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Biotechnology

Screening of Protease-Producing Microscopic Fungi Growing on Nutrient Media Similar to Natural Substrates

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(Presented by Academy Member Tinatin Sadunishvili)

Microscopic fungal cultures were isolated from spoiled milk products obtained out of the following products: milk, sour cream, cottage cheese, and yoghurt, in various soil-climatic regions of Georgia. Among the screened fungal cultures, three protease-producing strains were identified: *Mucor spp. 2-3, Penicillium candidum 5-1* and *Penicillium camemberti 7-5*. As a result of research, the nutrient medium similar to the natural substrate was selected – with the content of lactose, casein and potassium phosphate. Among the cultures, *Penicillium candidum 5-1* exhibited the highest protease production under submerged cultivation conditions. *© 2023 Bull. Georg. Natl. Acad. Sci.*

protease, microscopic fungi, extracellular, skim milk, nutrient media

Proteases are the main milk coagulation agents obtained from various plant, animal and microbial sources. The increased demand for enzymes in the cheese industry, religious and ethnic regulations against the use of enzymes of animal origin, have increased the interest in microbial-derived milk coagulation enzymes [1, 2]. In recent years, the prospect of using enzymes as industrial catalysts has been growing phenomenally. 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 [3]. 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 enzymes are secreted [4-6]. Microscopic fungi, unlike bacteria, have the ability to produce extracellular enzymes. That is why the use of fungal protease for industrial purposes is much more profitable, because in this case there is no need to break down the cell wall of microorganisms [7].

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Materials and Methods

Cultures of microscopic fungi were isolated from various spoiled milk products. A total of nine samples were collected and processed under sterile conditions within a laminar box. Fungi were isolated through direct inoculation on Sabouraud Dextrose Agar (SDA), a medium containing: 0.5% peptone, 4% casein, 4% glucose, and 1.5% agar. The samples were incubated at temperatures ranging from 25 to 30°C for a duration of one week to one month. Visible fungal growth was observed starting from the third day of incubation.

To obtain pure cultures, a part of the mycelium from each grown colony was transferred to nutrient media. Pure cultures were obtained and subsequently transferred to microbiological test tubes for further analysis.

For primary screening of protease-producing fungi, skimmed milk agar was used. 25 g of skimmed milk was dissolved in 250 ml of distilled water. The mixture was stirred thoroughly and regularly autoclaved. The pH of the medium adjusted by using the pH meter maintained at pH 6, the suspension of 2.5% agar used for solidification were autoclaved at 121°C for 15 min. For plating, skim milk and agar solutions were held in a water bath at 50°C and then the skim milk was poured into the agar flask and mixed thoroughly. To inhibit bacterial growth, 1 mg of ampicillin per 1000 ml of media was added. The skimmed milk agar was poured into plates after being held in a water bath at 50°C and mixed thoroughly. The plates were kept at 4°C until further use [8, 9]. Pure cultures of microscopic fungi were inoculated onto the surface of skim milk agar plates using sterile techniques. The inoculated plates were incubated at 27°C for four days. During incubation, the protease produced by the fungi degraded the skim milk protein, resulting formation of a clear zone around the fungal colonies. The appearance of a clear zone indicated protease activity.

The diameter of the clear zone surrounding each fungal colony was measured using a ruler. The measurements were recorded in millimeters (mm). The enzyme index (EI) was calculated as the ratio of the diameter of the degradation zone (R) to the diameter of the fungal colony (r), expressed as R/r. For further identification, fungal species were selected, which showed the diameter of the clear zone and, consequently, high protease activity.

The isolated fungal cultures were subjected to cultural-morphological and microscopic analyses. Macromorphological properties, such as colony appearance, color and growth rate, were examined. Microscopic examination involved the observation of fungal structures using various microscopic techniques. Species identification of the microscopic fungi was performed based on the study of their cultural-morphological and physiological properties, along with the utilization of different identification keys and taxonomic resources [10, 11].

The vitality and potential of a microorganism to produce enzymes intensively much depends upon the selection of the appropriate nutrient medium, in particular, carbon, nitrogen, phosphorus sources; For in-depth cultivation of active protease-producing strains, the following nutrients were used: glucose – 2%, casein – 2%, $MgSO_4$ – 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.

As carbon sources were used – Fructose, glucose and lactose at the amount of 2%. As mineral sources of nitrogen were tested Yeast extract, urea, and casein at the amount of 2%. As phosphorus sources were taken Dipotassium hydrogen phosphate, Potassium dihydrogen phosphate, and Disodium hydrogen phosphate at the amount of 0.2%.

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

Results and Discussion

In this research, a total of 17 cultures were isolated from 9 samples of spoiled milk products. These cultures were assigned serial numbers for identification purposes (Table 1).

Table 1. Cultures of microscopic fungi

	Isolated cultures	Samples
1	spp.1-1	**1**
2	spp.1-2	
3	**spp. 2-3**	**2**
4	spp. 3-3	3
5	spp. 3-4	
6	spp. 4-1	4
7	spp. 4-4	
8	**spp. 5 -1**	5
9	spp. 5-6	
10	spp. 6-3	6
11	spp. 7-1	7
12	spp. 7-3	
13	**spp. 7-5**	
14	spp.8-2	8
15	spp. 8-3	
16	spp. 9-1	9
17	spp. 9- 2	

Based on the skimmed milk agar experiment, 17 cultures of microscopic fungi were tested, of which 3 strains produced protease enzymes. The strain spp.5-1 exhibited the highest rate of protease activity (Table 2). Protease production increased from 48 hours to 96 hours, reaching its maximum level at 96 hours (Fig. 1).

The enzyme protease producer was identified - all three strains, isolated cultures belong to the genera of *Penicillium* and *Mucor.* In particular, the three selected strains are *Penicillium camemberti 7-5* (Fig. 2), *Mucor spp. 2-3* (Fig. 3) and *Penicillium candidum 5-1* (Fig. 4).

Fructose, glucose and lactose were tested as carbon sources under the conditions of deep cultivation of the selected strains. All three strains synthesized protease on all of these carbon sources, but the highest protease activity was observed when *Penicillium candidum-5-1* was cultivated in the nutrient medium using glucose and lactose as

Table 2. Enzyme index of the 3 positives isolated from dairy products

Isolates	Clear zone diameter (mm)			Colony diameter (mm)			Enzyme index (EI) = Clear zone diameter/Colony diameter		
	Incubation hours								
	48	**72**	**96**	**48**	**72**	**96**	**48**	**72**	**96**
spp. 2-3	7	10	12	6	8	9	1.16	1.25	1.3
spp. 5 -1	17	22	28	8	10	11	2.12	2.2	2.54
spp. 7-5	13	15	20	7	10	17	1.85	1.5	1.17

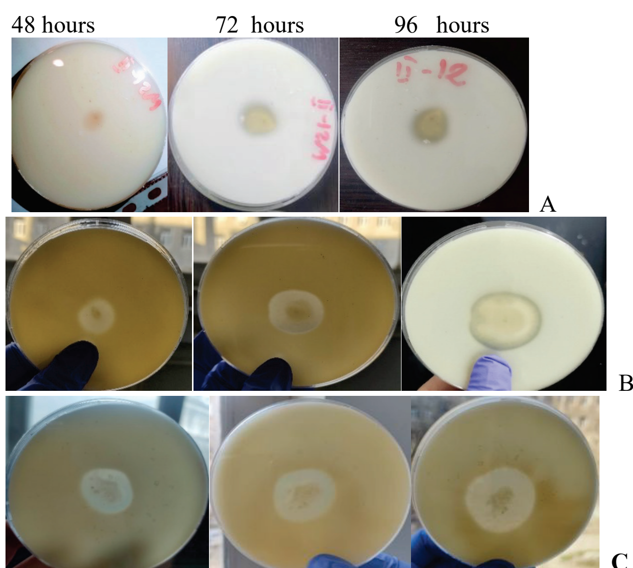

Fig. 1. Clearance zone diameter around the fungal isolates A) spp. 2-3 B) spp. 7-5 C) spp. 5-1.

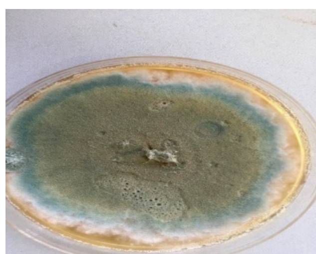

Fig. 2. *Penicillium camemberti* 7-5.

Fig. 3. *Mucor spp. 2-3.*

Fig. 4. *Penicillium candidum 5-1.*

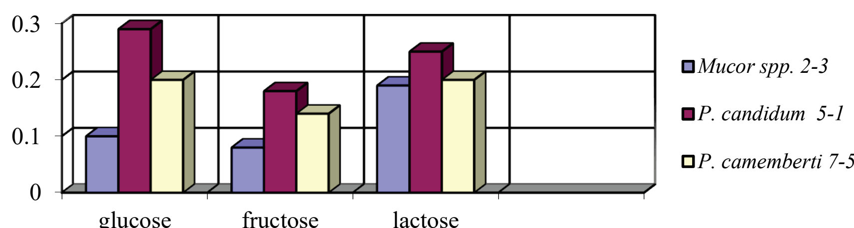

Fig. 5. Optimization of carbon sources for protease production by *mucor* spp.2-3, *p.candidum*-5-1 and *p.camemberti* 7-5.

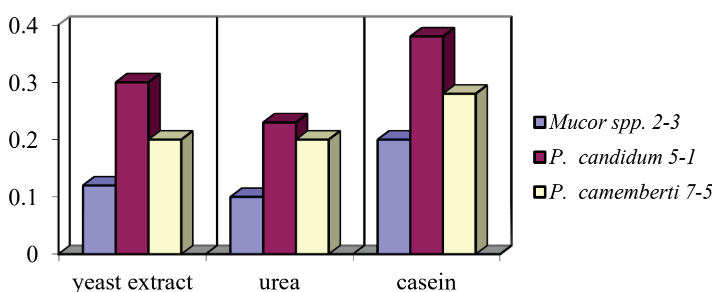

Fig. 6. Optimization of nitrogen sources for protease production by *mucor* spp.2-3*, p.candidum*-5-1 and *p.camemberti* 7-5.

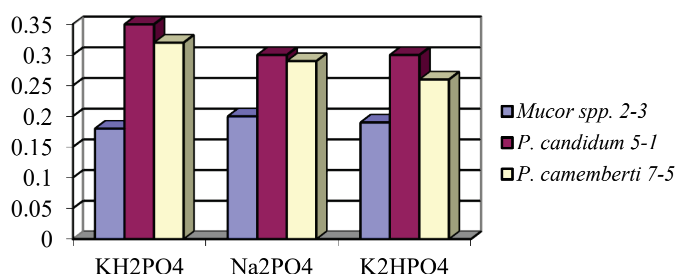

Fig. 7. Optimization of phosporus sources for protease production by *mucor* spp.2-3*, p. candidum*-5-1 *and p. camemberti* 7-5.

carbon sources (Fig. 5). Lactose was taken as the carbon source for future studies since our goal was to use medium close to the natural substrate, especially since no significant difference was observed between the use of these two sources.

Nitrogen sources were found to have an inducing effect on proteolytic enzymes. Yeast extract, urea, and casein were tested as nitrogen sources. While all three sources affected enzyme activity, casein resulted in higher protease activity for all three strains. Among them, *Penicillium candidum 5-1* exhibited the highest protease activity (Fig. 6).

Different phosphorus salts, including dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and disodium hydrogen phosphate, were tested to determine the optimal phosphorus

source. The strain *Penicillium candidum 5-1* showed the highest activity in the presence of potassium dihydrogen phosphate (Fig. 7).

Based on the analysis of the influence of carbon, nitrogen, and phosphorus sources on protease activity, *Penicillium candidum 5-1*, which exhibited relatively high proteolytic activity, was selected for further studies.

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ბუნებრივ სუბსტრატებთან მიახლოებულ საკვებ არეებზე მზარდი პროტეაზების პროდუცენტი - მიკროსკოპული სოკოების სკრინინგი

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საქართველოს სხვადასხვა რეგიონიდან აღებული რძის გაფუჭებული პროდუქტებიდან (თავად რძე, მაწონი, ხაჭო, იოგურტი და სხვა) გამოყოფილია მიკროსკოპული სოკოების კულტურები; სკრინინგის გზით გამოყოფილი სოკოების კულტურებს შორის გამოვლენილია პროტეაზას სამი პროდუცენტი. სამივე კულტურა იდენტიფიცირებულია სახეობამდე – *Mucor spp. 2-3, Penicillium candidum 5-1* და *Penicillium camemberti 7-5*. სამუშაოს ფარგლებში დადგინდა ნახშირბადის, აზოტისა და ფოსფორის წყაროები. შედეგად შეირჩა ბუნებრივ სუბსტრატთან მაქსიმალურად მიახლოებული საკვები არე – ლაქტოზას, კაზეინის და კალიუმის ფოსფატის შემცველობით. ამ არეზე სიღრმული კულტივირების პირობებში პროტეაზას მაქსიმალურ რაოდენობას პროდუცირებდა შტამი – *Penicillium candidum 5-1.*

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