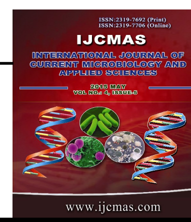
Int.J.Curr.Microbiol.App.Sci (2015) 4(5): 122-132



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 122-132**
http://www.ijcmas.com

Original Research Article

Extracellular Enzymatic Profile of Fungal Deteriogens of Historical Palace of Ujjain

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A B S T R A C T

Present study is aimed to evaluate extracellular enzymatic profiles of seven stone deteriorating fungi, isolated from historically significant Kaliadeh Palace, Ujjain so as to provide information about their mode of nutrition and industrial exploitation. The enzymatic activity of fungi wwas performed using specific substrates for the analysis of different enzymes such as amylases, cellulases, lipases, caseinase, catalase and urease. Among the seven test fungi, A. *niger*, A. *flavus,* Eurotium *amsteldomi* and Alternaria sp. were found to produce extracellular amylase enzyme. Eurotium *amsteldomi* and A. *flavus* were found positive for cellulase production and A. *niger* and A. *flavus* were positive for lipase production. Catalase activity was shown by *Eurotium amsteldomi*, *Curvularia sp* and *Talaromyces purpurogenus*. While the Urease activity is shown by only A. *flavus*.

Keywords

Extracellular enzyme, stone deterioration, amylase, cellulose

Introduction

Significant percentage of world's cultural heritage is made from stones say it be sandstone, limestone, marble, granite etc. The transformation of rocks or stone into sand and soil is a natural recycling process which is essential to sustain life on earth. However, their action on historically important artifacts in turn may lead to permanent loss of nation's heritage (Scheerer *et al.,* 2009). The stone surfaces present a complex ecosystem for a variety of biological forms like m.o. such as bacteria, fungi, algae, lichens as well as insects, mites and even higher plants once the colonization take place. Various studies have demonstrated the involvement of m.o. specially fungi in biodeterioration of museum collections (Valentin, 2010); library materials (Kowalik, 1980); lower denomination currency notes (Sharma and Sumbali, 2014) and in biodeterioration of even stones (Gadd, 2007). Fungi has the ability to decompose organic matter which leads to destruction of food, textiles, plastics, leathers and some other "unlikely" substances (Barron, 1977). Stone by itself does not favour the growth of fungi unless it has some organic residues of plant leaves, bird droppings guano or waste product of algae or bacteria on it (Sterflinger, 2000).

Evidence of fungi on material of varied composition explores their potential to spoil the materials aesthetically, physically & biochemically. The biochemical deteriorating effect of fungi depicts the involvement of extracellular enzymes secreted by them and causing degradation or deterioration of substrate.

Fungi cause deterioration of stone by means of both physical and chemical attack which more often result synergistic in deterioration. In fact, several cryptoendolithic fungi may actively bore into the stone and hence physically disrupt its integrity (Gadd, 2007). It can form cracks, fissures, and crevices, extend existing ones and lead to the detachment of crystals (Sterflinger, 2000). Biochemical action of fungi may lead to pitting and etching, mineral dislocation and dissolution (Gadd, 2007). Various metabolic substances excreted by fungi are colored, leading to staining of the substrate (Tiano, 2002). Hyphal sheath polysaccharides are directly involved in different aspects of stone biodeterioration such as adhesion of aerosols; solubilization of stone minerals; deposition of carbonates and bicarbonates; formation of complexes with melanin, and other organic and mineral compounds (Gutierrez *et al.,* 1995). The production of melanins by dematiaceous (dark pigmented mitosporic) fungi darkens the stone surface, leading to significant aesthetic alterations, and physical stress.

Number of extracellular hydrolytic enzymes excreted by fungi lead to the formation of acidic products that cause chemical changes of the material under attack (Koestler, 2000). The most prevalent enzymes involved in deterioration are Cellulases which catalyze hydrolysis of cellulose, break internal bonds to disrupt its crystalline structure (Bhat and Bhat, 1997). Proteases a complex group of hydrolytic enzymes, which add water across amide bonds and thus hydrolyze proteins into small peptides and amino acid. Previous studies report for synthesis of protease by *Aspergillus* & *Penicillium* strains (Chrzanowska *et al.,* 1993). Other m.o. like bacteria also produce protease but fungal proteases are active over a wide pH range and is not so substrate specific (Hankin, 1971). A single organism can produce more than one type of protease. Fungi are known to produce acid, neutral, alkaline, and metallo proteases (Teather and Wood, 1982). In a study on Physiological diversity of the first filamentous fungi isolated from the hypersaline dead sea intensity on the agar media, Molitoris *et al.* (2000) described that caseinase showed generally the highest activities under all conditions tested with little effect of salinity and temperature. Due to their ability to survive in this unique ecological niche, these fungi may possess novel enzymes with improved stability and a specific activity that could be exploited for industrial applications (Kobakhidze *et al.,* 2012). In the present study seven isolates obtained from under maintained and deteriorated historically significant Kaliadeh Palace, Ujjain were evaluated for their extracellular enzymatic profiles so as to provide information regarding their industrial exploitation (Table 1).

Materials and Methods

Fungal isolates and Inoculum preparation

Cultures of all the 7 test fungi, *A. niger*, *A. flavus, Eurotium amsteldomi, Curvularia sp*, *Fusarium sp, Talaromyces purpurogenus* and *Alternaria sp*. were maintained on PDA slants at 28°C. The method employed for inoculum preparation was according to Petrikkou *et al.* (2001). The isolates were subcultured on potato dextrose agar and all

isolates except members of the genus *Fusarium* were incubated at 35°C; *Fusarium* sp was incubated at 30°C. Inoculum suspensions were prepared from fresh, mature (7-days old) cultures grown on potato dextrose agar slants. The colonies were covered with 5 ml of sterile distilled water. 5% of Tween 20 was added to facilitate the preparation of inoculums of *Aspergillus* sp.

The inoculums of *Aspergillus sp* were prepared by gently rubbing the colonies with a sterile loop; the isolates were then shaken vigorously for 15 sec with a Vortex mixer and then transferred to a sterile tube. For slowly sporulating fungi, such as *Fusarium*, the suspensions were obtained by exhaustive scraping of the surface with a sterile loop. Afterwards, the inoculum was filtered using a sterile syringe with glass wool so as to remove the majority of the hyphae, producing an inoculum mainly composed of spore. Then the suspension was collected in a sterile tube. The inoculum size was adjusted to between 1.0×10^6 and 5.0×10^6 spores/ml by microscopic enumeration with Neubaurs chamber. Sterile 5 mm discs of Whatman No. 1 filter paper were soaked in the spore suspensions and left to dry and afterwards they were used as culture inoculums.

Enzyme assay

For each strain, enzyme hydrolysis was tested in duplicates. As per method of Janda-Ulfig *et al.* (2009), colony and hydrolysis zone diameters on different substrates were recorded in mm using a ruler.

Cellulase test

Cellulolytic activity of the test fungi isolates was determined by following the method of Pointing (1999) with some modification. In this method, inoculum discs were kept over minimal media plate supplemented with carboxymethyl cellulose (CMC) as substrate for cellulase. The petriplates were incubated in darkness for 7 days at 28°C. The petri plates were then flooded with 2% w/v aqueous Congo red and left for 15 minutes. After the stain was poured off, agar surface was rinsed with distilled water and again flooded with 1M NaCl to destain for about 15 minutes. Finally, NaCl solution was also poured off. The carboxymethyl cellulose degradation around the colony appeared as yellow opaque area against red colour for undegraded carboxymethyl cellulose test is considered as positive test.

Amylase test

Method adapted by Sanivada and Challa (2014) with some modifications has been used to detect amylase activity. A 1% soluble starch in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. The pH of the medium was adjusted to 7.0 just before autoclaving. The plates were inoculated with 5 mm inoculum disc with mycelia and incubated at 28 °C for 7 days. At the end of the incubation period, the plates were flooded with Lugol's iodine solution and a yellow colored halo around the colony could be seen in an otherwise blue medium indicating amylolytic activity.

Caseinase test

Method adapted by Sanivada and Challa (2014) with some modifications has been used to detect caseinase activity. A 1% milk powder in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. The pH of the medium was adjusted to 7.0 just before autoclaving and incubated at 28 °C for 7 days. At the end of the incubation period, the plates were observed for zone of

clearance after flooding with Coomassie brilliant blue solution.

Urease test

As described by Raghuramulu *et al,* 2011; Urease test medium (Christensen's urea agar medium) is used for urease activity. During the preparation of medium phenol red indicator is used and medium was inoculated with test fungi.

The scale for urease was the following:

(-) – no activity;
(+) – weak activity (hydrolysis zone width <2 mm);
(++) – moderate activity (hydrolysis zone width 2-5 mm);
(+++) – strong activity (hydrolysis zone width >5 mm).

Catalase test

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To determine the catalase producing potential of test fungi, small inoculums is mixed into hydrogen peroxide solution (3%).

Catalase activity was evaluated using the scale:
(-) – no activity;
(+) – weak reaction (single bubbles);
(++) – moderate reaction;
(+++) – strong reaction (abundant bubbles)

Results and Discussion

In present study, the main deteriorating fungal species isolated from deteriorating site, *A. niger, A. flavus, Eurotium amsteldomi*, *Talaromyces purpurogenus*, *Curvularia* sp, *Fusarium* sp and *Alternaria* sp were evaluated for presence of extracellular enzyme using specific substrate and the activity is detected by use of certain chemicals like Lugol's iodine for detection of amylase, Congo red for detection of cellulose and Coomassie brilliant blue for caseinase. Other tests performed were lipase test, catalase test and urease test. *A. niger* has shown highest colony diameter on starch agar & milk agar and zone of hydrolysis on them is also found to be more as compared to the other isolates. *A. niger*, *A. flavus, Eurotium amsteldomi* and *Alternaria* sp. were found to produce extracellular amylase enzyme (Table 1 & 2). *Talaromyces purpurogenus* and *A. flavus* were found positive for cellulase production and *A. niger* and *A. flavus* were also positive for producing lipase enzyme. Catalase activity was shown by *Eurotium amsteldomi*, *Curvularia* sp and *Talaromyces purpurogenus*. While the Urease activity is shown by *A. niger* and *A. flavus* (Table 3).

The fungi have shown growth on cellulose agar but hydrolysis zones were not formed by all test isolates. Slight hydrolytic activity of cellulose was observed only in *A. flavus* and *Talaromyces purpurogenus*. Whereas, other studies have demonstrated clear hydrolysis zones on cellulose agar by *A. flavus, A. fumigatus, A. nidulans, A. niger* and other species (Gopinath *et al.,* 2005; Krikstaponis *et al.,* 2001; Ulfig *et al.,* 2009). *Alternaria* sp is found to show amylase and caseinase activity. *Talaromyces purpurogenus* and *A. flavus* were showed some diffusion on CMC media, but no significant results obtained. Studies have shown that certain genera such as *Aspergillus*, *Penicillium* and *Fusarium* have been reported to produce neutral proteases. Some acidic and alkaline proteases producing fungi having commercial values are *Aspergillus* sp., *Penicillium* sp. and *Mucor* sp. (Sandhya *et al.,* 2005). All fungal isolates showed growth on media containing tributyrin as substrate. *A. flavus* was observed to produce good zone of hydrolysis

on media with tributyrin as substrate whereas *A. niger* produced comparatively shorter zone.

Urea hydrolysis was only visualized in case of *A. flavus* and *A. niger*. Urease activity in *A. niger* was first reported in 1903, yet the characteristics of this enzyme have not been determined (Pomar *et al.,* 1970). Smith *et al.* (1993) worked on first isolation and characterization of urease from *A. niger*. Deterioration of stored seeds by *A. flavus* and assessment of urease activity was reported by Dayal *et al.* (2001). *A. flavus* and *A. niger* and *Alternaria* sp. found to exhibit moderate Urease activity in comparison to *Fusarium* sp.

Catalase activity was found to be active in case of *E. amsteldomi* and *T. purpurogenus*. Catalase is used in sterilization and bleaching practices (Akertek and Tarhan, 1995). A cheap and readily available commercial source of catalase can be found in the extracellular production by several microorganisms, especially fungi strains (Chaouche *et al.,* 2013). Studies have reported that *A. fumigatus* produces three active catalases; one is produced by conidia, and two by mycelia (Paris *et al.,* 2003). *A. niger* has also been shown to produce catalase enzyme with high stability by Isobe *et al.* (2006).

E. amstelodami showed the lowest colony diameter on milk agar and no zone of hydrolysis were observed in *E. amstelodami* on milk agar (Table 1& 2). Whereas in case of *A. niger*, both the colony diameters and zone of hydrolysis on milk agar was highest. On tributyrin agar hydrolysis zones around the colonies were observed in *E. amstelodami.* No zones were observed on cellulose. Our finding of high amylase and lipase activities in *Aspergillus* partially agrees with Gopinath *et al.* (2005). They reported the same for *A. versicolor* from oil-rich environments. The *E. amstelodami* catalase reaction ranged from weak (single bubbles) to strong (abundant bubbles) (Table 2). The urease activities were found to be weak in *E. amstelodami* respectively.

Table.1 Colony diameters of various fungi isolated from deteriorating site after 7 days incubation at 25°C

Test fungi	Colony diameter* of test fungi on media containing			
	Starch	Milk	Cellulose	Tributyrin
A. niger	35.6 ± 3.2	34.4± 3	28.3± 2.6	14.1 ±1.5
A. flavus	29.8 ±0.5	32.8± 1.2	34.3 ± 3	19±0.8
Eurotium amsteldomi	30.2± 4.0	21.2 ±2.3	14.1±2.2	30.2± 3.1
Talaromyces purpurogenus	31.0±0.8	22±2.0	14±0.4	28.5±0.9
Curvularia sp	26.1±0.2	26±1.8	12±0.2	27.1±0.3
Fusarium sp	24.4±3.0	30±1.5	18±2.1	18.5±2.6
Alternaria sp	22.0±2.9	22±1.2	23±2.4	19.8±2.0

*Mean and standard deviation

Plate.1 Detection of Amylase on minimal agar+Starch (With / without glucose); (a)-(d) Growth of different isolates on media (e)-(i) Addition of iodine to the plate

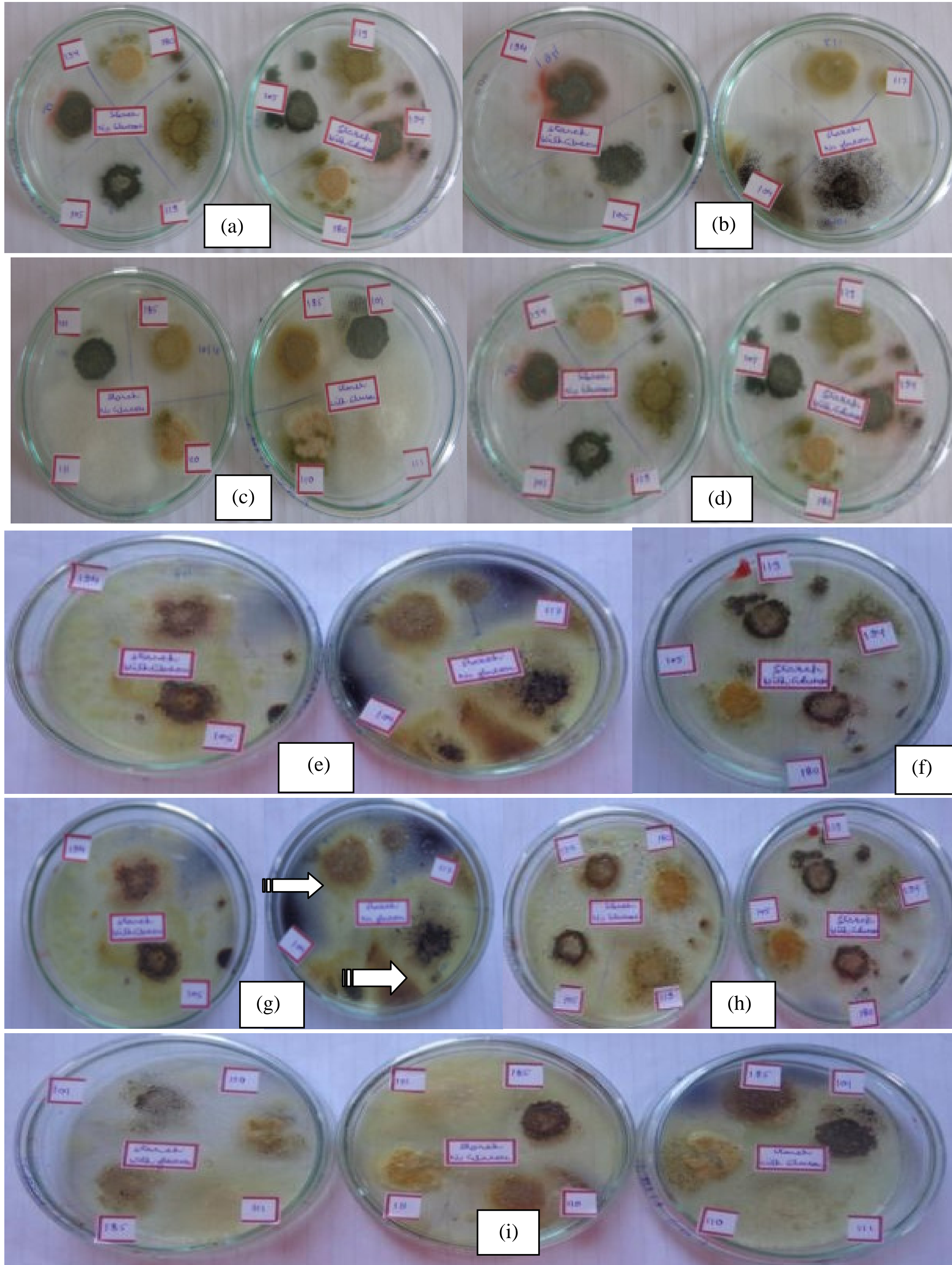


Plate.2 Detection of Casein hydrolyzing enzyme on minimal agar +casein (with/without glucose); (a)-(d) Growth of different isolates on media; (e)-(f) Addition of Coomassie brilliant blue to the plate

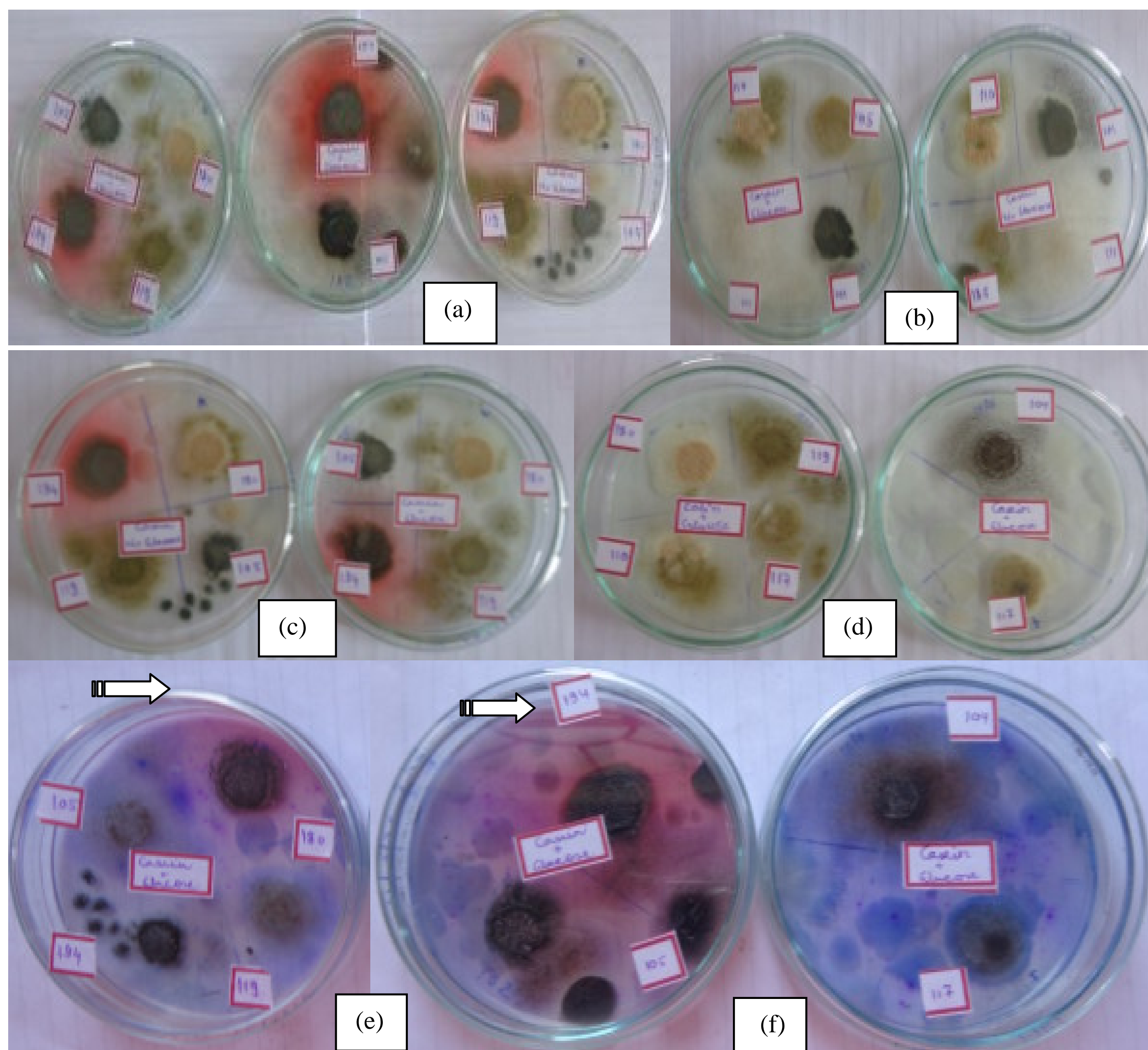
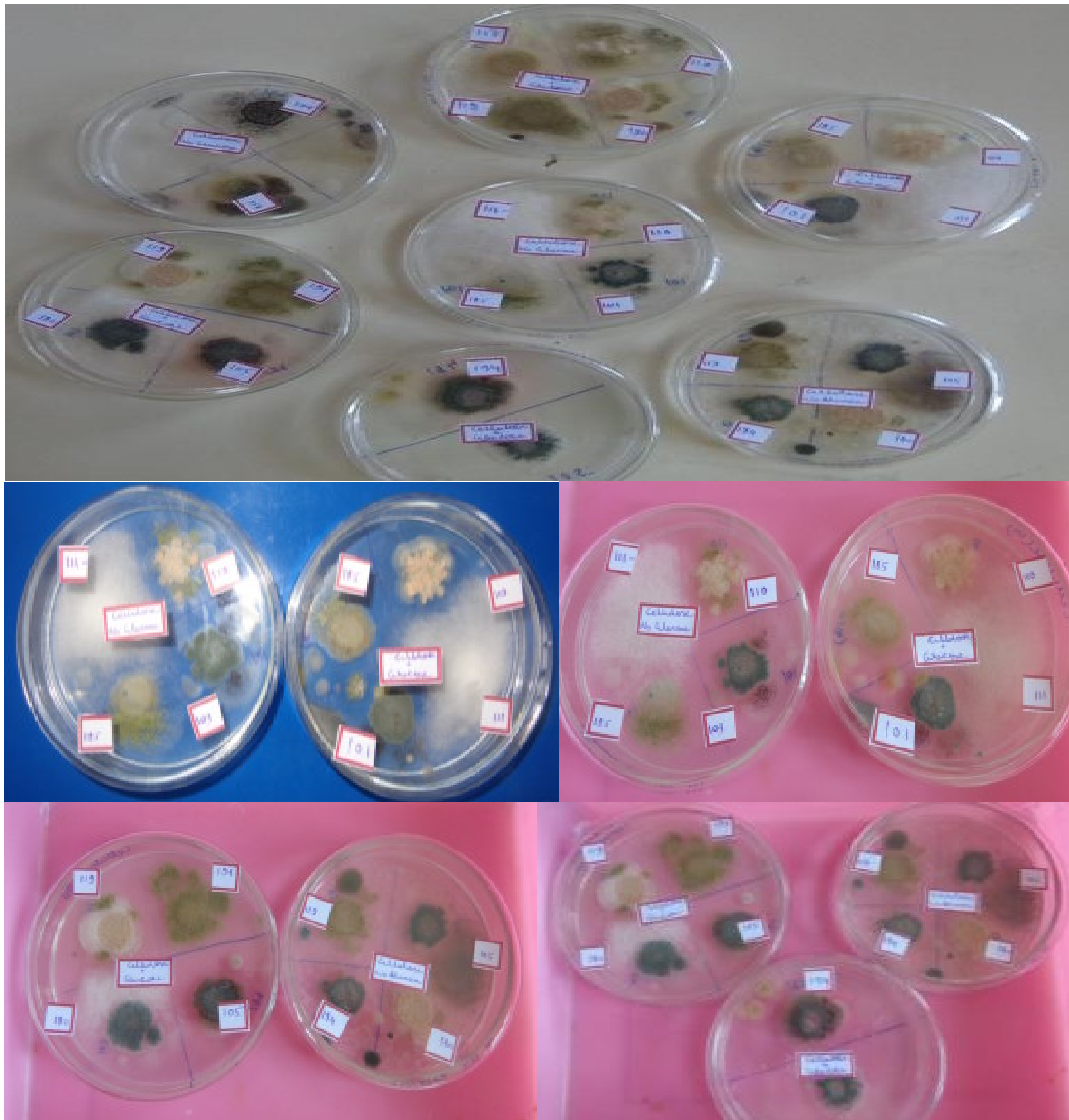


Table.2 Hydrolytic activity of stone deteriorating fungi after 7 days incubation at 25°C on agar containing different substrates

Test fungi	**Hydrolysis zone diameter on media containing**			
	Starch	Milk	Cellulose	Tributyrin
A. niger	0.9 ±0.1	0.9 ±0.0	0.0	0.7±0.2
A. flavus	1± 0.1	0.8± 0.1	0.8±0.1	1.1 ±0
Eurotium amsteldomi	0.6±0.2	0.0	0.0	0.0
Talaromyces purpurogenus	0.0	0.9±0.2	0.7±2.1	0.0
Curvularia sp	0.0	0.0	0.0	0.0
Fusarium sp	0.0	0.0	0.0	0.0
Alternaria sp	0.5±0.3	0.4±0.3	0.0	0.0

*Mean and standard deviation

Plate.3 Detection of Cellulase enzyme on minimal agar +CMC (with/without glucose)
(a)-(d) Growth of different isolates on media; (e)-(f) Addition of Coomassie brilliant blue to the plate



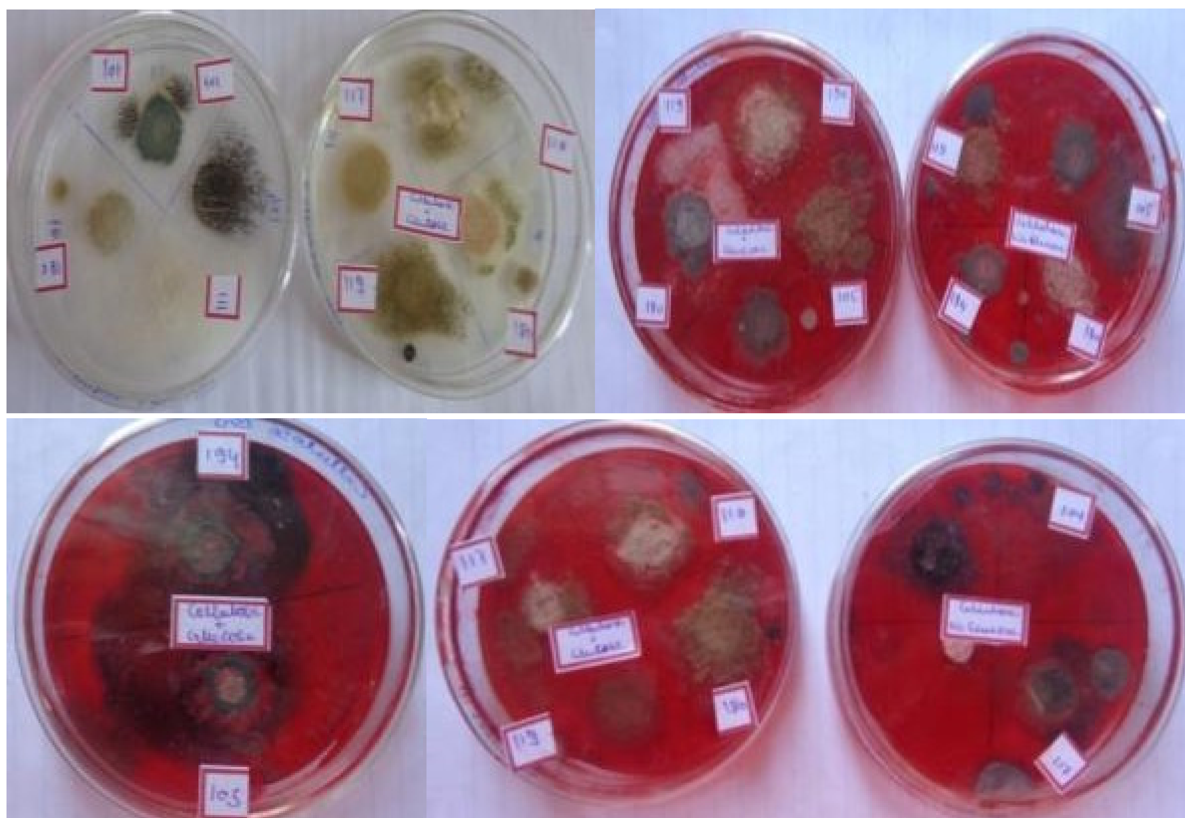


Table.3 Catalase and Urease tests

Test fungi	Catalase	Urease
A. niger	+	++
A. flavus	++	+++
Eurotium amsteldomi	+	-
Talaromyces purpurogenus	+	-
Curvularia sp	-	-
Fusarium sp	-	+
Alternaria sp	-	++

Assessed as - = none, + = weak, ++ = moderate, and +++= strong activity

The conclusion is that among seven stone deteriorating fungi, *Aspergillus sp and E. amstelodami* are the species with the highest biodeterioration potential to stone artifacts. Extracellular enzymes play an important role in degradation and solubiization of substrate and to make it available for m.o. for nutrition and growth. On the other hand extracellular enzymatic profile may provide information about the other microbial communities for providing varying substrate for heterotrophic fungi. Knowledge about the hydrolysis of various substrates will provide an insight for commercial exploitation of stone deteriorating fungi. Our study is aimed to exploit the enzymatic potential of fungal isolates from deteriorated historical palace of Ujjain. However further study is required to evaluate the effect of different pH, temperature, carbon source etc.

for commercial exploitation of enzymatic activities of stone deteriorating fungi.

Acknowledgement

The authors wish to extend sincere thanks to Dr. Mamta Srivastava, Principal, Mata Gujri College of Professional Studies, Indore, India for giving the permission to persue the research work in Institution. Also, would like to express deep gratitude towards Dr. Sadhana Nighojkar, Head, Biosciences Department for her suggestions during the work.

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