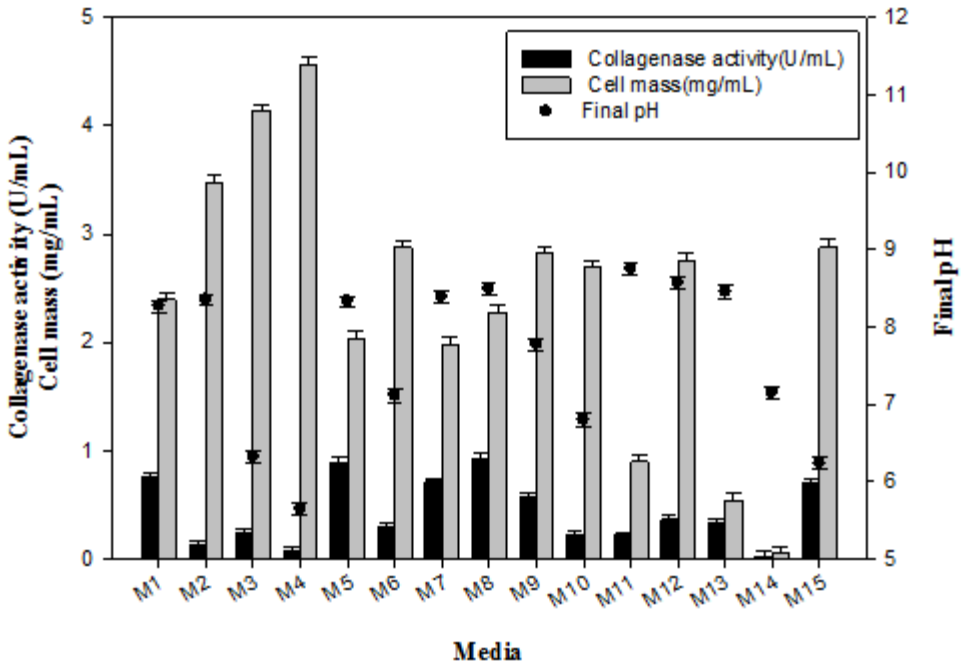

Fig. 3. Selection of medium for production of collagenase from *C. contaminans*

Table 2. Effect of inducer (gelatin/collagen) on collagenase production from *C. contaminans*

No.	Inducer (0.3%., w/v)	Collagenase activity (U/mL)	Cell mass (mg/mL)	Final pH
		Gelatin as inducer		
1	$S^+ P^+$	0.89±0.04	2.34±0.05	8.67±0.05
2	$S^+ P^-$	1.24±0.05	2.04±0.03	8.72±0.06
3	$S^- P^+$	0.67±0.03	2.16±0.04	8.75±0.04
4	$S^- P^-$	0.15±0.04	1.80±0.03	8.68±0.05
		Collagen as inducer		
5	$S^+ P^+$	0.98±0.03	2.40±0.05	8.65±0.05
6	$S^+ P^-$	0.82±0.05	2.10±0.05	8.76±0.06
7	$S^- P^+$	0.63±0.03	2.28±0.04	8.67±0.06
8	$S^- P^-$	0.15±0.04	1.80±0.03	8.68±0.05

'S'-seed medium
'P'-production medium
'+' and '-' indicates medium supplemented with or without inducer

(1.24±0.05 U/mL) was achieved when only seed medium was supplemented with gelatin whereas, in case of collagen as inducer, maximum collagenase production (0.98±0.03 U/mL) was attained when both seed and production medium were supplemented with collagen. Limited activity was seen when no inducer was present. This suggested that collagenase is an inducible enzyme and essentially requires gelatin/collagen in the medium. To attain cost effectiveness, gelatin was used an inducer in consecutive experiments. Collagen acts as most suitable inducer whereas gelatin was also found to be equally good for collagenase production from *Bacillus tequilensis* [21].

Effect of various physiological factors on collagenase production from *C. contaminans*

Effect of medium pH

Effect of pH on the production of collagenase was studied by varying initial medium pH at range 5.0 to 9.0. Trends in the production of extracellular collagenase at different initial pH of the medium have been exhibited in Fig. 4. The isolate *C. contaminans* grew well at acidic to neutral pH and its growth was restricted at alkaline pH. As far as, the production of collagenase is concerned, it was best yielded at a narrow range of pH (6.0-7.5) with optimum activity at pH 6.5 (1.67±0.03 U/mL). An initial medium pH lower than 6.0 and higher than 8.0 drastically inhibited the enzyme production. This could be the result of distortion or alternation of the 3D structure of enzyme at extreme pH [21]. Final pH of the culture broth was found to be increased. Usually, maximum collagenase activity from different bacterial isolates were obtained at neutral pH i.e., 7.0 -7.5. *B. cereus* MBL13 showed maximum activity within pH range of 7.0-7.2 [25]. Optimal collagenase production from *Streptomyces exfoliatus* CFS 1068 [17], *B. licheniformis* F11 [10] and *C. albicans* URM3622 [23] was reported at pH 7.0. *Bacillus megaterium* KM 369985 showed maximum activity pH 7.5 [20]. Similarly, Kaur and Azmi[21] and Nagano and To [27] reported maximum collagenase activity from *B. tequilensis* and *B. subtilus* at pH 7.5, respectively. Although extremely acidic and highly alkaline pH was deleterious for collagenase from *C. contaminans* but collagenase production from *Rhizoctonia solani* was facilitated at pH 5.5 [13], while collagenase production from *Bacillus* sp. DPUA 1728 was favored at pH 9.0 [24].

Effect of incubation temperature

To study the effect of incubation temperature on collagenase production, the isolate *C.*

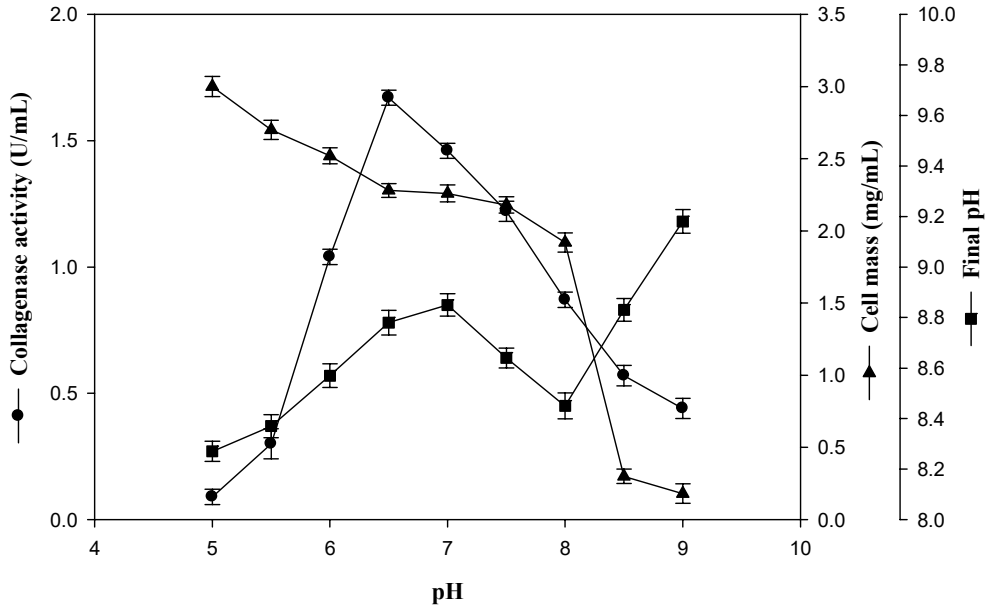

Fig. 4. Effect of medium pH on production of collagenase from *C. contaminans*

contaminans was grown on selected medium at optimized pH 6.5 and incubated at different temperatures ranging from 25 to 50°C for 24 h at 150 rpm. Bacterial growth and collagenase production was observed at a narrow range of 25°C-37°C with maximum enzymatic activity recorded at 30°C (1.80±0.06 U/mL) and maximum growth (2.22±0.05 mg/mL) at 35°C (Fig. 5). A drastic decrease in enzyme production was observed when the temperature was raised beyond 40°C. This vigorous reduction may be accredited to the denaturation of the enzyme at upraised temperatures. These results were in agreement with the findings of Hamdy [13] and Jain and Jain [17] where maximum collagenase production from *R. solani* and *S. exfoliatus* CFS 1068 was attained at 30°C, respectively. In various studies on collagenase from different *Bacillus* sp., the optimum temperature has been reported to be 37°C [20, 21, 24, 25]. Unlikely, *A. sendaiensis* [38] and *Geobacillus*

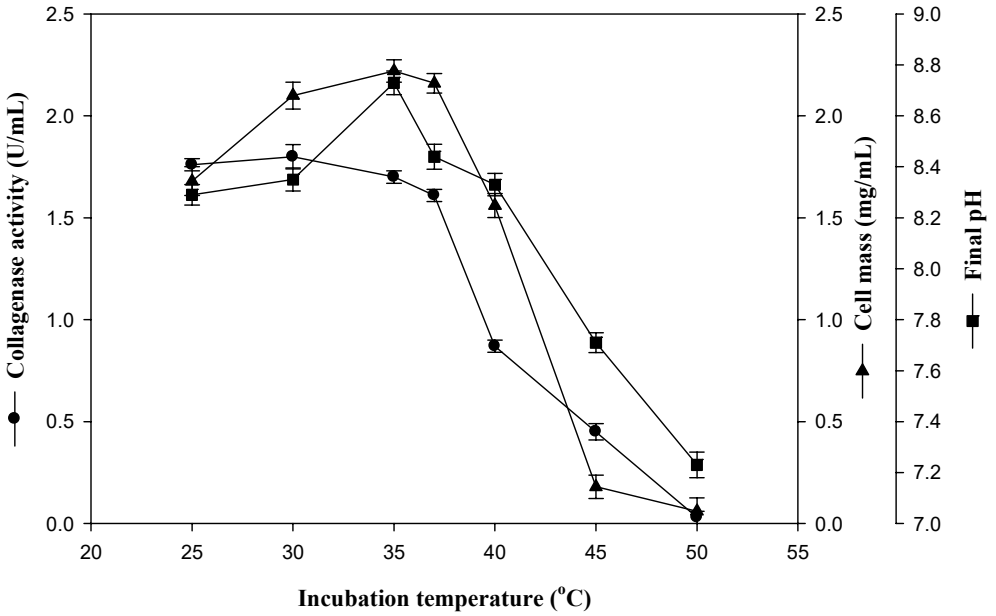

Fig. 5. Effect of incubation temperature on production of collagenase from *C. contaminans*

collagenovorans MO-1 [30] were reported to produce collagenase at temperatures 55°C and 50°C-70°C, respectively.

Effect of agitation rate

Agitation is an important parameter for microbial growth under submerged conditions. It maintains homogeneity and enhances the rate of oxygen transfer from bulk gas to the medium, which is basically utilized by the microorganism [5]. Agitation also results in proper mixing of nutrients. In present study, optimum agitation speed was determined by growing *C. contaminans* for production of collagenase in a temperature controlled orbital shaker at varying rotational speeds *viz*, static, 100, 150, 200, 250. Maximum collagenase production (1.82±0.03 U/mL) was observed at agitation rate of 150 rpm depicted in Fig. 6. Further increase of agitation led to a slight decrease in enzyme production. This might be the result of shearing force operative at elevated agitation. Negligible activity was observed at static condition. This result is in accordance with Jain and Jain [17] obtaining maximum collagenase production from *S. exfoliatus* CFS 1068 at 150 rpm. In different studies on collagenase production, optimum agitation rate attained by *C. albicans* URM3622 [23], *R. solani* [13], *B. cereus* MBL13 [25] and *B. tequilensis* [21] were 160 rpm, 175 rpm, 180 rpm and 200 rpm, respectively.

Time course analysis of collagenase production from *C. contaminans*

A time dependent study was done to determine the correlation between growth and collagenase activity of culture *C. contaminans* (Fig. 7). The production medium (pH 6.5) containing (%, w/v) Sucrose 1.0; Peptone 1.0; Yeast extract 0.2; Na_2HPO_4 0.20; Na_2CO_3 0.25 and $MgSO_4.7H_2O$ 0.04 was inoculated with 2 % (v/v) of 15 h old inoculum (seed containing 0.3 % gelatin) and incubated at 30°C and 150 rpm agitation. Collagenase production was detected in the logarithmic phase and after a 24 h of incubation, it reaches its optimum condition (2.00±0.05 U/mL). During the logarithmic phase of growth, the cell density was also high. However, with an increase in the incubation time beyond 24 h, a slight decrease in the collagenase activity was observed. This might be due to the fact that enzyme gets promptly inactivated with elongated incubation time. Similarly, optimum production time for collagenase from *B. subtilus* ATCC 6633 [34] and *B. tequilensis* [21] was also observed after 24 h of incubation. However, *B. megaterium* KM 369985 has shown maximum collagenase production after 12 h of incubation [20] and *B. cereus* MBL13 at 26 h [25]. Moreover, few strains exhibited a prolonged incubation time such as, *R. solani* showed optimum collagenase production after 108 h of submerged fermentation [13] and *S. exfoliatus* CFS 1068 produced maximum

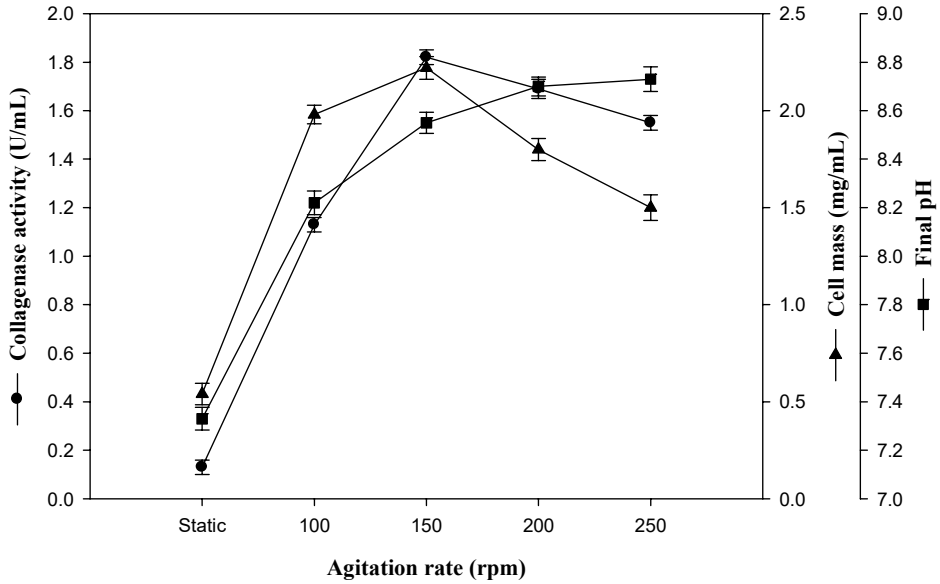

Fig. 6. Effect of agitation rate on production of collagenase from *C. contaminans*

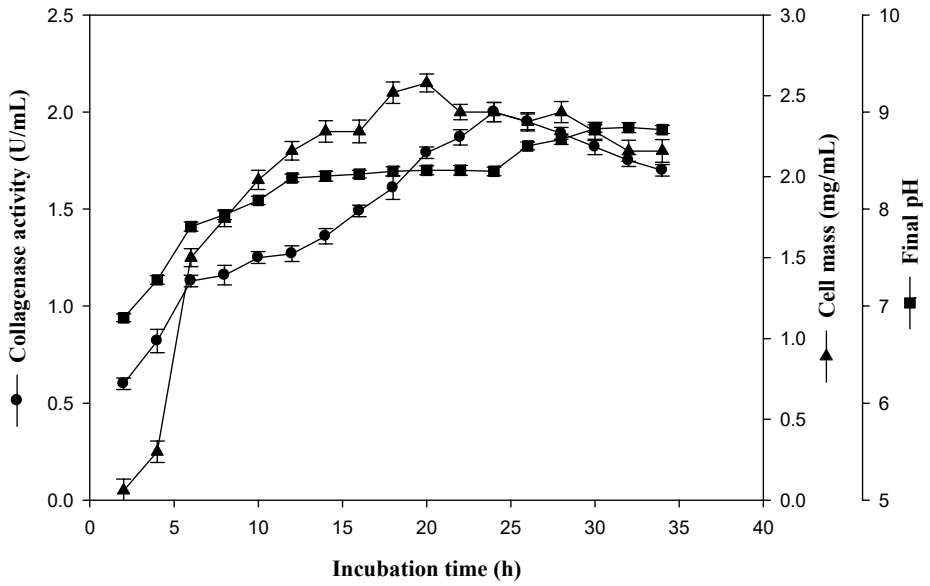

Fig. 7. Course of fermentation of *C. contaminans*

collagenase after 5 days of incubation [17].

Conclusion

Collagenases contribute to the pathogenicity of medically important bacteria. Primarily, collagenases have been obtained from pathogenic microorganisms that restrict the use of microbial collagenase in therapeutics and food industry. From present study, it is suggested that the novel isolate *Chryseobacterium contaminans* KU665299 could be used as alternative non-pathogenic source for production of collagenase. A 2.3-fold increase in enzymatic activity was attained after optimization of the process parameters for collagenase production from *C. contaminans*. This *C. contaminans* can be a potential source for commercial collagenase production with feasible applications in field of pharmaceutics, cosmetics and food industy.

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Conflict Of Interest

The authors confirm that this article content has no conflicts of interest.

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