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Protease Biosynthesis by Mutant Strain of *Penicillium griseoroseum* and Cheese Formation

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Abstract: The present study was conducted on proteases biosynthesis by *Penicillium griseoroseum* and its application to cheese formation. Thirty fungal strains of *Penicillium griseoroseum* were screened for the production of protease using solid substrate fermentation. Results indicated that *Penicillium griseoroseum* HUV-21 gave the best results for the biosynthesis of acidic protease. Different cultural conditions were employed to enhance protease production in the fermented mash. The enzyme synthesis was maximum (61.85 U g^{-1}), when soybean meal was used as substrate. The optimum temperature, incubation period and depth of substrate were found to be 30°C, 72 h and 20 mm, respectively. It was concluded that protease concentration of 6.0 U g^{-1} was the best for milk clotting.

Key words: *Penicillium griseoroseum,* protease, fermentation, cheese

INTRODUCTION

Proteolytic enzymes are the most important industrial enzymes, representing worldwide sales of about 60% of the total enzyme market[1]. The enzymes cause breakdown of proteins into smaller peptides and amino acids[2,3]. Proteases are mainly produced by microorganisms like bacteria and fungi, but fungi are more important[4]. Fungal proteases are acidic in nature. *Aspergillus niger, Rhizopus* spp. and *Penicillium* spp. are usually employed for the production of acidic proteases[5] .

Solid as well as submerged fermentation has been widely used for the production of proteases[6,7]. Solid substrate fermentation is the growth of microorganisms on a pre-dominantly insoluble substrate without a free liquid phase. The substrates used are usually agricultural by products such as soybean meal, almond meal, rice husk, wheat bran, sunflower meal, cottonseed meal, etc.[8]. The moisture level in solid substrate fermentation for enzyme production is typically in the range of 60%. This method is usually suitable for fungi because solid substrate fermentation is close to the natural habitat of fungi than submerged fermentation[9]. Acidic proteases are usually produced at temperature of 25-30°C[10].

The present study was undertaken to produce acidic proteases *in vitro*. Solid-state fermentation of *Penicillium griseoroseum*, using wheat bran, rice husk, almond, soybean and sunflower meals as substrate was employed. These by products are produced in large amounts in Pakistan. The application of proteases was also investigated on cheese formation.

MATERIALS AND METHODS

Present investigations were carried out at Biotechnology Research Laboratories, Department of Botany, Government College University, Lahore, during the year 2001-2002. The mould cultures were isolated from the soil samples of Lahore area by pour plate method[11]. These were maintained on potato-dextrose-agar slants. The slants (5-7 days old) were wetted by adding 10 mL of 0.005% solution of monoxol O.T. (Diacetyl ester of sodium sulpho succinic acid) to prepare the spore suspension. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension. Spore suspension of 1 mL ($1.5x10^6$) was used for inoculation. The spore count was made on haemocytometer slide bridge.

The 250 mL conical flasks containing 10 g substrate moistened with 15 mL of diluent (distilled water) were sterilized at 121°C (15 lbs/$inch^2$ pressure), cooled, inoculated and incubated at 30±1°C for 72 h. After incubation, 100 mL of distilled water was added to the flasks, which were shaken on rotary shaker (Gallenkamp UK) for 1 h at 160 rpm. The contents of flasks were then filtered and the filtrate was used for enzyme assay. Different agricultural by products such as sunflower and soybean meals, lupin cake, wheat bran and rice bran were evaluated for the production of proteases.

The method of McDonald and Chen[12] was used for the assay of proteases. Casein (1%) was used as a substrate, to which 1 mL of enzyme sample was added. The mixture was incubated at 30°C for 1 h. The reaction

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was arrested by adding 5 mL of 5% trichloroacetic acid (TCA). The mixture was centrifuged and 1 mL of supernatant was mixed with 5 mL of alkaline reagent. Then 1 mL of 1N NaOH was added to make the contents alkaline. After 10 min, 0.5 mL of Folin and Ciocalteau reagent were added to the test tubes and mixed. The blue colour produced was measured at 700 nm after 30 min.

In the present study Manchego cheese making procedure was used[13]. Different concentrations of crude enzyme were added in 100 mL cow milk. Casein was separated from cow milk by precipitation with acetic acid addition up to pH 4.6. The casein precipitated was washed repeatedly and reprecipitated. The samples were incubated at 32°C for 4 h after which the reaction was stopped. The samples were centrifuged for 15 min. Casein fractions thus obtained were washed and dried.

RESULTS

Thirty different fungal cultures of *Penicillium griseoroseum* were screened using soybean meal as substrate (Table 1). Of all the strains examined *Penicillium griseoroseum* HUV-21 gave maximum production of protease i.e. 54.00 U g^{-1}. The production of proteases by HUV-15 and HUV-20 was markedly low, i.e. 5.00 and 5.30 U g^{-1}, respectively. Eight strains of *Penicillium griseoroseum* gave low production of the enzyme in the range of 5.00-10.00 U g^{-1} and nine strains gave production in the range of 10.00-20.00 U g^{-1}. However, only two isolates produced enzyme ranging from 40.00-50.00 U g^{-1}. Since *Penicillium griseoroseum* HUV-21 gave maximum production, therefore, it was selected for further studies.

Different substrates i.e. sunflower, soybean and almond meals, rice husk or wheat bran were used for synthesis of proteases (Table 2). Of all the substrates examined, soybean meal gave maximum enzyme activity (58 U g^{-1}). Flasks were incubated at 30°C for different time intervals (24, 48, 72, 96, 120 h). The maximum production of protease (58 U g^{-1}) was reached after 72 h incubation period and further increase in incubation period resulted in the decrease of enzyme production. So incubation period of 72 h was optimized (Table 3).

The production of protease using *Penicillium griseoroseum* HUV-21 was studied using soybean meal as substrate. Flasks were incubated for 72 h at different temperature, i.e. 12, 20, 30, 37 and 40°C. It was noted that maximum protease (58 U g^{-1}) was obtained at 30°C (Table 4). Effect of varying depth ranging from 10-50 mm corresponding to the amount of substrate in the flask, i.e. 5-25 g, respectively, on the biosynthesis of enzyme was investigated (Table 5). It was found that at the depth of

Table 1: Screening of *Penicillium griseoroseum* isolates

Strains	Protease activity (U g^{-1})
HUV-1	25.00
HUV-2	29.00
HUV-3	20.50
HUV-4	11.00
HUV-5	15.50
HUV-6	9.70
HUV-7	7.00
HUV-8	7.55
HUV-9	23.00
HUV-10	30.00
HUV-11	29.50
HUV-12	13.25
HUV-13	9.50
HUV-14	21.55
HUV-15	5.00
HUV-16	11.20
HUV-17	30.30
HUV-18	33.50
HUV-19	23.00
HUV-20	5.30
HUV-21	54.00
HUV-22	12.00
HUV-23	18.10
HUV-24	52.50
HUV-25	9.50
HUV-26	11.50
HUV-27	8.10
HUV-28	20.10
HUV-29	16.20
HUV-30	25.40

Substrate= Soybean meal
Temperature= 30°C

Table 2: Selection of substrate for the production of protease by of *Penicillium griseoroseum* HUV-21

Substrate	U mg^{-1}
Soybean Meal	58
Almond Meal	30
Rice Husk	08
Wheat Bran	28
Sunflower Meal	08

Temperature= 30°C
Incubation period= 72 h

Table 3: Effect of incubation period on the biosynthesis of proteases by *Penicillium griseoroseum* HUV-21

Incubation period (h)	U mg^{-1}
24	24
48	30
72	58
96	35
120	15

Temperature = 30°C
Substrate = Soybean meal (10 g/250 mL conical flask)

Table 4: Effect of incubation temperature on the production of protease by *Penicillium griseoroseum* HUV-21

Temperature °C	U mg^{-1}
15	10
20	35
30	58
35	50
40	45

Substrate = Soybean meal (10 g/250 mL conical flask)
Incubation period = 72 h

Table 5: Effect of depth of substrate on the production of protease by of *Penicillium griseoroseum* HUV-21

Depth (mm)	U mL^{-1}
5	10
10	58
15	30
20	15
25	08

Temperature = 30°C Incubation period = 72 h

20 mm (10 g), the protease activity was found to be maximum (58 U g^{-1}).

Application of proteases to cheese formation: Effect of different concentrations of protease, i.e. 2.0, 4.0 and 6.0 U g^{-1} was studied on the clotting of milk enzyme samples. The concentrations were added to 100 mL of cow milk and the time required for the initiation of clot formation was noted. The results showed that time required for clot formation was 30 minutes, when 6.0 U g^{-1} of protease was used. However, the time required for clotting at 2.0 and 4.0 U g^{-1} was 2 and 1 h, respectively. Thus it was concluded that by increasing enzyme concentration, the time required for clotting was reduced.

DISCUSSION

Soybean meal gave better production of protease because it is an adequate source of proteins, carbohydrates and minerals required for growth of microorganisms. It was also noted that maximum production of proteases was reached after 72 h incubation; this is due to the fact that at this time the microorganisms were in stationary phase. Temperature plays an important role in metabolic processes of an organism. It was found that at 30°C the enzyme synthesis was maximum. The metabolic activities of micro organisms become slow at lower temperature[14] and at high temperature enzyme lost its catalytic properties due to stretching and final breaking of weak hydrogen bonds present in enzyme structure, which result in denaturation of the enzyme[15].

Effect of varying depth of substrate has a critical role on production of protease. Enzyme production was maximum at the depth of 20 mm (10 g) i.e. 58 U g^{-1}. As the depth of substrate increases the supply of oxygen to the fermentation medium decreases, which resulted decreased proteases activity. Ramamurthy and Kothari[16] also achieved the best production (20.00 U g^{-1}) at the depth of 20 mm of substrate. As the concentration of the enzyme used for the clotting of milk increased, it was noted that the time required for clotting was decreased. It might be due to the fact that milk contains fats, proteins, milk sugar (lactose), mineral salts and water. Casein (the principal protein) combined with calcium exists in a colloidal form. Fernendaz *et al.*[13] suggested that with the addition of protease in milk the casein got free from calcium and no longer appeared as fine particles distributed throughout the medium. But instead of solution casein accumulated in larger lumps. By increasing concentration the clotting was enhanced. These results are in agreement with the work of Channe and Shewale[17].

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