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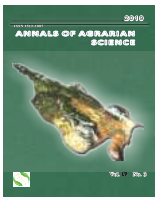

Optimizing of nutrient media for Penicillium candidum 5-1 to increase the biosynthesis of casein-specific protease

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A B S T R A C T

This study aimed to optimize the nutrient media of the protease-producing strain *Penicillium candidum 5-1,* the strain was isolated from spoiled dairy products. The nutrient medium closest to the natural substrate was selected. Accordingly, the percentage composition of lactose, casein, and potassium dihydrogen phosphate was determined. Optimal conditions for deep cultivation, including temperature, pH, and cultivation time, were established. The results showed that by optimizing the components of the nutrient media and determining the optimal conditions, the protease activity of the strain increased approximately by 50% (0.37 U/MG).

The effect of the enzyme preparation on milk showed that the protease preparation curdles the milk instead of coagulating.

This fact indicates the presence of a different type of protease, which is synthesized in nutrient media close to natural conditions, this enzyme may be a peripheral, auxiliary (minor) component, and requires further research.

Key words: protease, microscopic fungi, extracellular protease, natural conditions, nutrient media.

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INTRODUCTION

Proteases are a very large and complex group of enzymes that are widely used in various industries. Proteases make up about 60% of the enzymes sold in the world [1, 2]. They differ in properties such as substrate specificity, active site and catalytic mechanism, temperature and pH optima. It should be noted that microorganisms of different taxonomic groups have the ability to synthesize proteases. Various species of microscopic fungi are known from which milk coagulating proteases are secreted (*Talaromyces leycettanus, Rhizomucor miehei, R. pusillus, Endothia parasitica, Mucor circinelloides, Aspergillus oryzae, Amylomyces rouxii, Rhizopus microsporus, Mucor mucedo, Mucor pusillu, Mucor racemosus, and Iprex lactis*) [3-11].

Microscopic fungi have the ability to produce extracellular proteolytic enzymes. By establishing optimum cultivation conditions of microscopic fungi, it is possible to obtain proteolytic enzymes preparation with high protease activity.

MATERIALS AND METHODS

The vitality and potential of a microorganism to produce enzymes intensively much depends upon the selection of the appropriate nutrient media, in particular, carbon, nitrogen, phosphorus sources; For in-depth cultivation of active protease-producing strain *Penicillium candidum-5-1*, the following nutrients were used: lactose - 2%, casein - 2%, KH2PO4 -0.2 %, MgSO4 - 0.1%; Deep cultivation of individual strains was carried out in 250 ml Erlenmeyer conical

flasks on temperature-controlled rotary shaker (180-200 rpm), at 30°C for 72-96 hours. 10-day conidia culture suspension served as the cultivation material.

To determine the optimal concentrations of the selected carbon, nitrogen, and phosphorus sources, various percentage compositions of each source were used. The nitrogen source, casein, was tested at the following concentrations: 0.5%, 1%, 1.5%, 2%, and 2.5%. The carbon source, lactose, was taken at the following concentrations: 1%, 2%, 3%, 4%, and 5%. The phosphorus source, Potassium dihydrogen phosphate, was used at the following concentrations: 0.05%, 0.1%, 0.5%, and 1%.

Protease activity was determined in the culture fluid by Sigma's method [12].

Temperature is the most important factor for detecting the growth and physiological activity of microorganisms, first of all, the influence of temperature on the activity of the enzyme produced by the selected strain was studied. *Penicillium candidum-5-1* was cultivated in the temperature range of 25-40^0 C, with a temperature interval of 5^{0}C.

Also, the physiological activity of microorganisms is greatly influenced by nutrient media pH. The strain *Penicillium candidum-5-1* was cultivated at pH in the range from 5.0 to 9.0 with an interval value of 0.5 in the starting nutrient medium.

To study the duration of deep cultivation, the strain *Penicillium candidum-5-1* was grown in a selected liquid medium (pH 6.5) at 30^{0}C (taking into account the optimal conditions of the strain). In order to reveal the dynamics of protease accumulation in a nutrient medium, the producer of this enzyme was grown for 8 days, and the activity of this enzyme was determined every second day.

To obtain the technical preparation, cooled alcohol (-15°C) was slowly added to the supernatant at a ratio of 1:4. The solution was kept in the refrigerator at 2°C for 4 hours. Centrifugation was performed at 6000 rpm for 20 minutes to obtain the precipitate. The resulting enzyme preparation was then dried using a freez dryer, and the enzyme activity was measured.

For the milk coagulation activity assay (MCA), 0.5 mL of enzyme solution was added to 5 mL of skim milk in a 12 mL test tube pre-incubated at 35 °C for 10 min. The skim milk solution consisted of 10 g dry skim milk/100 mL of 0.01 M CaCl2. The clotting time was quantified in Soxhlet units (SU) according to the following formula: SU=(2400∗5∗D)/(T∗0.5) where T is the clotting time (s) and D is the crude enzyme dilution. One SU is expressed as the amount of enzyme required to coagulate 1 mL of a solution containing 0.1 g skim milk powder and 0.01 M calcium chloride at 35 °C for 40 min[13].

RESULTS AND DISCUSSION

The optimization of liquid nutrient media for *Penicillium candidum-5-1* was an important stage. In addition to the type of carbon source, its concentration was also found to be important for increasing proteolytic activity. The highest enzyme activity was observed when the nutrient medium contained 4% lactose. (Fig. 1).

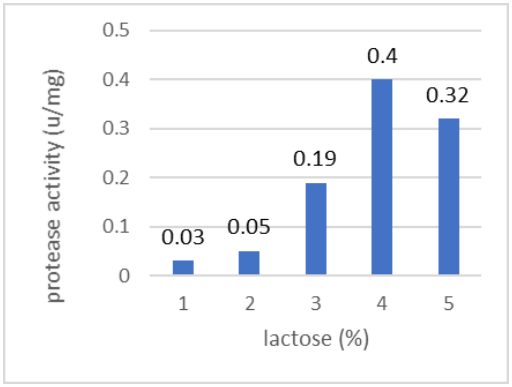

Fig.1. *Effect of different concentration lactose on protease activity Strain-Penicillium candidum- 5-1*

When different percentages of casein were added to the nutrient medium, the highest proteolytic activity was achieved at a concentration of 1.5% casein (Fig. 2).

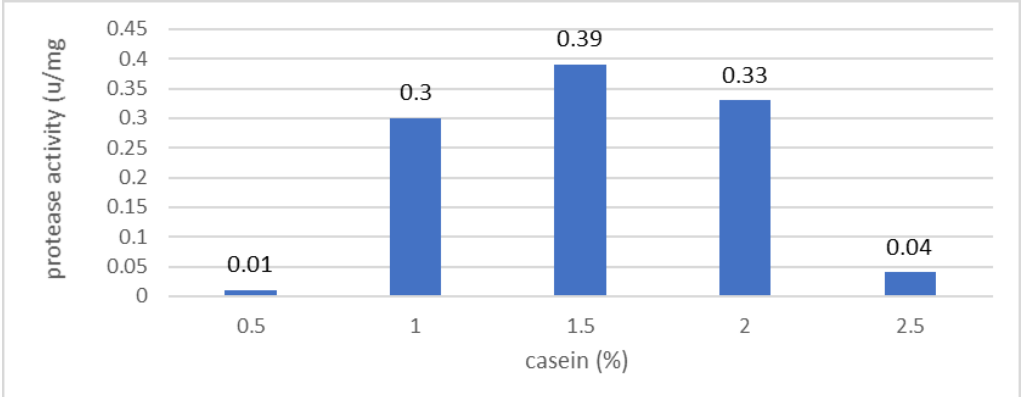

Fig. 2. Effect of different concentration casein on protease activity Strain-*Penicillium candidum*-5-1

For Potassium dihydrogen phosphate, the highest enzyme activity was observed at a concentration of 0.1% in the nutrient medium (Fig. 3).

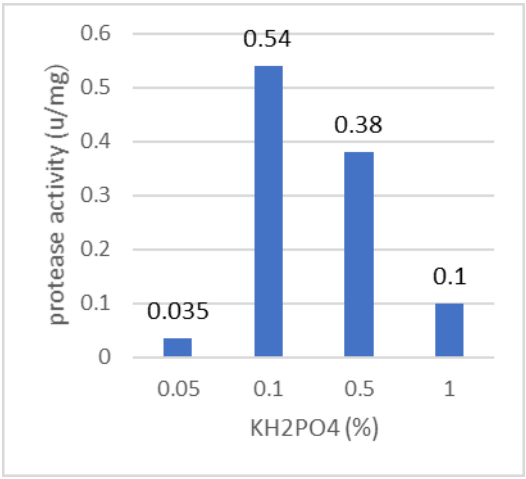

Fig. 3. Effect of different concentration KH-2PO4 on protease activity Strain-*Penicillium candidum*-5-1

The optimal temperature for protease production by *Penicillium candidum 5-1* was determined to be 30°C, at which the maximum amount of protease was produced (Fig. 4).

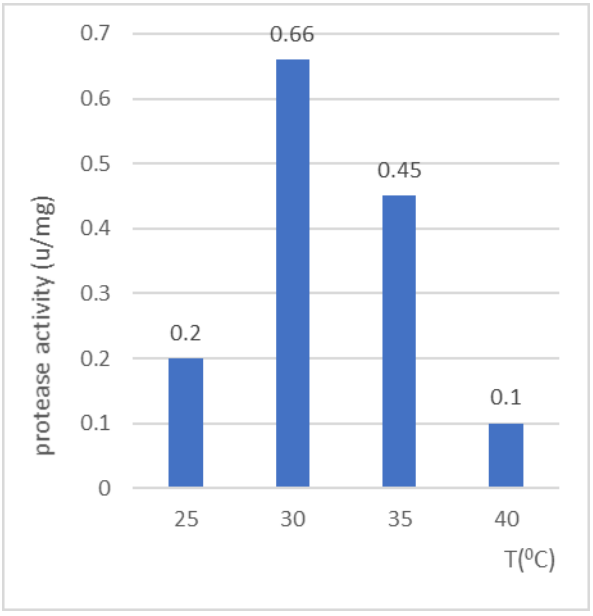

Fig. 4 Influence of cultivation temperature on protease biosynthesis Strain-*Penicillium candidum*- 5-1

The optimum pH for the cultivation of *Penicillium candidum 5-1* was found to be 6.5 (Fig. 5).

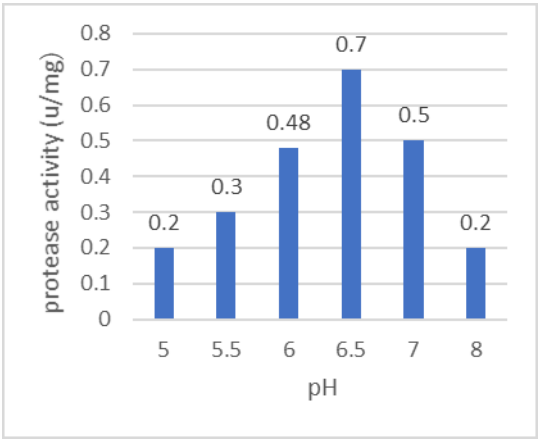

Fig. 5 Effect of medium pH on the formation of protease Strain-*Penicillium candidum*- 5-1

According to the obtained results, the production of extracellular protease started 24 hours after *Penicillium candidum-5-1* strain cultivation, and the highest activity was reached on the 6th day (144 hours). After that, a decrease in proteolytic activity was observed (Fig. 6).

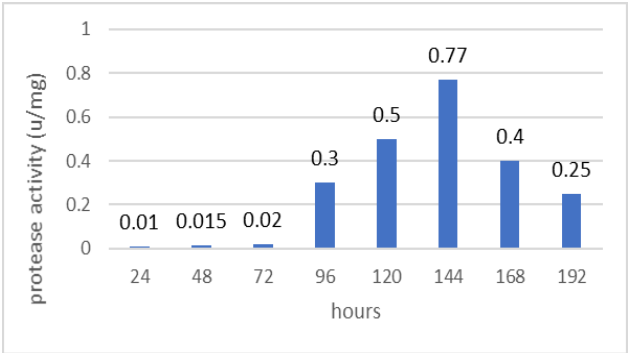

Fig. 6 *Effect of cultivation duration on the action of protease Strain-Penicillium candidum- 5-1*

As a result of the optimization of the nutrient media, the activity of protease increased by 0.37 U/MG and 50%, respectively.

Analysis of milk coagulation activity (MCA) was performed on the commercial enzyme - chymosin as a control and on the enzyme preparation obtained from *Penicillium candidum 5-1*. As a result, it was determined that the obtained enzyme preparation has a different effect from chymosin, it can curdle milk instead of coagulate (Table 1).

Table 1. *Milk-clotting activity of different concentrations of enzymes.*

Enzymes *U/MG*	Dilution factor protease	Clotting time (s)	*SU*
182	1	300	80
90,9	2	1200	40
45,4	4	4800	20
23	8	-	-
11	16	-	-
470	Commercial enzyme (chymosin) 1	300	80

This fact indicates the presence of a different type of protease, which is synthesized in nutrient media close to natural conditions, this enzyme may be a peripheral (minor) component, which is characterized by a different mechanism of action on casein and requires research.

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