



Bio-prospecting of marine-derived fungi with special reference to production of keratinase enzyme - A need-based optimization study

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ABSTRACT

Keratin derived from poultry, and other keratinous waste remains a headache to the human society and also to the nature. Marine fungi - an untapped resource to be exploited specifically for the production of keratinase. In this connection, marine sediment samples were randomly collected from Kovalam located along the south west coast of Tamil Nadu. Two marine-derived fungi were isolated from the sediment samples. The internal transcribed spacer region of the isolates was sequenced, submitted to the GenBank and an individual accession number obtained for *Colletotrichum capsici* (KY817475) and *Curvularia lunata* (KY828216). Keratinase enzyme was produced with *C. capsici* and *C. lunata* at pH 8.5, and total protein was estimated. The amount of protein produced by *C. capsici* was found to be 0.91 mg/ml and by *C. lunata* was 1.06 mg/ml. The molecular weight of keratinase was determined as 35kDa. Four parameters through pH, concentration of feather meal, carbon source, and nitrogen source were optimized during the present study. Keratinase production media inoculated with *C. capsici* and *C. lunata* exhibit OD_{max} with respect to feather meal concentration and corresponding OD was 1.264 mg/ml and 0.639 mg/ml. The OD_{max} for *C. capsici* and *C. lunata* supplemented with maltose as carbon source was 1.281 mg/ml and 1.426 mg/ml. The OD_{max} for *C. capsici* and *C. lunata* supplemented with yeast extract as nitrogen source and corresponding OD was 1.858 mg/ml and 1.613 mg/ml. Thus, we conclude that marine-derived fungi *C. capsici* and *C. lunata* are potential producers of ecovaluable keratinase which could be used up to treat the ecological burden created by keratinous waste.

1. INTRODUCTION

Soil-borne microfungi are reported to synthesize a wide variety of hydrolytic enzymes. Still several species are exploited in the industries toward the production of industrially important enzymes such as cellulase, pectinase, carbohydrase, and lipase. A unique class of enzyme called keratinase produced by microfungi population has both biotechnological, biomedical potentials, and ecological benefits. Some clinically important pathogenic dermatophytes such as *Trichophyton* spp. [1] produce this enzyme to invade the skin tissues. On the other hand, the microbial keratinase has ecovaluable role since it is being applied in the bioconversion of poultry and other keratinous waste. For this purpose keratinases trapped from *Streptomyces* spp. [2,3] and *Bacillus* spp. [4,5] have been investigated. Around 24 billion chickens were killed annually consequently 8.5 billion tone of poultry feather

waste are generated. As per a recent citation from India's leading newspaper that our country alone contributes 350 million tone of poultry waste. Discarded feather causes several ailments to human beings such as chlorosis, mycoplasmosis, and fowl cholera [6]. Poultry waste drenched during the rainy season gradually percolates into the soil subsequently affecting the groundwater. Microbial mediated degradation of keratinous waste could pave a way for the development of ecofriendly method. Handling a right method of treatment of poultry waste would considerably reduce the development of pathogenic microbes. Through microbial degradation, some of the essential amino acid (lysine, histidine, and methionine) composition of the soil could be preserved [7]. After an intensive literature survey, it was found that the availability of reports with respect to marine-derived fungi mediated keratinase production is very less. Hence, an attempt has been made to isolate marine-derived fungi and subject them toward the production of keratinase. During the course of study, media optimization was carried out, and isolates were individually evaluated for the production of ecovaluable enzyme.

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2. MATERIALS AND METHODS

2.1. Collection of Marine Sediment

The sediment sample was collected randomly from the sampling spots (Lat: 8° 4'43.95"N Long: 77°31'38.71"E) along the south west coast of Tamil Nadu [Figure 1] located along the south west coast of Tamil Nadu. Sediment samples were collected by hand-pushing plastic core tubes (7 cm diameter) as far as possible into the sediment. The sediments were kept cool in icebox during the transportation to the laboratory [8], while sediment sampling usually, 50–500 ml of sea water from the sampling spot was also collected for preparation of fungus isolation media since to supply native soluble nutrients to support and enhance the growth of fungus during the time of isolation.

2.2. Isolation and Identification of Marine-Derived Fungi

Dilution plating technique [9] was used to isolate the fungi from marine sediment. After the isolation, a small tuft from the well grown fungal cultures was transferred; subcultured and individual axenic cultures were maintained. The semi-permanent slides were prepared using lactophenol cotton blue staining [10] for morphological characterization. Colony color and morphology were noted beside hyphal structure, spore size, shapes, and spore-bearing structures. They were compared with the standard works of manual of soil fungi [11]; hyphomycetes [12]; a manual of penicillin and [13]; higher fungi [14].

2.3. Molecular Characterization of Marine Fungal Isolates – Internal Transcribed Spacer (ITS) Sequencing

The fungal DNA was isolated by adapting the protocol [15] with minor modifications. The resulting genomic DNA was used as templates to amplify the 18s rDNA [16] subsequently the ITS region of the fungal DNA was amplified [17].

2.4. Preparation of Keratinase Production Medium

Large amount of chicken feather was collected from the poultry farm located in Thiruthangal, Virudhunagar district, Sivakasi, and washed well with chloroform-methanol mixture (1:1, v/v) and finally with distilled water. Chicken feather was then dried in sunlight, tyndallized at 100°C for 20 min for 5 successive days and then powdered [Figure 2]. It was moistened with a mineral salt solution in the ratio of 1:2. 10 g of feather keratin powder prepared was taken in 250 ml Erlenmeyer flasks and mixed thoroughly with 20 ml mineral salt solution [18].

2.5. Protein Estimation

The amount of total protein in the culture filtrate was quantified [19].

2.6. Molecular Weight Determination of Keratinase

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE): To determine molecular weight, keratinase enzyme and known molecular weight markers were subjected to electrophoresis. SDS-PAGE was performed with 12% polyacrylamide gels, and the protein bands were stained with a solution of Coomassie blue R-250 [20].

2.7. Optimization of Keratinase Production

2.7.1. Effect of pH on keratinase production

The effect of pH of the medium on keratinase production was observed by adjusting pH of the medium through 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5, respectively.



Figure 1: Sampling spot along the south west coast of Tamil Nadu.



Figure 2: Powdered chicken feather.

2.7.2. Effect of feather meal concentration on keratinase production

Feather minimal medium was prepared and added with various feather meal concentrations 0.5, 1, 1.5, 2, and 2.5 g, respectively.

2.7.3. Effect of N₂ source on keratinase production

Addition of different nitrogen sources such as yeast extract, urea, and ammonium sulfate on keratinase production was also studied.

2.7.4. Effect of carbon source on keratinase production

The feather basal medium was supplemented with 1% (W/V) fructose, maltose, and sucrose to study the effects of different carbon source on keratinase production.

3. RESULTS AND DISCUSSION

3.1. Analysis of Mycoflora

The study revealed the presence of two predominant fungal species. The fungi were *Colletotrichum capsici* [Figure 3] and *Curvularia lunata* [Figure 4], respectively, on potato dextrose agar medium. The presence of *C. lunata* in the marine aquatic ecosystem was confirmed [21]. Another study confirms the existence of *Curvularia* spp. as an endophytic fungus of coral reef [22] in the marine ecosystem. The percentage of distribution of *Curvularia* spp. was determined as 0.2% this clearly indicates that *Curvularia* sp is facultative to marine ecosystem [23]. The biopotentials of *Curvularia* spp. were assessed and reported to contain novel secondary metabolites called



Figure 3: *Colletotrichum capsici* on potato dextrose agar medium.



Figure 4: *Curvularia lunata* on potato dextrose agar medium.

“Curvularin” [24]. On the other hand, *Colletotrichum* spp. was already isolated and identified as saprophyte with a plethora of antifungal compounds [25]. *Colletotrichum* spp. was found to be the source of “phytosterols” [26]. Thus, the analysis confirms marine fungi are potential due to their adaptation in salt-rich environment. With respect to fungal distribution across the coastal region, it is decided by the climate of the microenvironment and several other parameters through physiochemical properties of the sediment. Further, the study confirms that distribution is quite uneven as well as prone to be altered across the geological area being studied.

3.2. Molecular Characterization of the Marine Fungi Isolates

The DNA from the isolates was resolved and analysis revealed the presence of prominent 1Kbs bands [Figure 5].

3.3. Polymerase Chain Reaction (PCR) Amplification Study

The isolated DNA was subjected to PCR study and the amplified products were resolved on agarose gel electrophoresis. The study revealed the presence of 650 bps of ITS region [Figure 6] of marine isolates.

3.4 BLAST Analysis of the ITS Sequences from Marine Isolates

The obtained sequences (ITS1 and ITS2) of *C. capsici* and *C. lunata* were submitted in the GenBank, and corresponding accession numbers were obtained KY817475 and KY828216. The BLAST analysis revealed that the sequences showed 91% identity to *C. capsici* isolate

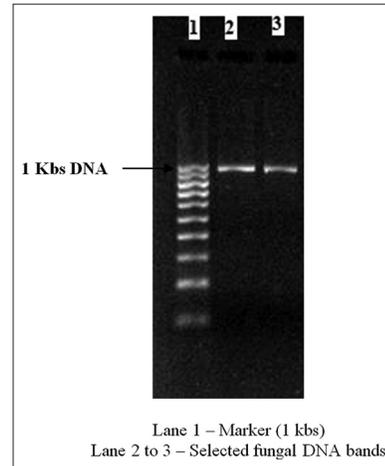


Figure 5: 1Kbs bands of fungal DNA. Lane 1 - Marker (1 kbs). Lane 2 to 3 - selected fungal DNA bands.

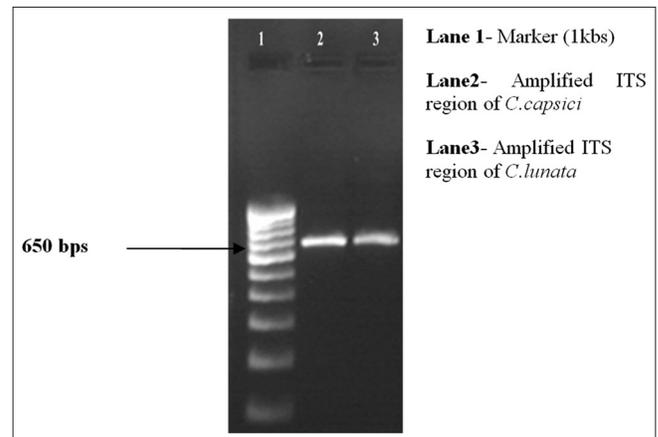


Figure 6: Polymerase chain reaction-amplified 650 bps internal transcribed spacer (ITS) region of marine isolates. Lane 1 - marker (1 kbs). Lane 2- amplified ITS region of *Colletotrichum capsici*, Lane 3 - amplified ITS region of *Curvularia lunata*.

cc2 with a query cover of 97% and showed the same 91% identity to *Fusarium chlamydosporum* strain vkm with a query cover of 97%. Hence, the preliminary BLAST analysis led us to confirm the fungal isolate. For the second isolate, the BLAST analysis revealed that the sequences showed 94% identity to *C. lunata* isolate UFMGCB4427 with a query cover of 100% and showed 100% identity to *Phoma tropica* with a query cover of 100%.

3.5. Phylogenetic Tree Analysis

A phylogenetic tree was constructed. All the two isolates were subjected to analysis. The study led us to confirm the isolate 1 designated with the code M1YSANJACBT2017 showed 100% relatedness to *C. capsici* [Figure 7]. In the same way, isolate 2 designated with the code M2YSANJACBT2017 showed 100% relatedness to *C. lunata* [Figure 8]. The identification of marine fungi isolated under ideal laboratory condition rely solely on morphological features leads to several ambiguities induced by environmental conditions hence ITS sequencing based identification is recommended [27, 28]. Combining the value-added features obtained from both microscopical examination and molecular

characterization would clear the misleading features associated with classification and identification of marine fungi. ITS domain is being considered an important tool used by several mycologist to classify marine fungi [29-32]. Thus, the molecular study concludes ITS sequences are target sequences for the species level characterization of the diversity of fungi obtained from marine environmental samples. The present study revealed that no significant divergence was exhibited by frequent fungal isolates and the gene pool remains inviolated by environmental changes. The sampling stations along the south west coast of Tamil Nadu remains an ideal place for the isolation of inviolated marine-derived fungi [33].

3.6. Production of Keratinase

Both the marine isolates (*C. capsici* and *C. lunata*) were tested for keratinase production. The pH was set at 8.5 and incubated for 5 days. After 5 days, the production medium was filtered, and the filtrate was tested for the amount of Keratinase. *C. capsici* exhibit OD value of 1.505 which corresponds to 0.91 mg/ml of keratinase. Similarly, *C. lunata* exhibit OD of 1.366 which corresponds to 1.06 mg/ml of keratinase.

3.7. Molecular Weight Determination of Keratinase by SDS-PAGE

The keratinase was resolved, and its molecular weight was determined as 35kDa [Figure 9] on SDS-PAGE. Studies based on production and purification of Keratinase using *Bacillus licheniformis* [4] revealed the molecular weight of the enzyme was 33 kDa similarly in another study were *Bacillus cereus* [34] revealed the molecular weight of keratinase was 70 kDa. According to a study using *Streptomyces pactum* a filamentous bacteria [3] the molecular weight of the protein was 30 kDa. Using fungi for the production of keratinase had revealed dramatic variations in the molecular weight of keratinase. In the same way, two different fungal species used for the production of keratinases [35] its molecular weight was reported as 33 kDa and 30 kDa, respectively. A wide range of variations can be observed in fungal keratinases (18–200 kDa) [36]. Even though the present study was contradictory to the above reports [3,4,34] later major variations are existing even among the fungal keratinases are confirmed with the report [36].

3.8. Effect of pH on Keratinase Production

The different pH values used in the present study are shown in Table 1. Among both the isolates, OD_{max} was obtained near the alkali pH. In case of *C. capsici* maximum keratinase production was obtained at the pH 7.5 [Figure 10a] and similarly in the case of *C. lunata* maximum keratinase production was recorded at pH 7.5 [Figure 10b]. Hence, it leads to the conclusion that pH 7.5 was found to be optimum for the production of Keratinase by marine fungal isolates.

The keratinase produced from the two different fungi were found to be active with a broad pH range from 3.5 to 9.5, respectively, with the optimum pH of 6.5. This result is in good agreement with keratinases obtained from other fungal sources such as *Trichophyton granulosum* [37] and *Trichophyton rubrum* [38]. However, the optimum pH of some of the keratinolytic enzymes from other sources was toward alkaline side, e.g. pH 8.0 for *Paecilomyces marquandii* and *Aspergillus fumigatus* keratinase [39]. The enzyme obtained from both the media was found to be stable at a wide range of pH's from 5 to 9. However, in the present study, quantification of the keratinase protein in the cell-free extract was made according to Lowry's method, and the concentration is directly proportional to the OD value. The greater OD value corresponds to greater protein level. Both the isolates were seems

to yield maximum amount of protein at the pH 7.5. Hence, this is the pioneering report emphasizing the production of Keratinase at pH 7.5. Further, the significant reduction in keratinase production might be due to non-availability of nutrients due to pH shift or complete depletion of nutrients or feedback inhibition as correlated with the findings [42].

3.9. Effect of Feather Meal Concentration on Keratinase Production

The bioavailability of feather meal as a substrate for the production of Keratinase as described in the [Table 2]. Among both the isolates, OD_{max} was obtained near the highest concentration of feather meal (2.5 g/100 ml). In case of *C. capsici* maximum keratinase production was obtained at the 2.5 g of feather meal [Figure 11a] and similarly in the case of *C. lunata* maximum keratinase production was recorded at 2.5 g of feather meal [Figure 11b]. Second, optimization is based on the concentration of feather meal on the production of keratinase. The report revealed that the increase in production with an increase in the concentration of feather meal. The maximum OD was obtained at 2.5 g/100 ml feather meal was used. *C. capsici* showed 1.264 and 0.639 showed by *C. lunata*.

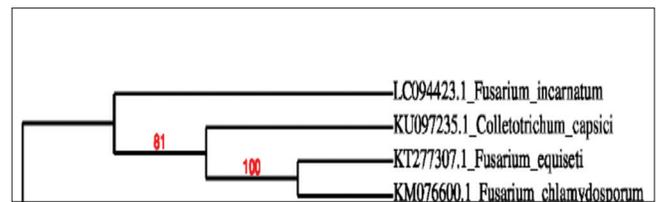


Figure 7: Isolate showed 100% relatedness to *Colletotrichum capsici*.

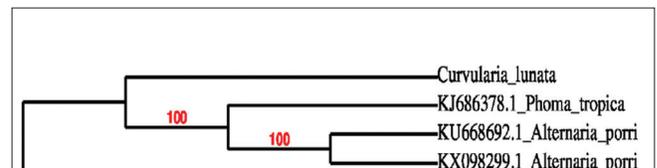


Figure 8: Isolate showed 100% relatedness to *Curvularia lunata*.

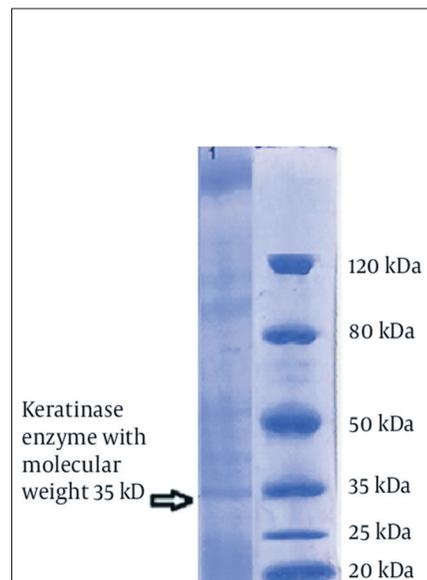


Figure 9: 35 kDa keratinase on sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

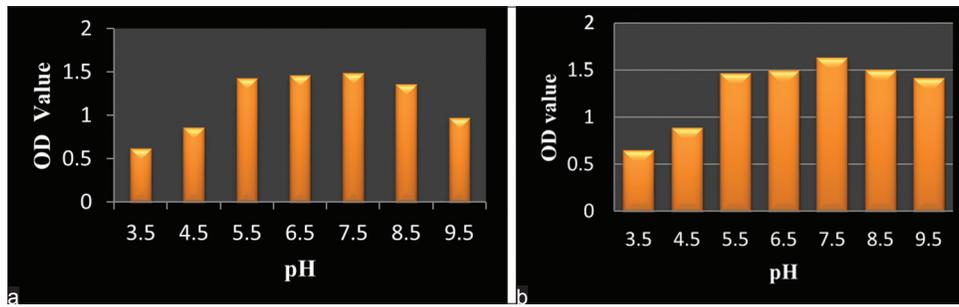


Figure 10: (a) Maximum keratinase production at pH 7.5 by *Colletotrichum capsici*, (b) Maximum keratinase production at pH 7.5 by *Curvularia lunata*.

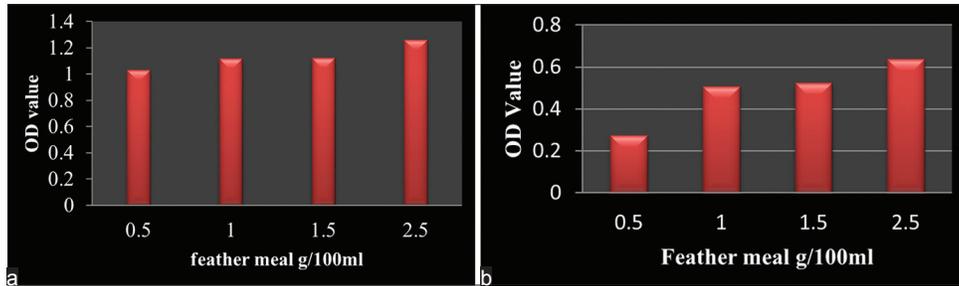


Figure 11: (a) Maximum keratinase production at 2.5 g of feather meal by *Colletotrichum capsici*, (b) Maximum keratinase production at 2.5 grams of feather meal by *Curvularia lunata*.

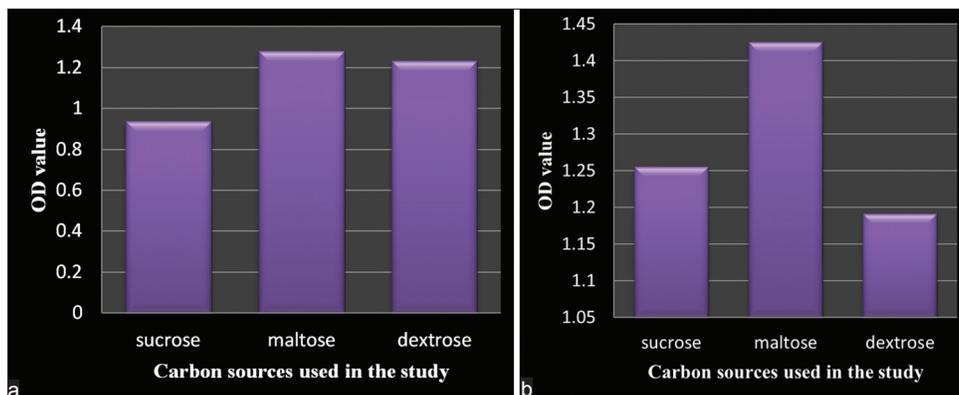


Figure 12: (a) Maximum keratinase production in maltose by *Colletotrichum capsici*, (b) Maximum keratinase production in maltose by *Curvularia lunata*

Table 1: Effect of pH and corresponding OD value for keratinase concentration

pH	<i>C. capsici</i>	<i>C. lunata</i>
3.5	0.65	0.62
4.5	0.89	0.862
5.5	1.473	1.43
6.5	1.498	1.461
7.5	1.639	1.491
8.5	1.505	1.366
9.5	1.423	0.974

C. capsici: *Colletotrichum capsici*, *C. lunata*: *Curvularia lunata*

Table 2: Effect of feather meal concentration and corresponding OD value for keratinase production

Feather meal concentration (g/100 ml)	<i>C. capsici</i>	<i>C. lunata</i>
0.5	1.034	0.275
1	1.12	0.509
1.5	1.125	0.524
2.5	1.264	0.639

C. capsici: *Colletotrichum capsici*, *C. lunata*: *Curvularia lunata*

3.10. Effect of Carbon Source on Keratinase Production

The types of carbon sources used in the present study are listed in Table 3. Among both the isolates, OD_{max} was obtained in the presence of maltose. In case of *C. capsici* maximum keratinase production was obtained in the presence of maltose [Figure 12a] and similarly in the case of *C. lunata* maximum keratinase production was recorded in the presence of maltose [Figure 12b]. In a study using *Streptomyces*

The present study was correlated with the study using amazonian bacterium *Bacillus* spp. [40] reported a yield of 1.8–8.6 g/L⁻¹ keratinase. Thus, the study concludes that the keratinase concentration increase with the increase in the concentration of feather meal.

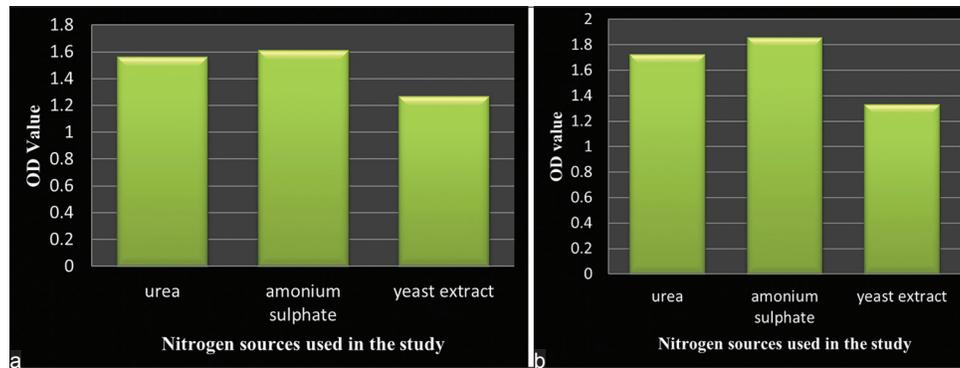


Figure 13: (a) Maximum keratinase production in ammonium sulfate by *Colletotrichum capsici*, (b) Maximum keratinase production in ammonium sulfate by *Curvularia lunata*.

Table 3: Effect of different carbon sources and corresponding OD value for keratinase production

Carbon sources	<i>C. capsici</i>	<i>C. lunata</i>
Sucrose	0.939	1.256
Maltose	1.281	1.426
Dextrose	1.234	1.192

C. capsici: *Colletotrichum capsici*, *C. lunata*: *Curvularia lunata*

Table 4: Effect of different nitrogen sources and corresponding OD value for keratinase production

Nitrogen sources	<i>C. capsici</i>	<i>C. lunata</i>
Urea	1.726	1.564
Ammonium sulfate	1.858	1.613
Yeast extract	1.334	1.269

C. capsici: *Colletotrichum capsici*, *C. lunata*: *Curvularia lunata*

gulbargensis [41] revealed the addition of starch as carbon source would result in the notable increase in the extracellular secretion of keratinase. The study further confirms there was 10-fold (14.3 U/ml) increase in the keratinase secretion in the presence of 3 g of starch per liter of the medium. In the present study, simple sugars are being used as a carbon source by marine fungi further they were able to use simple sugars to produce keratinase when compared to the above-mentioned report was starch was used as the principal carbon source which is a polysaccharide.

3.11. Effect of Nitrogen Source on Keratinase Production

The types of nitrogen sources used in the present study are listed in Table 4. Among both the isolates, OD_{max} was obtained in the presence of ammonium sulfate. In case of *C. capsici* maximum keratinase production was obtained in the presence of ammonium sulfate 3.

CONCLUSION

The present study leads to the isolation of two marine-derived fungi *C. capsici* and *C. lunata*. The ITS sequences of both the fungi were sequenced, and individual accession numbers were obtained KY817475 and KY828216. Ecovaluable keratinase enzyme was produced during the study, and its molecular weight was also determined as 35 kDa. Optimization was made with different parameters through pH of the medium, the effect of feather meal concentration, the effect of carbon sources and effect of nitrogen sources keratinase production. Based on

the observation of all the optimization studies both the isolates could be recommended as production entities.

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