Production and Characterization of a Collagenolytic Serine Proteinase by *Penicillium aurantiogriseum* URM 4622: A Factorial Study

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Abstract A $2^4$ full factorial design was used to identify the main effects and interactions of the initial medium pH, soybean flour concentration, temperature and orbital agitation speed on extracellular collagenase production by *Penicillium aurantiogriseum* URM4622. The most significant variables for collagenase production were soybean flour concentration and initial medium pH that had positive main effects, and temperature that had a negative one. Protein concentration in soybean flour revealed to be a significant factor for the production of a collagenase serine proteinase. The most favorable production conditions were found to be 0.75% soybean flour, pH 8.0, 200 rpm, and 28ºC, which led to a collagenase activity of 164 U. The enzyme showed an optimum activity at 37ºC and pH 9.0, was stable over wide ranges of pH and temperature (6.0 – 10.0 and 25 – 45ºC, respectively) and was strongly inhibited by 10 mM phenylmethylsulphonyl fluoride. The first-order rate constants for collagenase inactivation in the crude extract, calculated from semi-log plots of the residual activity versus time, were used in Arrhenius and Eyring plots to estimate the main thermodynamic parameters of thermal inactivation ($E_a^* = 107.4$ kJ/mol and $\Delta H^*_d = 104.7$ kJ/mol). The enzyme is probably an extracellular neutral serine collagenase effective on azocoll, gelatin and collagen decomposition.

Keywords: collagenase, enzyme production, *Penicillium aurantiogriseum*, submerged culture, factorial design

1. Introduction

Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism, and active site [1]. These enzymes, which are able to hydrolyze the peptide bond of proteins, represent one of the largest groups of industrial enzymes, with increasing market demand due to their usefulness in various industrial sectors and in basic research [2].

Collagenolytic proteases, which degrade the native triple helix of collagen, are involved in various physiological and pathological situations, such as fetal bone development, embryonic development, wound repair, rheumatoid arthritis, malignant tumor invasion, intestinal ulceration and chronic periodontal inflammation [3]. These proteases are classified into two major groups: Metallocollagenases and serine collagenases. Hydrolysis by metalloproteases takes place mostly between the peptide bond of residue X and Gly-Pro. Metallocollagenases, first discovered in tissue explants of tadpole, are zinc-containing enzymes, but usually also require calcium for their optimum activity and stability. They are involved in remodeling the extracellular matrix. On the other hand, serine collagenases were first isolated...
from the hepatopancreas of the fiddler crab (*Uca pugilator*) and are probably involved in food digestion rather than morphogenesis, along with in the production of hormones and pharmacologically active peptides, besides various cellular functions, such as protein digestion, blood-clotting, fibrinolysis and fertilization [4].

The collagenolytic activity of these enzymes has industrial, biotechnological, medicinal and commercial applications. Collagenase hydrolyzes bovine trachea cartilage, allowing for the preparation of intact mammalian cells in culture and cleaning blood cells for improved screening in medical diagnostics [5]. Potential therapeutic applications include wound healing [6] and predilutional therapeutic studies on various types of destructive fibrosis, such as liver cirrhosis [7].

Collagens are the major protein constituents of extracellular matrix and the most abundant proteins in all higher organisms. The triple helix, tightly coiled, of collagen molecule assembles into water-insoluble fibers or sheets, which are cleaved only by collagenases, being resistant to other proteinases [8]. Collagen peptides produced by collagenolytic enzymes have been used in the chemical, medical, cosmetic and food industries, as well as in experimental applications of molecular biology [9]. They can be used as seasonings, non-allergic preservatives for drugs, as ingredients for dietary materials and parentally-fed products, and for the treatment of diseases such as collagen-induced arthritis [10].

Collagenolytic proteases are ubiquitously found in plants [5], animals [11] and microorganisms [12]. However, the microorganisms are the preferred sources of these proteases because of their broad biochemical diversity and their susceptibility to genetic manipulation. Among the microbes, fungi have a distinct advantage as enzyme producers, because their hydrolytic enzymes are released extracellularly, which makes their recovery from the fermented broth particularly easy [2]. Reports are available on collagenase biosynthesis by fungi belonging to the genera *Aspergillus*, *Cladosporium*, *Alternaria* and *Penicillium* [13].

It is well known that extracellular protease production in microorganisms is greatly influenced by medium components, especially carbon and nitrogen sources, physical factors such as pH, temperature, inoculum size, orbital agitation speed and incubation time [14]. However, no single medium has been established for optimal production of protease from different microbial sources, because each organism requires different conditions for maximum production [15].

Usually 30 – 40% of industrial production cost of enzymes is related with the cost of the growth medium, making the search for cost-effective media particularly relevant [16]. Soybean flour has been recognized as a potentially useful and cost-effective ingredient, because it is a by-product from oil extraction. It consists of approximately 40% proteins and is rich in other organic and inorganic compounds, thus being a good candidate for a culture medium [17].

Factorial statistical designs are useful to study enzyme production conditions. When conventional “one-factor-at-a-time” designs are used, they usually require a large number of experimental runs. Worse still, they are likely to miss possible interaction effects between production parameters [18].

Environmental conditions can play an important role in the induction or repression of extracellular proteases [19]. The aims of this work were to study by a statistical approach the influence of soybean flour concentration, pH, temperature and agitation speed on collagenase production by *P. aurantiogriseum* URM4622 and to characterize the enzyme obtained under the most favorable conditions.

### 2. Material and Methods

#### 2.1. Microorganism and culture medium

The *P. aurantiogriseum* dierchx (URM4622) strain was obtained from the Culture Collection of the Department of Mycology of the Federal University of Pernambuco (Micoteca). The fungi was isolated from the microscopy-room air of “Micoteca” and at the moment qualitatively characterized, on a solid medium, only for its ability to produce protease (data not published). The full characterization of this strain is currently under way.

The strain was maintained at 4°C in a malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose, and 1.5% (w/v) agar.

The soybean flour medium described by Porto et al. [20], composed of 0.5% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH₄Cl, 0.06% (w/v) MgSO₄·7H₂O, 0.435% (w/v) K₂HPO₄, 0.01% (w/v) glucose, and 1.0% (w/v) mineral solution, was used for collagenase production. The mineral solution was prepared adding, per 100 mL of distilled water, 100 mg FeSO₄·7H₂O, 100 mg MnCl₂·4H₂O, 100 mg ZnSO₄·H₂O, and 100 mg CaCl₂·H₂O. This fermentation medium was sterilized in an autoclave at 121°C for 20 min.

#### 2.2. Screening of significant variables for collagenase production

The 2⁴ full design mentioned above was carried out at all combinations of the levels given in Table 1. The central point was run in quadruplicate, to provide an estimate of the pure error variance in the experimental responses. From that, experimental errors of the effects were estimated [18] and used to assess the significance of the effects and interactions of the independent variables – initial medium