JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 39-44

Screening of Extracellular Protease (Gelatinase) at Different pH by Keratinophilic Fungi

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(Received: 20 October 2012; accepted: 28 November 2012)

Totally 35 fungi were isolated from poultry farm and feather dumping soil from Namakkal and Chennai (India), respectively. These isolated fungi were screened for the production of protease (gelatinase) on agar plates containing gelatin as the substrate in different pH 6, 7 and 8. The enzyme activity showed the clear zone around the fungal colonies due to hydrolysis of gelatin. This study suggests that the fungal strains were able to produce different amount of protease at acid, neutral and alkaline pH. The non-dermatophytic fungi were able to secrete high protease activity in all the pH than dermatophytes and closely related species. The fungi such as *Paecilomyces carneus* and *Scopulariopsis brevicaulis* were produced highest protease activities in all the pH.

Key words: Keratinophilic fungi, plate assay, protease, screening.

The organic substances such as cellulose, fat, proteins and starch are quite resistant for enzyme attack, but some microorganisms can produce the enzymes that can hydrolyze into simple substances (Choudhary & Jain, 2012). Particularly, the proteolytic enzyme catalyzes the hydrolysis of proteins (Rao *et al.,* 1998). Proteases constitute the most important industrial enzymes and their applications have recently increased in fields such as baking, brewing, food production, leather processing, pharmaceutical manufacture, and the recovery of silver from photographic film (Rao *et al.,* 1998). Currently, majority of the microbial proteases are used in industrial purpose than plant and animal proteases, because of their stability. Plate assay screening is used as a qualitative method for estimation of the enzyme activity from a large number of microorganisms. Singh & Agrawal (1982) have developed a rapid method for the detection of extracellular enzymes using solid media. Different protein substrates such as casein, gelatin and skim milk were used for screening of protease activity (Buzzini & Martini, 2002).

Physico-chemical conditions are very important, particularly pH and temperature to select the potent enzyme producer. Many researchers have screened the dermatophytes and closely related species for the production of keratinase (Friedrich *et al.,* 1999; Wawrzkiewicz *et al.,* 1991). The purpose of this study was to screen the isolated fungi for their ability to produce the extracellular protease at different pH.

MATERIALS AND METHODS

The keratinophilic fungi were isolated from poultry farm and feather dumping soil from Namakkal and Chennai, respectively (Anbu *et al.,* 2004). All fungi were maintained on Sabouraud's dextrose agar slants (dextrose, 40 g/l; peptone, 10 g/l; agar, 20 g/l; pH 5.6).

Protease screening (Hislop *et al.,* 1982)

The medium contained 2% (w/v) agar and 1% (w/v) gelatin in Mcllavaine's buffer was

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adjusted to different pH namely 6.0, 7.0 and 8.0. They were autoclaved separately cooled and then mixed. About 20 ml of the media were poured into each sterile Petriplate and allowed to solidify. The fungal cultures were inoculated at the centre of the plate using a pinpoint inoculum and incubated at room temperature for a period of 7 days. After the incubation period, the plates were developed with 15% (w/v) mercuric chloride in 20% HCl solution. In each plate the clear zone was measured.

RESULTS AND DISCUSSION

Fungal species, isolated from poultry farm and feather dumping soil, produced extracellular enzymes for their survival in the ambient environment. Fungi represented by 3 species of dermatophytes, 4 species of closely related fungi and 28 species of other fungi were screened for an extracellular protease. The production of extracellular protease was assayed on solid media,

Table 1. The proteolytic activity of kertinophilic fungi at pH 6.0

S. No	Name of the fungi	pH 6.0		
		Mycelial growth (mm)	Lysed zone (mm)	Enzymatic Index-EI (mm)
1	Dermatophytes and closely related species			
	Microsporum gypseum	19	26	7
2	*Trichophyton mentagrophytes*	14	23	9
3	*T. mentagrophytes* var. *introdugi*	15	20	5
4	*Chrysosporium keratinophilum*	22	26	4
5	*C.* state of *Arthroderma tuberculatum*	36	39	3
6	*Geomyces pannorum*	27	36	9
7	*Myceliopthora vellerea*	28	0	0
8	Other species*Aspergillus flavus*	55	72	17
9	*A. fumigatus*	18	24	6
10	*A. glaucus*	21	29	8
11	*A. nidulans*	29	44	15
12	*A. niger*	44	48	4
13	*A. terreus*	54	62	8
14	*A. ustus*	28	33	5
15	*A. versicolor*	17	39	22
16	*Cladosporium cladosporioides*	24	0	0
17	*C. oxysporum*	21	0	0
18	*Cunninghamella echinulata*	77	81	4
19	*Curvularia lunata*	50	71	21
20	*Drechslera australiensis*	56	58	2
21	*Fusarium solani*	52	60	8
22	*Geotrichum candidum*	45	52	7
23	*Humicola grisea*	14	18	4
24	*Paecilomyces carneus*	34	60	26
25	*Penicillium citrinum*	17	21	4
26	*P. frequentans*	31	36	5
27	*P. funiculosum*	22	45	23
28	*P. oxalicum*	14	0	0
29	*P. purpurogenum*	21	26	5
30	*Phialophora* sp.	65	72	7
31	*Rhizopus stolonifer*	90	0	0
32	*Scopulariopsis brevicaulis*	26	51	25
33	*Trichoderma viride*	68	71	3
34	*Trichoderma* sp.	80	82	2
35	Non-sporulating fungus	38	0	0

semiquantitatively by measuring the diameter of the lysed zone and the growth of the colony.

To determine the protease activity, the fungi were grown on the agar plates containing gelatin, at acid, neutral and alkaline pH and incubated for a period of 7 days. The diameter of the mycelial growth and lysed zone were measured and the value was taken as an enzymatic index (EI) of the protease activity.

Acid pH

Data on the degree of lysis of the gelatin and the diameter of the colony growth at the acid pH for a period of 7 days of incubation are presented in Table 1. Of the 35 species, the maximum EI (above 15 mm) was registered in *Aspergillus flavus, A. nidulans, A. versicolor, Curvularia lunata, Paecilomyces carneus, Penicillium funiculosum* and *Scopulariopsis brevicaulis*. The

Table 2. The proteolytic activity of kertinophilic fungi at pH 7.0

S. No	Name of the fungi	pH 7.0		
		Mycelial growth (mm)	Lysed zone (mm)	Enzymatic Index-EI (mm)
	Dermatophytes and closely related species			
1	*Microsporum gypseum*	14	34	20
2	*Trichophyton mentagrophytes*	11	19	8
3	*T. mentagrophytes* var. *introdugi*	13	16	3
4	*Chrysosporium keratinophilum*	24	30	6
5	*C.* state of *Arthroderma tuberculatum*	27	38	11
6	*Geomyces pannorum*	13	23	10
7	*Myceliopthora vellerea*	19	0	0
8	Other species*Aspergillus flavus*	46	58	12
9	*A. fumigatus*	27	41	14
10	*A. glaucus*	14	27	13
11	*A. nidulans*	17	27	10
12	*A. niger*	28	31	3
13	*A. terreus*	51	58	7
14	*A. ustus*	36	42	6
15	*A. versicolor*	13	23	10
16	*Cladosporium cladosporioides*	20	0	0
17	*C. oxysporum*	17	0	0
18	*Cunninghamella echinulata*	66	70	4
19	*Curvularia lunata*	44	61	17
20	*Drechslera australiensis*	48	50	2
21	*Fusarium solani*	37	51	14
22	*Geotrichum candidum*	32	42	10
23	*Humicola grisea*	12	14	2
24	*Paecilomyces carneus*	24	53	29
25	*Penicillium citrinum*	24	28	4
26	*P. frequentans*	27	33	6
27	*P. funiculosum*	19	37	18
28	*P. oxalicum*	10	0	0
29	*P. purpurogenum*	19	23	4
30	*Phialophora* sp.	45	53	8
31	*Rhizopus stolonifer*	88	0	0
32	*Scopulariopsis brevicaulis*	20	46	26
33	*Trichoderma viride*	50	55	5
34	*Trichoderma* sp.	69	72	3
35	Non-sporulating fungus	30	0	0

remaining fungi produced low EI. The two fungal species *Paecilomyces carneus* and *S. brevicaulis* exhibited minimal biomass with high enzyme activity 26 and 25 mm, respectively. The following fungi such as *Cladosporium cladosporioides, Cladosporium oxysporum, Myceliopthora vellerea, Penicillium oxalicum, R. stolonifer* and non-sporulating fungus produced no protease at pH 6.0, even with the growth of the colony. The remaining fungal species showed mycelial growth as well as enzyme production.

Neutral pH

Data on the degree of lysis of the gelatin and the diameter of the colony growth at the neutral pH for a period of 7 days of incubation are presented in table 2. High EI was registered in *Curvularia lunata, Microsporum gypseum, Paecilomyces carneus, Penicillium funiculosum*

Table 3. The proteolytic activity of kertinophilic fungi at pH 8.0

S. No	Name of the fungi	pH 8.0		
		Mycelial growth (mm)	Lysed zone (mm)	Enzymatic Index-EI (mm)
	Dermatophytes and closely related species			
1	*Microsporum gypseum*	20	31	11
2	*Trichophyton mentagrophytes*	9	13	4
3	*T. mentagrophytes* var. *introdugi*	11	14	3
4	*Chrysosporium keratinophilum*	12	27	15
5	*C.* state of *Arthroderma tuberculatum*	24	28	4
6	*Geomyces pannorum*	23	32	9
7	*Myceliopthora vellerea*	15	0	0
8	Other species*Aspergillus flavus*	40	48	8
9	*A. fumigatus*	19	28	9
10	*A. glaucus*	19	37	18
11	*A. nidulans*	22	38	16
12	*A. niger*	34	37	3
13	*A. terreus*	33	37	4
14	*A. ustus*	34	40	6
15	*A. versicolor*	15	36	21
16	*Cladosporium cladosporioides*	17	0	0
17	*C. oxysporum*	15	0	0
18	*Cunninghamella echinulata*	70	79	9
19	*Curvularia lunata*	49	53	4
20	*Drechslera australiensis*	50	52	2
21	*Fusarium solani*	40	51	11
22	*Geotrichum candidum*	29	38	9
23	*Humicola grisea*	19	26	7
24	*Paecilomyces carneus*	20	48	28
25	*Penicillium citrinum*	14	30	16
26	*P. frequentans*	17	30	13
27	*P. funiculosum*	21	25	4
28	*P. oxalicum*	7	0	0
29	*P. purpurogenum*	11	14	3
30	*Phialophora* sp.	34	45	11
31	*Rhizopus stolonifer*	85	0	0
32	*Scopulariopsis brevicaulis*	13	48	35
33	*Trichoderma viride*	49	53	4
34	*Trichoderma* sp.	45	47	2
35	Non-sporulating fungus	26	0	0

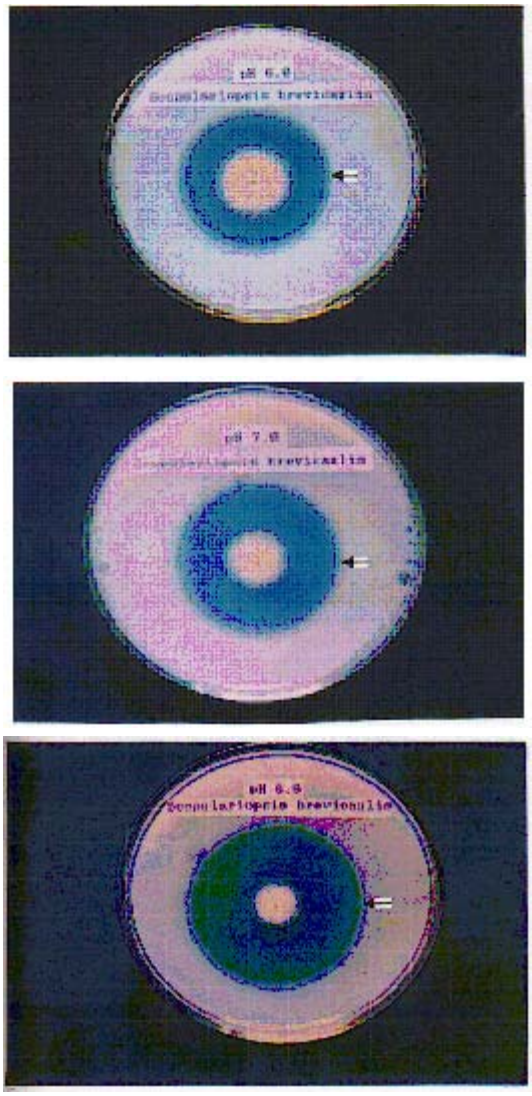

Fig. 1. Protease activity of *S. brevicaulis* at different pH

and *S. brevicaulis*. However, *Paecilomyces carneus* (29 mm) and *S. brevicaulis* (26 mm) were efficient to secrete the protease. Moderate EI was recorded in *A. flavus, A. fumigatus, A. glaucus, A. nidulans, A. versicolor, C.* state of *Arthroderma tuberculatum, Fusarium solani, Geomyces pannorum,*and *Geotrichum candidum,* whereas in the remaining fungi low EI was registered.

Alkaline pH

Data on the degree of lysis of the gelatin and the diameter of the colony growth at the alkaline pH for a period of 7 days of incubation are presented in Table 3. High EI was registered in the case of *A. glaucus, A. nidulans, A. versicolor, Chrysosporium keratinophilum, Paecilomyces carneus, Penicillium citrinum* and *S. brevicaulis*. Moderate EI was registered in *F. solani, Microsporum gypseum, Penicillium frequentans* and *Phialophora* sp. The rest of the species exhibited low EI. The fungus *S. brevicaulis* was most efficient to synthesis the protease (35 mm) in alkaline condition.

Of the dermatophytes and related species, *Microsporum gypseum* showed high activity in pH 7.0, moderate activity in pH 8.0 and low activity in pH 6.0. The fungus *Chrysosporium keratinophilum* showed high activity in pH 8.0 and low activity in pH 6.0 and 7.0. The remaining dermatophytes and related species were produced moderate or low activity. The fungus *Myceliopthora vellerea* did not produce the protease at any pHs. Several investigators have reported that the dermatophytes and closely related species were mainly producer of keratinase (Yu *et al.,* 1968; Raju *et al.,* 2007). However, in the present study, *Microsporum gypseum* and *Chrysosporium keratinophilum* could produce the high level of protease at neutral and alkaline pH, respectively.

Among the other species, the high protease activities were showed by *Paecilomyces carneus* and *S. brevicaulis* in all the pH. However, *Paecilomyces carneus* was produced highest activity at acid and neutral pH and *S. brevicaulis* was produced the highest activity at alkaline pH. The fungus *Paecilomyces carneus* showed almost similar levels of activity (26 to 29 mm) in all the pH. This result confirmed that the enzyme was stable in wide range of pH. It is interesting to note that the *S. brevicaulis* growth was minimal and enzyme secretion was high in alkaline pH when compared with acid and neutral pH (Fig. 1 and Table 1, 2, 3). However, the fungus *S. brevicaulis* was also a keratinase producer (Anbu *et al.,* 2007). The present result also confirmed that there was no correlation between growth rate and protease activity.

ACKNOWLEDGEMENTS

This work was supported by Inha University Grant, Inha University, Republic of Korea.

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