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Optimization of physiochemical requirements on collagenase production from *Aspergillus* fungi

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Abstract
Collagenolytic enzymes are one of the most important industrial enzymes which have a wide variety of applications in medical, pharmaceuticals, food, cosmetics, textiles industries etc. The industrial processes of enzymes production are performed under specific conditions, required for the activity and stability of the enzyme. The parameters such as temperature, substrate, pH, cincubation time and inoculum percentage strongly affect many enzymatic processes. In this study, the optimization of physiochemical parameters such as pH and temperature for the collagenase production was carried out in *Aspergillus awamori 16* and *Aspergillus awamori 22* mixed cultures. The physiochemical parameters were found to be optimal in pH 6.0 and temperature 35ºC.

Keywords: collagenase, *Aspergillus* fungi, optimization, temperature, pH

1. Introduction
Proteases represent an important group of enzymes produced industrially and account for 60% of total worldwide sale of enzymes [1, 2]. Collagenases are the proteolytic enzymes responsible for degradation of native collagen to small peptide without affecting the other proteins [3-6]. The collagenase is responsible for the degradation of native collagen to small peptide fragments, without affecting the other proteins. Some proteolytic enzymes like collagenases have a number of industrial applications in fur, leather, meet processing, etc. industries. The most extensive works have been reported on the bacterial collagenases because of their broad substrate specificities and abilities to degrade both native and denatured collagens (genera of *Clostridium*, *Pseudomonas*, *Vibrio* and *Streptomyces*). However in the recent period micromycetes got wide application because of high productivity. Given the high demand of collagenolytic enzyme, there is an interest in finding new microbial strains able to produce collagenases with novel properties, and in developing low-cost industrial media formulations. Extracellular collagenase production in microorganisms is strongly influenced by media components, e.g. carbon and nitrogen sources, C/N ratio etc. Besides, several other physical factors, such as aeration, pH, temperature, incubation time, and dissolved oxygen also affect collagenase biosynthesis [7]. pH influences many enzymatic processes, such as enzyme production, cell transport across membranes and extracellular proteases expression [8]. Temperature can regulate some components as enzymatic synthesis, enzyme secretion and length of the enzyme's synthesis phase [9, 10]. The aim of this work was to study the influence of pH and temperature on collagenase production by fungal association of *Aspergillus awamori 16* and *Aspergillus awamori 22.*

2. Materials and Methods
Growth media
Fungal association of *Aspergillus awamori 16* and *Aspergillus awamori 22* was maintained on potato dextrose agar medium: potato infusion – 200g; dextrose – 20g; agar – 20g per liter. The nutrient broth used was KH_2PO_4 – 0,1; $MgSO_4$ – 0,05; KCL – 0,05; $FeSO_4$ – 0,001; peptone -1,0%; sucrose – 2,0%.
For inoculum preparation, 25 ml of sterile distilled water was added to the 5-day-old culture and scraped aseptically with inoculating loop. This suspension containing $1.3 \cdot 10^7$ of fungal cells in 1 ml solution served as inoculum.

Effect of pH
pH range from 3.0 to 10.0 was used to study the effect of pH on collagenase activity. The particular pH was adjusted by using 0.1N HCl and 0.1N NaOH at 30ºC. 250 ml Erlenmeyer flasks containing 50 ml of culture medium containing fungal inoculum were incubated for 72h on rotary shaker (180 rpm).

Effect of Temperature
The optimum temperature was determined by incubating the culture medium containing fungal inoculum for 72 h at different temperatures from 20 to 50ºC (at regular intervals of 10ºC) on rotary shaker (180 rpm).

Collagenase assay
Collagenase activity was assayed by ninhydrin-based method [11]. 20 mg collagen from bovine tendon (Sigma) was suspended in 3.8 ml Tris buffer (0.02 M Tris, 0.005 M $CaCl_2$, pH 7.4) and added to 200 µl collagenase solution (1 mg/ml in Tris buffer) to make a total volume of 4.0 ml. The mixture was incubated at 40°C for 3 h or 70°C for 30 min. The mixture was centrifuged in a microfuge for 10 min at 14,000

rpm. 1.5 ml of supernatant was mixed with 4.5 ml of 5N HCl and kept in a drying oven at 110°C for 16h for complete hydrolysis of soluble peptides. The hydrolysate was diluted 25 times with distilled water. To 1.00 ml of diluted hydrolysate 1.00 ml of chloramine-T solution was added and the mixture was allowed to stand at room temperature for 20 min. After that 1.00 ml of color reagent was added and the reaction mixture was transferred to a 60°C water bath and incubated for 15 min. Tubes were removed and allowed to cool down to room temperature. Absorbance at 600 nm was measured

3. Results and discussion

The process of extracellular enzymes biosynthesis can be affected by environmental factors, such as pH and temperature. The pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The pH is known to affect the secretion of collagenolytic enzyme and its stability.

Optimization of pH for collagenase production

The collagenase activity gradually increased when increasing the pH up to 6.0 (Fig. 1). It was also noted that the collagenase activity was stable at pH range of 6.0 – 9.0. The enzyme was active at alkaline pH and activity decreased at the acidic range. Any change in pH affects the protein structure and a decrease in enzyme activity beyond the optimum pH could be due to the enzyme inactivation or instability. It could be concluded from the results that collagenase from the association of *A. awamori 16* and *A. awamori 22* needed an alkaline protein environment to be active.

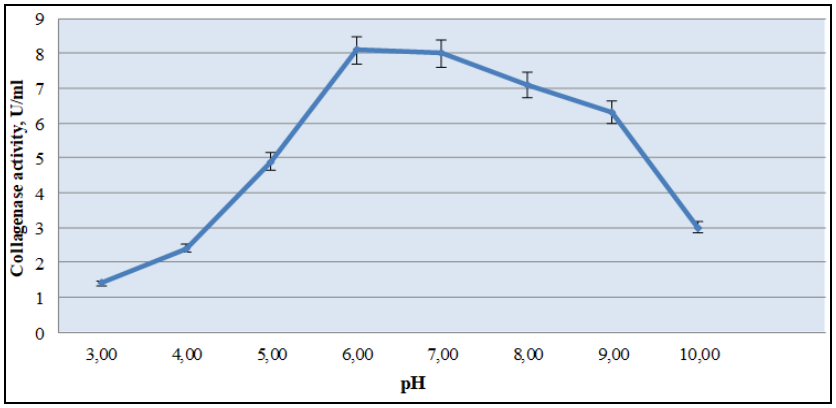

Fig 1: Effect of pH on collagenase production in *A. awamori 16* and *A. awamori 22* mixed cultures

Optimization of Temperature for Collagenase production

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms. The effect of temperature on enzyme activity was assayed at different temperatures ranging from 20 to 50°C at pH 6.0. The results of the test made at different temperatures value showed that the optimal temperature for collagenase production by fungal association of *A. awamori 16* and *A. awamori 22* was between 30˚C and 40˚C (Fig. 2).

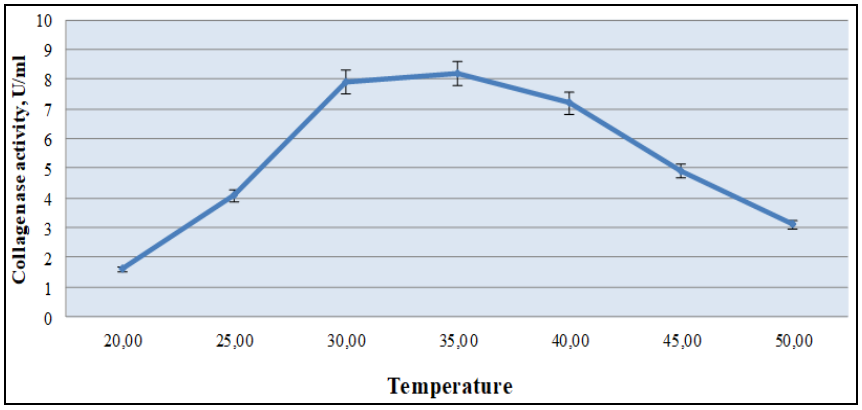

Fig 2: Effect of temperature on collagenase production in *A. awamori 16* and *A. awamori 22* mixed cultures

It was observed that at 20°C enzyme activities were low and showed a gradual increase with the increase in temperature to 35°C. Further increase in temperature resulted in decrease in collagenase production. Thus, optimum temperature for collagenase production by *A. awamori 16* and *A. awamori 22* mixed culture is 35˚C.

4. Conclusion

The optimum pH and temperature for collagenase activity in *A. awamori 16* and *A. awamori 22* mixed cultures were determined as 6.0 and 35°C, respectively. The optimal fermentation medium for the production of collagenase by *A. awamori 16* и *A. awamori 22* mixed culture was as a follow:

sucrose – 1,0%; KH_2PO_4 – 0,1%; $MgSO_4$ – 0,05%; KCL – 0,05%; $FeSO_4$ – 0,001%; peptone – 1,0%, pH 6.0 at 35°C in submerged cultivation. This enzyme is also stable over a broad pH range, 6 to 9, for a period at 35°C. Thus, collagenase from the association of *A. awamori 16* and *A. awamori 22* needed an alkaline protein environment to be active.

5. Acknowledgements
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