Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Archives of Applied Science Research, 2014, 6 (1):73-78
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-508X
CODEN (USA) AASRC9

Screening of fungi efficient in feather degradation and keratinase production

Jitendra Kumar[1*] and R. K. S. Kushwaha[2]

[1]Department of Botany, Dolphin PG College of Life Sciences, Chunni Kalan, Fatehgarh Sahib (Punjab), India
[2]Shree Shakti College, Ghatampur Kanpur(UP), India

ABSTRACT

Two hundred thirty four fungal strains were isolated by baiting method, for their feather degradation and keratinase producing ability. Fungi were tested on solid milk agar plates and in submerged culture. Maximum clearing zone was made by Chrysosporium indicum (7mm) on solid agar plates. The highest keratinase production was found in case of Acremonium strictum (74.40Unit/mL & 124.72 Unit/ml in 8 & 12 day incubation respectively, while Chrysosporium indicum 110.10U/mL and Chrysosporium tropicum 78.64U/mL was found next to it.

Keyword: Keratinase, Keratinophilic fungi

INTRODUCTION

The huge amount of poultry waste is spawned in the form of feathers that cannot degrade easily in nature. Keratinophilic fungi can degrade feathers as they can produce enzyme keratinase. Keratinases are serine or metallo proteases capable of degrading the structure forming keratin proteins. Most of the purified keratinases known to date cannot completely solubilize native keratin [1, 2]. Their exact nature and uniqueness for keratinloysis is still a mystery in the world for proteases. Nonetheless, keratinases in the nature have been continuously contributing to the valorization of voluminous keratin containing waste in the form of hair, feather, dead birds and animals [3]. Keratinase from *Bacillus* sp. [4], *Bacillus licheniformis* [2, 5], *Bacillus subtilis* KD-N2 [6], *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium* sp. [7] *Chryseobacterium* sp. [8], *Streptomyces* sp. [9] and *Acremonium strictum* [10] were isolated and studied with respect to various parameters.

Although keratinases from dermatophytic fungi have been well known due to their notorious pathogenic nature. The best studied are keratinases from the dermatophytic genera *Microsporum* [11] and *Trichophyton* [12, 13] as well as *Streptomyces* [14, 15]. These enzymes have only recently gained biotechnological impetus. Their growing importance mainly contributes to the isolation of keratinase from nonpathogenic microorganism and their ability to degrade the tough insoluble keratin of feather and convert it into the economically useful feather meal. Nitrogenous fertilizer, biodegradable films, glues, foils [16, 17].

There are relatively only few reports on characterization of the keratinases from nondermatophytic fungi [18-20]. It is now well established that the breakdown of keratin is carried out by the action of extracellular enzymes keratinases [21-22]. Biochemically keratin differs from other proteins in having a higher content of sulfur containing amino acids. The disulphide bonds between these amino acids make it resistant to most proteases. Developing upon the origin, the keratin is always found associated with lipids of varied nature. These lipids, which make keratin resistant to microbial degradation, are utilized as nutrients by keratinophilic fungi [23]. Therefore there is need to search for new keratinase producing nondermatophytic and related fungi that is efficient in feather degradation from soil.

MATERIALS AND METHODS

Isolation of fungi

Keratinophilic fungi were isolated from different soil habitats by hair baiting method.

Screening of fungi on milk agar plates

The method of Riffel and Brandelli [7] was to use to screen on solid medium Milk agar plates 5 g L^{-1} peptones, 3 g L^{-1} yeast extract, 100 mL $L^{-1,}$ sterile UHT nonfat milk and 12 g L^{-} 1 agar) were prepared for primary screening of proteolytic activity. Fungi were inoculated onto plates and incubated for 3, 6, 9 and 12 days for 28 $\pm$ 2^0C. Fungal strains that produced clearing zone in this medium were selected. Keratinase activity of fungus was detected as a clear zone around the colony. The diameter of clear zone was measured to quantify activity.

Keratinase production

The fungal culture were grown on two sets of modified production medium out of which media (M1) contained the following- 05g whole chicken feather; 2g glucose; 05g peptone; 05g yeast extract; 01g K_2HPO_4; 03g KH_2PO_4; 01g $Cacl_2$ and 01g $MgSO_4$ in 1000ml [24], and media (M2) containing - 5g feather; 0.5g $MgSO4.7H_2O$; 0.01g $FeSO4.7H_2O$ and 0.005g $ZnSO_4.7H_2O$ in 1000ml pH-7.8 [25] were employed for keratinase production. Each flask was grown in 250 ml capacity Erlmeyer flask containing 50 ml of sterilized basal medium with 200 mg of pre sterilized feather as the only source of keratin were inoculated with inoculum disc 6 mm in diameter obtained with a sterile circular cutter from the periphery of actively growing seven days old culture on PDA. Flask containing medium inoculated with a disc of agar without the fungus served as control. All the experiments were performed in triplicates. Flasks were incubated at 28±2^OC for 4, 8, 12, 16, 20 days in static and shaking conditions. At the end of growth period, the fungal mat and feather were separated from culture medium by filtering through glass wool. The culture filtrates were used for further analysis of keratinase assay. pH change was recorded by glass electrode.

Analytical method for keratinase production

Keratinase activity was measured by the method described by Dozie *et al*., [26]. The reaction mixture containing 1 ml. of appropriately diluted enzyme, 4 ml of NaOH buffer (0.05 M, pH 10) and 20 mg of feather were incubated at 60^0C for 60 min. The reaction was terminated by adding 4 ml of 5% (w/v) trichloroacetic acid and the tubes were incubated for 60 min at room temp. The feather and insoluble residue were removed by filtration through glass wool and the control was prepared in a similar manner, except that 1 ml of 5% Trichloroacetic acid and 3 ml of the buffer were added instead of 4 ml. of the buffer used in the test. Proteolytic products in the supernatant were determined by reading at 280nm against controls using UV-1700 Shimadzu UV-Visible spectrophotometer. An increase of 0.01 in the absorbance was considered as equivalents to 1 unit of enzyme activity per mL.

RESULTS AND DISCUSSION

Isolation of fungi

Two hundred thirty four nondermatophytic and related fungal strains isolated from different habitats, belongs to 29 genera and 73 species were tested on solid milk agar plates and in submerged culture to test their keratinase producing ability.

Solid plate screening

About 52% of fungi exihibited growth and made clearing zone on Skimed milk agar (Table 1). The maximum clearing zone was made by *Chrysosporium indicum* (7mm), *Microsporum gypseum* (7mm), Acremonium *strictum* (8mm) and *Acremonium* Sp. (6mm). While *Malbranchea* sp. (4mm), *Crysosporium tropicum* (4mm), *Aspergillus candidus* (4mm) was found moderated (Fig.1&2, Table 1).

Friedrich *et al*., [25] found about 54% fungi exhibited growth and keratin degradation by the excreted enzymes. In the compendium of soil fungi some data indicating the possible keratinolytic potential of those species could be found, thus confirming our results. Some *Acremonium* species were reported to produce proteinase, and grow on hair, feather or wool, and *Fusarium culmorum* mycelial extract caused skin lesions in rabbits. All these properties might be connected with the keratinolytic activity of these fungi [27]. CHAO Ya-Peng *et al*., [28] obtained more than 200 microorganisms from different sources (*Aspergillus*, *Streptomyces*, *Bacillus* and *Vibrio*) screened.

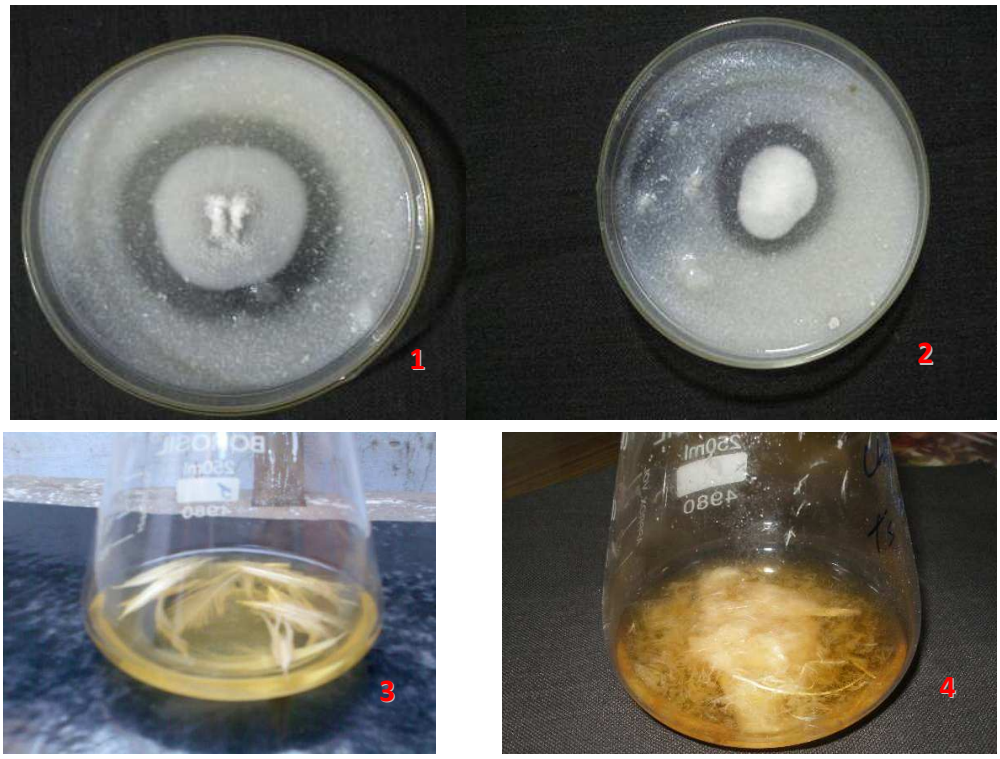

Fig. 1. Clearing zone by *Acremonium* sp. on Skimmed Milk Agar
Fig. 2. Clearing zone by *Chrysosporium indicum* on Skimmed Milk Agar
Fig. 3. Control flask
Fig. 4. Degradation of feather by *Chrysosporium indicum*

Screening of fungi submerged condition

The highest keratinase production in 8 day incubation was *Acremonium strictum* (74.40Unit/ml), *Microsporum gypseum* (70.18), *Chrysosporium indicum* (67.02Unit/ml), *Chrysosporium tropicum* (65.67Unit/ml), *Penicillium griseofulvum* (49.62Unit/ml), *Malbranchea* sp. (43.56Unit/ml) *Myceliophthora fergusii* (42.34) and *Gymnoascus intermedius* (39.60Unit/ml). While genera *Cheatomium*, *Eurotium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phoma and Rhizopus* were not shown any production of keratinase in submerged culture condition (Table 1). Other fungi were found moderate in production of keratinase. Twelve fungi were selected on the basis of results of solid plate screening and preliminary screening in submerged culture (8d), were grown in production medium for 12 day incubation for observation of keratinase, % wt loss, biomass and final pH of the medium (Table 2). *Acremonium strictum* was found to produce 124.72 Unit/ml while *Chrysosporium indicum* 110.10Unit/ml and *Microsporum gypseum* 104.60Unit/ml were next to it. Three maximum producer of keratinase were tested on 2 production media. *Chrysosporium indicum*, *Microsporum gypseum*, *Acremonium strictum* were incubated for of 4, 8,12, 16, 20 days Maximum production was achieved on 12 day in medium 1st by all three fungi (Table 3). Maximum percent weight loss in feather was achieved by *Chrysosporium indicum* 76.54% due to degradation (Fig.3-4). The maximum change in pH of the medium is induced by *Acremonium strictum* 9.4 followed by *Microsporum gypseum* and *Chrysosporium indicum* (Table 2).

Table 1: Keratinase production and clearing zone on skimmed milk agar plate

Fungi	Keratinase production	Clearing zone (mm) Incubation period			
	U/mL (8Day)	3	6	9	12
Acremonium kiliense (GPCK 3725)	36.06	0	1	2	2
Acremonium recifei (GPCK 3510)	45.04	0	1	2	3
Acremonium sp. (GPCK 3619)	55.58	2	3	5	6
Acremonium strictum (GPCK 3629)	74.40	3	5	7	8
Alternaria alternata (GPCK 3512)	29.12	1	2	2	2
Amauroascus mutates (GPCK 3708)	16.87	0	0	1	1
Aphanoascus fulvescens (GPCK 3700)	23.54	0	0	0	0
Aphanoascus keratinophilus(GPCK 3765)	30.78	0	1	2	2
Aphanoascus terreus (GPCK 3759)	41.56	2	3	3	3
Arthoroderma simii (GPCK 3724)	23.00	0	0	1	2
Aspergillus candidus (GPCK 3995)	37.62	2	3	4	4
Aspergillus fumigates(GPCK 3629)	14.89	0	1	1	1
Aspergillus sparsus (GPCK 3645)	0.0	0	0	0	0
Aspergillus sydowii (GPCK 3737)	09.0	0	0	0	0
Aspergillus terreus (GPCK 3761)	12.89	0	0	0	0
Aspergillus ustus (GPCK 3525)	0.0	0	0	0	0
Auxarthron conjugatum(GPCK 3732)	0.0	0	0	0	0
Cheatomium globosum(GPCK 3729)	0.0	0	0	0	0
Chrysosporium indicum (GPCK 3733)	67.02	2	4	5	7
Chrysosporium keratinophilum(GPCK 3661)	34.53	0	1	2	2
Chrysosporium merdarium(GPCK 3707)	14.56	0	0	0	0
Chrysosporium pannicola(GPCK 3699)	17.65	0	0	0	0
Chrysosporium queenslandicum(GPCK 3731)	15.76	0	0	1	1
Chrysosporium sp.1	0.0	0	0	0	0
Chrysosporium sp. 2(GPCK 3530)	21.76	1	2	2	2
Chrysosporium sulphureum(GPCK 3596)	5.00	0	0	0	0
Chrysosporium tropicum(GPCK 3527)	65.67	2	3	4	4
Chrysosporium zonatum(GPCK 3588)	0.0	0	0	0	0
Ctenomyces serratus(GPCK 3524)	0.0	0	0	0	0
Curvularia geniculata (GPCK 3728)	13.67	0	0	0	0
Curvularia lunata(GPCK 3740)	32.20	0	0	0	0
Epidermophyton sp. (GPCK 3565)	35.87	1	2	3	4
Eurotium repens (GPCK 3671)	0.0	0	0	0	0
Fusarium proliferatum (GPCK 3753)	24.87	0	0	0	0
Fusarium oxysporum(GPCK 3736)	37.60	1	2	3	3
Geomyces pannorum(GPCK 3756)	27.29	1	2	2	2
Gymnoascus intermedius(GPCK 3528)	39.60	0	1	1	1
Gymnoascus reessii(GPCK 3556)	0.00	0	0	0	0
Humicola griesa(GPCK 3758)	15.48	0	0	0	0
Malbranchea aurantiaca(GPCK 3551)	35.70	1	2	2	2
Malbranchea chrysosporoidea(GPCK 3764)	0.00	0	0	0	0
Malbranchea flava(GPCK 3616)	17.00	0	0	0	0
Malbranchea gypsea(GPCK 3629)	32.85	1	2	2	2
Malbranchea pulchella(GPCK 3578)	36.78	0	1	2	2
Malbranchea sp.	43.56	0	2	3	4
Microsporum canis (GPCK 3755)	18.56	0	1	1	1
Microsporum cookie(GPCK 3705)	12.76	0	0	0	0
Microsporum equinum(GPCK 3657)	29.43	1	2	2	2
Microsporum fulvum(GPCK 3676)	10.67	0	0	0	0
Microsporum gypseum (GPCK 3730)	70.18	2	3	5	7
Microsporum nanum(GPCK 3542)	0.00	0	0	0	0
Microsporum vanbreuseghemii(GPCK 3767)	12.67	0	1	1	1
Mucor sp. (GPCK 3506)	0.00	0	0	0	0
Myceliophthora fergusii(GPCK 3505)	42.34	0	2	3	3
Myceliophthora vellerea(GPCK 3766)	34.56	0	1	1	1
Paecilomyces javanicus (GPCK 3663)	0.0	0	0	0	0
Paecilomyces crustaceous(GPCK 3620)	0.0	0	0	0	0
Paecilomyces fusisporus(GPCK 3526)	0.0	0	0	0	0
Paecilomyces sp. (GPCK 3637)	0.0	0	0	0	0
Penicillium chrysogenum(GPCK 3701)	09.89	0	0	1	1
Penicillium griseofulvum(GPCK 3709)	38.62	0	1	2	2
Penicillium pusillus(GPCK 3540)	0.00	0	0	0	0
Phoma humicola (GPCK 3779)	0.0	0	0	0	0
Rhizomucor sp. (GPCK 3659)	09.63	0	1	1	1
Rhizopus sp. (GPCK 3688)	0.0	0	0	0	0
Trichoderma viride (GPCK 3664)	14.60	0	1	1	1
Trichophyton ajelloi (GPCK 3735)	23.50	0	1	2	2
Trichophyton mentagrophytes(GPCK 3674)	38.79	0	1	2	3
Trichophyton oryzae (GPCK 3584)	29.21	0	0	1	1
Trichophyton rubrum(GPCK 3722)	19.57	0	1	1	1
Trichophyton simii (GPCK 3537)	29.67	1	2	3	3
Trichophyton terrestre (GPCK 3543)	14.76	0	0	0	0
Verticillium sp. (GPCK 3634)	12.15	0	0	0	0

Table 2: Keratinase production by nondermatophytic keratinophilic fungi (12Days)

Fungus	Keratinase production (U/mL)	Final pH	% Wt loss	Biomass
Acremonium strictum (GPCK 3629)	124.72	9.2	62.50	308
Aphanoascus terreus (GPCK 3759)	67.80	8.4	55.00	278
Chrysosporium indicum (GPCK 3733)	110.10	9.1	67.00	182
Chrysosporium keratinophilum(GPCK 3661)	53.67	7.9	48.00	243
Chrysosporium tropicum(GPCK 3527)	78.64	8.8	57.00	302
Fusarium oxysporum(GPCK 3736)	49.75	7.8	45.00	394
Gymnoascus intermedius(GPCK 3528)	53.35	7.9	38.00	293
Malbranchea aurantiaca(GPCK 3551)	46.56	7.5	35.00	283
Malbranchea pulchella(GPCK 3578)	47.67	7.6	41.50	308
Microsporum gypseum (GPCK 3730)	104.60	8.9	60.50	297
Myceliophthora fergusii(GPCK 3505)	53.90	7.9	35.00	538
Penicillium griseofulvum(GPCK 3709)	58.65	8.0	44.00	297
Verticillium sp. (GPCK 3634)	35.23	7.2	32.00	256

Table 3: Keratinase production by fungi on selected media

Fungi	Media	Keratinase (U/mL) Incubation time (Days) 4	8	12	16	20	% wt. loss (20 D)
Acremonium strictum (GPCK 3629)	M1	55.67	74.78	124.72	68.54	53.43	74.23
	M2	46.56	56.76	80.35	57.87	43.45	65.76
Chrysosporium indicum (GPCK 3733)	M1	56.30	68.4	117.10	64.8	54.17	76.56
	M2	38.90	57.50	79.33	58.20	43.20	61.35
Microsporum gypseum (GPCK 3730)	M1	74.90	76.90	104.60	76.75	39.50	71.90
	M2	40.40	64.95	73.94	69.62	57.60	58.78

M1& M2 are two different media

However, Biswas *et al*., [31] screened 31 isolates of species belonging to the family Gymnoascaceae for keratinase activity using human hair as substrate. Pissuwan and Suntornsuk [32] 52 keratinase-producing bacterial strains isolated from soils in Thailand and screened on a semi-solid agar medium containing 5% feather powder as a substrate at 37^0C. They produced keratinase in a range of 0.7-2.6 Unit/ml by shaking cultivation. Elíades *et al*., [33] was carried out screening on 69 fungal to determine their ability to grow at alkaline pH. A total of 32 fungi were supplemented with soybean meal (SM) and tryptone and on cow hair (CH) under solid state fermentation conditions. Although several fungal strains produced keratinolytic activity on both SM and CH, they differed in the levels detected.

CONCLUSION

Screening tests on agar plates showed that about 20% of the fungi were unable to grow in the given environmental conditions. About 28% were capable of some growth but did not make clear zone on milk agar, presumably due to the lack of extracellular enzymes or specific protease activity. Only about 52% of fungi exhibited growth and made clearing zone on Skimed milk agar plate. The highest keratinase production in 8 day incubation was founded by *Acremonium strictum* (74.40Unit/ml), while genera *Cheatomium*, *Eurotium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phoma and Rhizopus* were not shown any production of keratinase in submerged culture condition. Other fungi were found moderate in production of keratinase. There is a change in the pH of medium towards alkalinity after the release of cysteine, protein and keratinase by fungi. It has been proposed that the basis of keratinolysis is high quantity and as a result the spent media becomes alkaline

Acknowledgement
One of us RKSK is thankful to University Grant Ccommision New Delhi.

REFERENCES

[1] Ignatova Z; Gousterova A; Spassov G; Nedkov P. *Can J Microbiol*. **1999**, 45(3), 217-222.
[2] Ramnani P; Singh R; Gupta R. *Can J Microbiol*. **2005**,51: 191-196
[3] Onifade AA; Al-Sane NA; Al-Musallam AA; Al-Zarban S. *Biores. Technol*.**1998**, 66:1-11.
[4] Zerdani IM, Faid and Malki. *Afr. J. Biotechnol*. **2004**, 3: 67-70.
[5] Korkmaz H; Hür H; Dinçer S. *Annals of Microbiology*. **2004**, 54(2): 201-211.
[6] Cai C; Zheng X. *J. Ind Microbiol. Biotechnol.* **2009**, 36:875–883.
[7] Riffel A; Brandelli A. *Brazilian J Microbiol*. **2006**, 37: 395-399.
[8] Brandelli A; Riffel A. *Electronic J. of Biotechnology*. **2005**, 8(1): 35-42.
[9] Montero-Barrientos M; Rivas R; Velazquez E; Monte E; Roig MG. *Int. J Syst. Evol. Microbiol*.**2005**, 55(6): 2491-2495.
[10] Kumar J; Kushwaha RKS. *Advances in Applied Science Research*.**2012**, 3(5):3233-3242.

[11] Mignon B; Swinnen M; Bouchara JP; Hofinger M; Nikkles A; Pierard G; Gerday C; Losson B. *Med Mycol*.**1998**, 36:395-404.
[12] Qin LM; Dehio S; Jidoi J. *Zentralbl Bacteriol*.**1992**, 277:236-244.
[13] Tsuboi R; Ko IJ; Takamori K; Ogawa H. *Infect. Immun*. **1989**, 57:3479-3483.
[14] Bressollier P; Letourneau F; Urdaci M; Verneuil B. *Applied and Environ Microbio*.**1999**, 65:2570–2576.
[15] Bockle B; Galunsky B; Muller R. *Applied and Environ Microbiol*. **1995**, 61:3705–3710.
[16] Schrooyen PMM; Meer RV; Kruiftc G. *Proc. Nut. Soc*. **2001**, 60: 475-479.
[17] De Toni CH; Richter MF; Chagas JR; Henriques JA; Termignoni C. *Can J Microbiol*. **2002**, 48:342–348.
[18] Gradisar H; Kern S; Friedrich J. *Appl Microbiol Biotechnol*. **2000**, 53:196–200.
[19] Farag AM; Hassan MA. *Enzyme Microb. Technol*. **2004**, 34: 85-93.
[20] Upadhyay MK; Kumar R; Kumar A; Gupta S; Kumari M; Singh A; Jain D; Verma HN. *African Journal of Agri. Research*. **2010**, 5 (14):1845-1850.
[21] Takiuchi I; Higuchi D; Sei Y; Koga M. *Sabouraudia*. **1982**, 20:281-288.
[22] Rajak RC; Parweakar S; Malviya HK; Hasija SK. *Mycopathologia*. **1991**, 114: 83-87.
[23] Kunert J. In: Kushwaha RKS, Guarro J (eds). *Biology of Dermatophytes and Other Keratinophilic Fungi*. Bilbao: *Revista Iberoamericana de Micologıa*. **2000**, 77-85.
[24] Ramnani P; Gupta. *Biotechnol. Apl. Biochem*. **2004**, 40:191-196
[25] Friedrich J; Gradisar H; Mandin D; Chaumont JP. *Letters in Applied Microbiology*.**1999**, 28:127-130.
[26] Dozie INS; Okeke CN; Unaeze NC. *World Journal of Microbiology and Biotechnology*.**1994**, 10:563–567.
[27] Domesch KH; Gams WV; Anderson TH. Compendium of soil fungi. Academic Press, London-San Francisco. **1980**
[28] Chao YP; Xi FH; Yang J; Lu JH; Qian SJ. J *Environ Sci. (China)*.**2007**, 19 (9):1125-8.
[29] Cai C; Lou B; Zheng X. *J Zhejiang Univ. Sci*. **2008**, 9:60-67.
[30] Kaul S; Sumbali G. *Mycopathologia*. **1999**, 146:19–24.
[31] Biswas SB; Gupta M; Ghosh GR. *Kavaka*.**1988**, 14:81-85.
[32] Pissuwan D; Suntornsuk W. *Kasetsart Journal*. **2001**, 35(2):171-178.
[33] Elíades LE; Marta C; Claudio V; Betina G; Mario S. *World Journal of Microbiology and Biotechnology*.**2010**, 26:2105-2111.