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Production of Protease from Soil Fungi by Submerged Fermentation

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Abstract

The demand of proteases is increasing regularly because of their numerous applications in the biotechnological industries. It promotes isolation and identification of potent protease producing fungi from soil samples. Therefore, the objective of the present investigation was to screen soil fungi in submerged fermentation (SmF) for detection of hyperproducer isolate and production of protease in SmF. Fungal strains from three diverse soil samples were isolated on potato dextrose agar (PDA) plates by serial dilution agar plate technique, purified by point inoculation and twelve isolates utilized for production of extracellular protease in SmF. Results were examined both in terms of protease activity and specific activity. Among all 12 fungal strains, isolate S2St1 (isolated from medicinal garden soil) exhibited maximum activity of protease (256.63 ± 3.18 U/ml/min) at 72 h of incubation.

Isolate S2St1 was identified as potent producer of protease in SmF. Further this isolate can be used for production of large quantity of protease in SmF within very short time period of 3 days.

Keywords: Proteases, potato dextrose agar, fungi, soil samples, submerged fermentation.

Introduction

Microbes are recognized to play a very important role in the industries for production of enzymes of intracellular as well as extracellular origin [1]. For greatest productivity, chosen microbes are cultivated in fermenters under most favorable conditions and can be further utilized to formulate products such as bread, cheese, beer and wine [2, 3]. Proteases or peptidases are well recognized enzymes that stimulate cleavage of peptide bonds presents within the protein and peptide molecules. The product of protease action is few free amino acids and small polypeptide chain [4]. Proteases can be classified according to (1): sources of secretion: plant, animal and microbial; (2): site of peptide bond cleavage-endopeptidases and exopeptidases; (3) mode of action; (4): optimum pH and (4) substrate chain length [4, 5, 6, 7].

Microbial sources of proteases are preferred than other sources due to stability and chemical properties. Microbial proteases have drawn a huge deal of consideration in the last decade due to their biotechnology potential in different industries such as leather, pharmaceutical, detergent, textile and dairy [8]. Among all enzymes, microbial proteases are important industrial enzymes and contribute 60% of the world enzyme sale [9]. Among the microbes, fungal proteases have attracted the consideration of biotechnologists since fungi can cultivate on inexpensive substrates and produce great quantity of enzymes into culture medium which further can easy the downstream processing [10].

Soil is a good habitat of number of enzyme producing microbes Therefore, several researchers reported isolation and screening of protease producing fungi from soil samples. Industrial production of microbial proteases can be carried out by two methods: solid state fermentation (SSF) and submerged fermentation (SmF) [11]. For industrial enzyme production, SmF is a widely utilized process than SSF because of number of advantages

(regulation of process, easy downstream process etc.) offered by SmF [12]. Considering industrial importance of proteases, the purpose of present study was screening of soil fungi in SmF for detection of fungal isolate with increased productivity of protease.

Materials and methods

Collection of soil samples

Soil samples were collected from three diverse areas (medicinal garden, mustard crop field and wheat crop field of Banasthali University).

Isolation of fungi

Fungi were obtained on PDA plates by serial dilution of soil samples as explained previously by Waksman [13] and fungal colonies were purified by point inoculation on PDA plates.

Production of extracellular protease

Preparation of spore suspension

Spore suspension was made by adding three ml of sterile distilled water in 6 days old slant culture of purified fungal isolates followed by vigorous shaking for liberation of spores [14].

Transferring spore suspension in fermentation broth

One ml of spore suspension was transferred in 100 ml of autoclaved fermentation broth which was prepared in 250 ml of Erlenmeyer flask. The composition of fermentation broth (g/100 ml) is as follows: glucose, 2.0; yeast extract, 1.0; K_2HPO_4, 0.1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.02 and pH was adjusted to 7.0. Flasks were placed at 28 $^{\circ}$C, 150 rpm for 6 days in an incubator shaker [15].

Separation of crude protein mixture from fermentation broth

Fermentation broth from the each inoculated flask was filtered via Whatmann filter paper No. 1. Mycelium was removed by centrifugation of filtrate at 7,000 rpm at 4 ˚C for 10

minutes. Supernatant was recovered from centrifugation tube after discarding pellet [16]. This supernatant was considered as crude protein lysate for the measurement of protein contents [17] and extracellular protease activity [18].

Measurement of protease activity from crude protein extract

Preparation of standard curve of L-tyrosine

Standard curve of L-tyrosine was prepared within the concentration range of 25-275 µM, in 50 mM Potassium Phosphate buffer, pH 7.5 [19].

Protease assay

Activity of protease in the mycelium free culture supernatant was measured according to the technique of Tsuchida et al. [18] in which casein was used as a substrate. A mixture of 0.5 ml of 1% (w/v) of casein in 50 mM phosphate buffer, and 0.2 ml of crude enzyme extract (source of protease) were incubated in a water bath at 40 °C for 30 minutes. After 30 minutes reaction was ceased by addition of 1 ml of 10 % tricholoro acetic acid (TCA) reagent and was placed for 30 minutes at room temperature for precipitation of unreacted proteins. Thereafter, precipitated casein was removed by centrifugation of reaction mixture at 10000 rpm for 5 minutes at 4 ºC. Then filtrate was mixed with 2.5 ml of 0.4 M Na_2CO_3 followed by addition of 3-fold diluted Folin reagent. The resultant solution was incubated in dark for 30 minutes. After 30 minutes amount of blue color complex was determined at 660 nm against the reagent blank using standard curve of tyrosine. All experiments were performed in triplicates and mean values and standard deviation of them is presented in tables. One unit of enzyme will hydrolyze casein to produce colour equal to 1.0 µM of tyrosine per minute at 40 ˚C, pH 8.0.

$$\text{Protease activity} = \frac{\text{µM of L-tyrosine released}}{\text{Volume of protease taken} \times \text{Incubation time}}$$

Estimation of protein contents from crude protein lysate

Quantity of protein in culture supernatant was measured by Lowry's technique [17].

Preparation of standard curve of BSA (bovine serum albumin)

Standard curve of BSA was made within the concentration range of 40 to 200 µg/ml.

Protein quantification

0.1 ml of crude protein lysate was transferred into a test tube followed by addition of 0.9 ml of distilled water to adjust the final volume to 1 ml. Thereafter, 5 ml of alkaline copper reagent was added and tubes were kept for 10 minutes at room temperature. Test tubes were placed in dark for 30 minutes after addition of 0.5 ml of Folin's reagent. Blue colour complex was developed at the end of incubation and quantity of this blue colour compound was determined at 660 nm with reference to blank.

Results and Discussions

Isolation of fungi

Fungi were isolated on PDA plates. Fungi with different colony characteristics were appeared on PDA plates (Results are not shown here). Isolated fungi were purified by point inoculation on PDA plates. Results of purification are presented in Figure 1.

Protease production in SmF

Results of quantitative screening are given in Table 1-3. Most of the isolates demonstrated higher protease activity at 3 days of incubation. Table 1 presents that among all the 4 strains of wheat field soil, isolate S1St2 exhibited maximum protease activity (200.08 ± 6.50 U/ml/min) and specific activity (40.38 ± 1.5 U/mg) at 72 h of incubation. Table 2 represents that isolate S2St1 (isolated from medicinal garden soil) demonstrated highest production of protease (256.63 ± 3.18 U/ml/min) at 72 h of incubation. Thereafter, it started to decline (229.65 ± 7.18 U/ml/min at 96 h) and reached to minimum (196.7 ± 12.72 U/ml/min) at 120 h of incubation. Table 3 presents that isolate S3St2 exhibited highest protease activity (154.70 ± 5.02 U/ml/min) and specific activity (31.22 ± 1.21 U/mg) at 3 days of incubation among all 4 isolates of mustard field soil.

If we compare protease activity of all isolates then highest activity of protease (256.63 ± 3.18 U/ml/min) was obtained from isolate S2St1 at 3 days of incubation, which indicate that this fungal isolate can be utilized to produce large quantity of protease in very short period of time (within 3 days of fermentation period). Isolate S2St1 was found efficient producer of protease in SmF because it was growing well and consumed all ingredients for

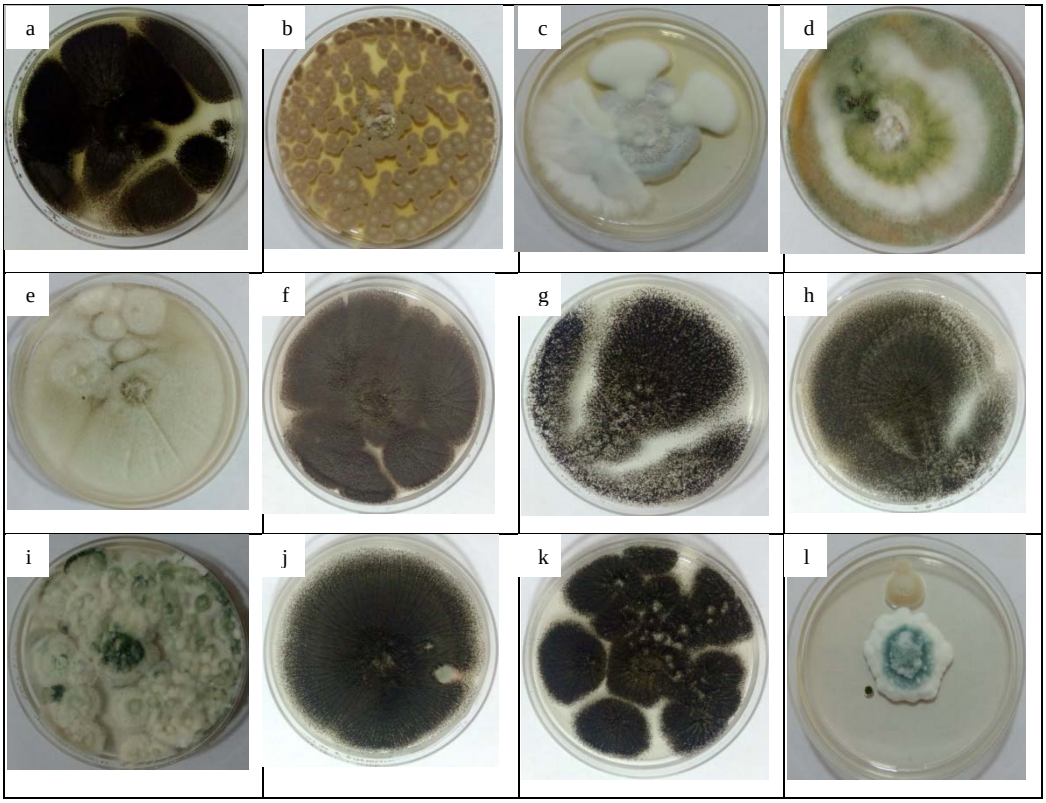

Figure 1. PDA plates showing purified fungal colonies.

Table 1: Protease production and specific activity from different fungal strains of soil sample 1 (wheat field).

Time (days)	Protease activity (U/ml/min)				Total protein content (mg/ml)				Specific activity (U/mg)			
	St1	**St2**	**St3**	**St4**	**St1**	**St2**	**St3**	**St4**	**St1**	**St2**	**St3**	**St4**
Day 2	81.55±3.83	169.21±3.54	99.06±7.52	83.93±3.17	4.7	4.29	0.45	4.47	17.36±0.3	**39.49±1.4**	21.85±1.4	18.97±2.40
Day 3	87.96±1.75	**200.08±6.50**	86.55±3.95	126.08±50.9	6.2	4.96	0.46	4.54	14.30±1.6	**40.38±1.5**	18.42±0.3	28.85±14.28
Day 4	82.99±0.82	90.85±7.59	102.57±1.5	84.82±3.75	1.8	1.85	1.23	2.39	45.18±5.3	48.99±4.22	83.06±1.59	35.73±2.68
Day 5	95.22±8.72	106.68±7.71	85.46±21.4	99.30±10.91	6.3	4.91	3.61	4.14	16.14±4.5	21.87±2.8	23.77±6.2	23.88±1.08

St1: Strain 1; St2: Strain 2; St3: Strain 3; St4: Strain 4.

Table 2: Protease production and specific activity from different fungal strains of soil sample 2 (medicinal garden).

Time (days)	Protease activity (U/ml/min)				Total protein content (mg/ml)				Specific activity (U/mg)			
	St1	St2	St3	St4	St1	St2	St3	St4	St1	St2	St3	St4
Day 2	88.79±3.80	51.36±5.02	50.04±2.13	50.23±0.30	3.57	4.3	4.5	4.2	22.70±1.8	11.08±0.95	10.90±0.39	11.83±0.80
Day 3	**256.63±3.18**	129.37±1.35	129.425±6.34	123.76±6.26	3.9	4.1	4.7	5.0	64.4±3.30	30.94±0.88	26.99±0.72	24.72±1.12
Day 4	229.65±7.18	135.98±7.51	133.05±5.96	127.82±5.90	2.7	3.1	3.5	2.6	**82.23±3.6**	44.25±3.63	37.47±3.02	48.34±1.48
Day 5	196.7±12.72	76.92±3.06	60.79±6.04	40.28±3.52	4.6	4.5	3.5	3.7	42.12±2.4	16.94±1.08	17.33±1.98	10.74±1.11

St1: Strain 1; St2: Strain 2; St3: Strain 3; St4: Strain 4.

Table 3: Protease production and specific activity from different fungal strains of soil sample 3 (mustard field).

Time (days)	Protease activity (U/ml/min)				Total protein content (mg/ml)				Specific activity (U/mg)			
	St1	St2	St3	St4	St1	St2	St3	St4	St1	St2	St3	St4
Day 2	63.06±2.96	130.84±2.73	76.59±5.81	64.90±2.45	4.6	4.2	4.5	4.4	13.44±0.2	30.53±1.13	16.90±1.11	14.67±1.8
Day 3	68.01±1.35	**154.70±5.02**	66.92±3.05	69.99±0.78	6.2	4.9	4.6	4.5	11.06±1.2	**31.22±1.21**	14.24±0.28	15.50±1.4
Day 4	22.73±4.20	27.26±7.99	26.08±0.43	23.20±5.96	3.5	3.5	2.0	3.2	6.42±1.31	7.82±2.52	13.13±2.48	7.01±1.13
Day 5	73.62±6.74	82.49±5.96	66.08±16.61	58.67±3.76	5.3	4.9	3.6	5.8	13.88±1.5	16.91±2.19	18.38±4.83	10.05±0.1

St1: Strain 1; St2: Strain 2; St3: Strain 3; St4: Strain 4.

maximum production of protease. In our study, lowest protease production (22.73 ± 4.20 U/ml/min) was found with isolate S3St1 at 96 h of incubation. Our results showed that the production of protease may be directly related to the organism concerned and effectiveness and suitability of the fermentation broth.

Similar to our results, Anand [20] reported that maximum protease production was observed from *Aspergillus niger* (18.64 µg/ml) and *Aspergillus flavus* (13.73 µg/ml) after 3 days of incubation. Oseni et al. [21] reported that fungal isolates of forest soil samples produced protease maximally between 72 h to 120 h of incubation, which is similar to our results. Ali and Vidhale [22] reported highest protease production (72 U/mg) from *Fusarium*

oxysporum after 3 days of incubation. On the other hand, Irfan et al. [23] used SSF for acidic protease production from *Rhizopus oligosporus* and *Rhizopus arrhizus*. Kamath et al. [24] reported maximum yield of protease from *Aspergillus niger* after 96 h of incubation.

Opposite to our results, Muthulakshmi et al. [25] reported highest protease activity (49.3 U/ml/min) at 7^{th} day of incubation.

Conclusion

Proteases are classified in Hydrolase class of enzymes. Proteases are produced by microbial sources at industrial scale especially by fungal sources using the technique of SmF. Protease production was carried out from 12 soil fungal isolates in SmF, where glucose and yeast extract were used as carbon source and nitrogen source, respectively. Among all the fungi, isolate S2St1 (isolated from medicinal garden soil) showed maximum protease activity (256.63 ± 3.18 U/ml/min) at 72 h of incubation. Therefore, in our investigation isolate (S2St1) was recognized as potent producer of extracellular protease, which can be further used for mass production of protease in SmF. Protease activity of this isolate can be further increased by optimization studies.

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