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Full Length Research Paper

Production of amylase enzyme from mangrove fungal isolates

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The mangrove ecosystem serves as a bioresource for various industrially important microorganisms. The use of fungi as a source of industrially relevant enzymes led to an increased interest in the application of microbial enzymes in various industrial processes. Fungal colonies were isolated from sediments of five different mangrove ecosystems of Odisha such as Bhitarakanika, Dhamra, Mahanadi, Devi and Budhabalanga areas. Forty (40) fungal colonies were isolated and screened for amylase activity out of which five strains were found to be more active. Among the isolates I_{Famy} value was maximum; that is, 5.2 for MSF-9. The amylase enzyme activity of MSF-9 was maximum at pH-5.0, 1% NaCl, 1% substrate and Inositol as carbon source. The most potent fungi was identified through morphological, microscopical and 18S rDNA sequence methods and identified as *Penicillium citrinum*-JQ249898. This strain can be better utilized in large scale industries for enzyme production. Hence further study is suggested on enzyme purification for various value-added product formation.

Key words: Mangrove, fungi, I_{Famy}, *Penicillium citrinum*, microscopical study.

INTRODUCTION

Marine fungi is an important target group for various useful products of industrial importance such as enzymes, sugars, antibiotics, alcohols, beverages, food products etc (Gupta et al., 2007). Study based on screening of fungal resources of mangroves for enzyme and their application has been done to accomplish environment friendly technological development (Maria et al., 2005). Microbial enzymes have completely replaced the chemical hydrolysis of starch in starch process industry (Pandey et al., 2000). Amylases are among the most important enzymes in present-day biotechnology. The enzyme has found numerous applications in commercial processes, including thinning and liquefaction of starch in alcohol, brewing and sugar industries. α-Amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants (Octávio et al, 2000). In fungi, detailed studies on α-amylase purification have largely been limited to a few species of fungi (AbouZeid, 1997; Khoo et al., 1994). The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain

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enzymes of desired characteristics (Lonsane and Ramesh, 1990).

In the present study fungal strains were isolated from five different mangrove ecosystems of Odisha, India. The screening of the fungal isolates and enzyme assay of the most active strain will be carried out. Finally the identification of potent fungal strain was done by morphological, microscopical and molecular study.

MATERIALS AND METHODS

Collection of sediment

Sediment samples were collected from five different mangrove ecosystems such as Bhitarakanika, Dhamra, Mahanadi, Devi and Budhabalanga areas of Odisha, India. Sediment samples were collected with an air dried Beckman's grab. The sediment collected were aseptically transferred to labeled polythene bags and kept in an ice-chest box before transferring to the laboratory. The collected sediments were air dried for 7-10 days at room temperature in the laboratory for further study.

Isolation of microorganism

Isolation of fungi has been was done through serial dilution technique by taking one gram of air-dried sediment samples. The air-dried sediment sample was serially diluted with 50% sea water and plated on the Potato Dextrose Agar (PDA) media (Himedia) by spread plate method with the addition of 100 mg/L of ampicillin to avoid unwanted growth of bacteria and the culture plates were incubated at 28°C for 36-48 h. The total viable count (TVC) was estimated from the mixed culture plate. The pure culture was obtained by repeated sub-culturing of the fungal isolates from the mix culture plates. Then these pure cultures were maintained in fresh PDA slants and preserved at 4°C for further study.

Amylase enzyme assay

Screening of isolates

Primary screening was done by using 1% of starch soluble in the media plate. The results were recorded by using Gram's iodide in the agar plate and the strains showing hydrolysis were selected and index value (I_{Famy}) has been determines for the isolates.

Production medium

The production medium used for growth of the fungal isolate was soluble starch 50 g, yeast extract 0.5 g, KH_2PO_4 10 g, $(NH_4)_2SO_4$ 10.5 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.5 g, $FeSO_4$ 0.013 g, $MnSO_4$ 0.004 g, $ZnSO_4$ 0.004 g, CoCl 0.0067 g, 50% seawater. The pH was adjusted to 6.5 and the media were sterilized in an autoclave for 15 min at 121°C. The media were inoculated with a loop-full of fungal spore suspension and incubated at 30°C in an orbital shaker set at 100 rpm for 72 hrs. The media were centrifuged at 5,000 g for 15 min to obtain crude enzyme solution.

Enzyme assay

Amylase assay has been was carried out by taking the reaction mixture (4 mL) which consisted of 1 mL of centrifuged enzyme solution and 2 mL of soluble starch in phosphate buffer, pH 6.5 (Wood and Bhat, 1988). The mixture was incubated for 10 min at 30°C. The amount of reducing sugars released was determined by dinitrosalicylate method (Miller, 1959) at 540 nm and is expressed in units (one unit is the amount of enzyme releasing 1 mg of glucose per mL per minute).

Optimization of culture conditions

The factors such as pH, salinity, carbon sources and different substrate conc. concentrations and affecting production of amylase were optimized by varying the parameters one at a time. The experiments were conducted in 250 mL Erlenmeyer flask containing 150 mL of production medium for each parameter. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various culture conditions such as pH (3.0, 5.0, 7.0, 9.0 and 11.0), carbon source (D-glucose, lactose, dextrose, sucrose and inositol at 1%), salinity (0, 0.5, 1, 1.5, 2.0 and 2.5% of NaCl) and substrate conc. (0, 0.5, 1, 1.5, 2.0 and 2.5% of starch). After 72 hr (expect for incubation period effect), 4 mL of the culture filtrate was assayed in triplicate to study the enzyme activity.

Statistical analysis

All experiments were carried out in triplicates, and repeated three times. The samples collected from each replicate were tested for amylase production and activity. Means and standard errors of amylase activity and production were calculated, respectively. and significant differences were calculated by determining standard error. The significance level of the individual parameters was done through The data were statistically analyzed using one way analysis of variance (ANOVA) and Tukey's multiple comparision test with the aid of the software, Graph pad 5.0; data with p-values lesser than 0.05 were considered significant.

Identification of potent strains

Morphological analysis

The fungal isolates were identified by studying the morphological and microscopical study. Morphological characterization was carried done by studying the upper and lower surface of the culture plate.

Microscopical analysis

The microscopical analysis has been done by using Lactophenol cotton blue (LPCB) staining method and were observed under compound microscope. The fungal spores were also observed under the SEM to observe the spore surface and structure.

Molecular taxonomy of the potent isolates

Phylogenetic (18S rDNA) analysis of the strain the potent the most amylase-producing strain possessing maximum I_{FAMY} value was carried out using following procedures

Genomic DNA isolation

Genomic DNA was isolated by using approximately 50mg of mycelium (wet weight) from a fresh culture plate as described by Hapwood et al. (1985).

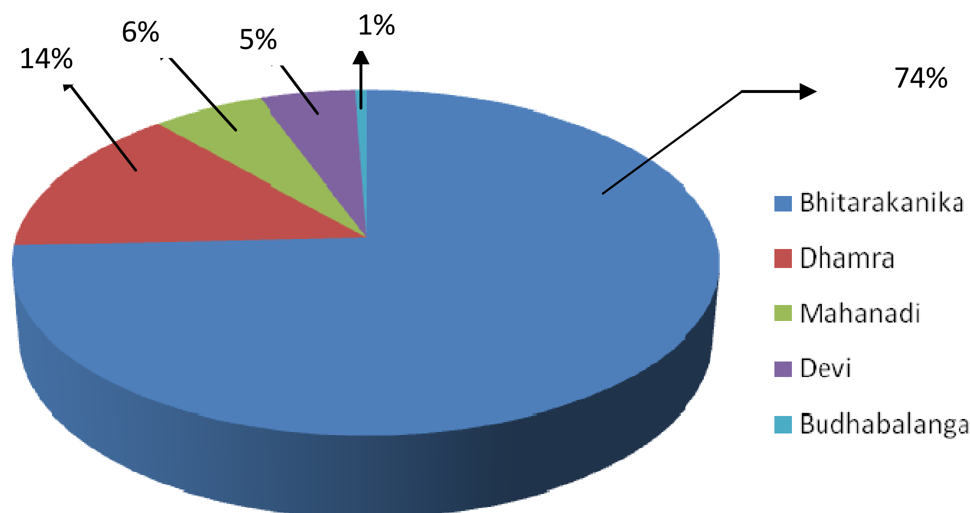

Figure 1. Comparative study of fungal diversity of mangrove ecosystems of Orissa.

PCR amplification of the 18S rDNA

Genomic DNA from the fungal cultures was used to perform PCR reaction. The 500bp of rDNA fragment was amplified using high fidelity PCR polymerase. 10 µL of the amplifications were analyzed on 1% agarose gel.

18S rDNA sequencing

The purified fragments were directly sequenced bidirectionally. The data was analyzed using applied biosystem DNA editing and assembly software and sequence comparisons were obtained using the micro Seq Software.

Sample identification

A distance matrix was generated using the Jukes-Cantor corrected distance model. Identification was done on the pair wise alignment algorithms and phylogenetic tree.

Phylogenetic analysis

Sequence similarity search was made for the 18S rDNA sequence of the fungal strain MSF-9 by applying its sequence to BLAST search for in NCBI. The Evolutionary tree was inferred by using neighbor-joining method (Thompson et al., 1997). The CLUSTAL X package was used for multiple alignment and identification of the strains.

RESULTS AND DISCUSSION

Diversity analysis

The total fungal diversity was found to be maximum that is, 74% at Bhitarakanika, 14% at Dhamra, 6% at Mahanadi, 5% at Devi and least 1% at Budhabalanga mangrove sediments of Orissa (Figure 1).

Amylase screening

Maximum index value I_{Famy} was observed in case of MSF-9 that is, 5.2 whereas in MSF-3, MSF-7, MSF-13 and MSF-28 it was 3.09, 2.68, 3.63 and 4.75, respectively (Figure 2).

Amylase assay

Effect of pH

The most potent amylase-producing fungal culture (MSF-9) was inoculated into the flask containing amylase assay media at different pH such as 3.0, 5.0, 7.0, 9.0 and 11.0. The enzyme activity was maximum at pH-5.0, that is, 80.68±0.09U mL^{-1} whereas minimum activity; that is, 22.67±0.33 U mL^{-1} was observed at pH-3.0. At pH-7.0 and 9.0 the enzyme activity was 67.44±0.23 U mL^{-1} and 58.58±0.41 U mL^{-1} respectively. The enzyme activity was first increased up to pH 5.0 and then gradually decreased with the increase of pH (Figure 3).

Effect of NaCl

The isolate MSF-9 was inoculated into the flask containing amylase assay broth at different NaCl concentrations such as 0, 0.5, 1, 1.5, 2 and 2.5%. The enzyme activity was maximum at 1% NaCl; that is, 36.89±0.26 U mL^{-1}, whereas minimum activity (6.76±0.063 U mL^{-1}) at 0% NaCl. At various concen-

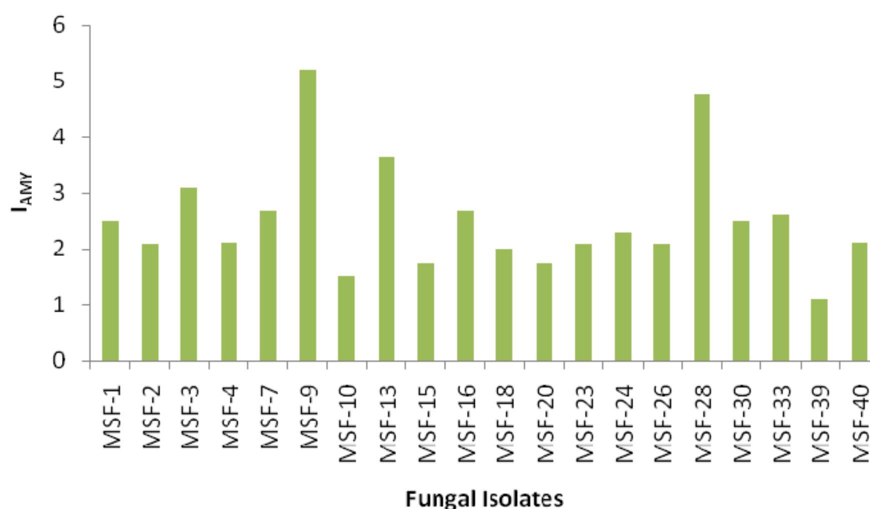

Figure 2. Screening for amylase enzyme activity of mangrove fungal isolates.

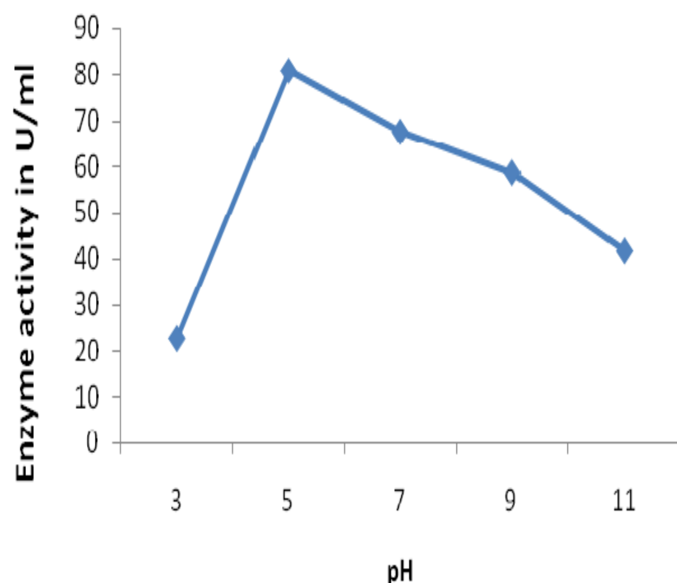

Figure 3. Effect of pH on amylse enzyme activity of MSF-9 strain.

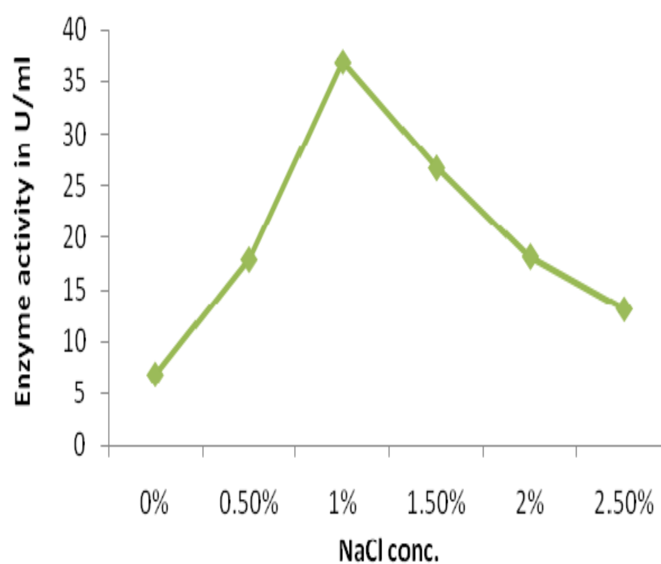

Figure 4. Effect of NaCl conc. (in %) on amylse enzyme activity of MSF-9 strain.

trations of NaCl such as 0.50, 1.50, 2 and 2.5%, the enzyme activity was 17.84±0.14 U mL^{-1}, 26.66±0.18 U mL^{-1}, 18.13±0.38 U mL^{-1} and 13.06±0.30 U mL^{-1} respectively. The enzyme activity first increased up to 1% NaCl and then gradually decreased (Figure 4).

Effect of substrate concentration

The selected strain MSF-9 was inoculated into the flask containing amylase assay broth at different substrate levels such as 0, 0.5, 1, 1.5, 2 and 2.5%. The enzyme activity was maximum at 1% starch soluble; that is, 98.25±0.23 U mL^{-1}, whereas minimum activity (12.64±0.711 U mL^{-1}) at 0% substrate. At various concentrations of 0.50, 1.50, 2 and 2.5%, the enzyme activity was 49.02±0.54 U mL^{-1}, 65.42±0.45 U mL^{-1}, 54.86±0.69 U mL^{-1} and 45.51±0.34 U mL^{-1} respectively. The enzyme activity first increased up to 1% starch soluble and then gradually decreased (Figure 5).

Effect of carbon sources

The amylase enzyme activity of MSF-9 was recorded at

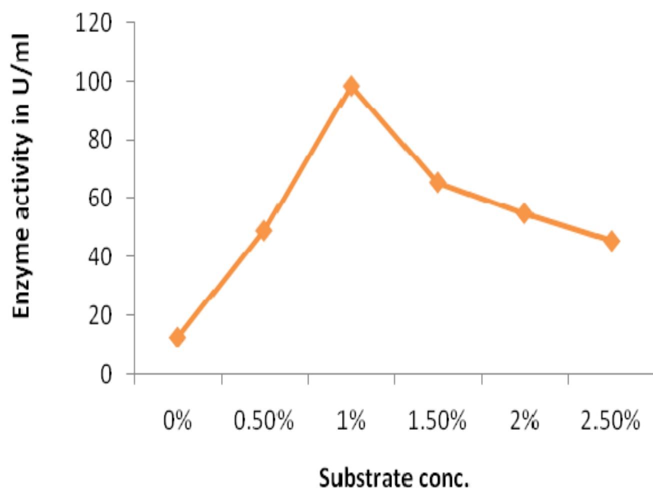

Figure 5. Effect of substrate conc. (starch) on amylse enzyme activity of MSF-9 strain.

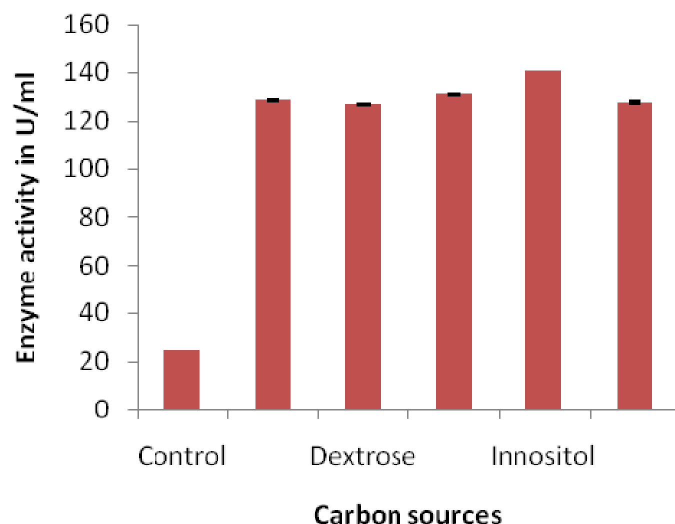

Figure 6. Effect of carbon sources on enzyme activity.

different carbon sources such as D-glucose, Dextrose, Sucrose, Inositol, and Lactose. The enzyme activity of MSF-9 was found to be minimum in lactose (127.58±0.754 U mL^{-1}) but maximum in inositol (140.58±0.22 U mL^{-1}). In case of control, D-glucose, dextrose and sucrose, the activity was 24.84±0.05 U mL^{-1}, 128.664±0.39 U mL^{-1}, 126.74±0.079 U mL^{-1}, and 131.042±0.079 U mL^{-1} respectively (Figure 6).

Significant relationship between individual of variables such as pH, NaCl conc. concentration, substrate concentration and carbon sources was observed through the one way analysis of variance (ANOVA) and Tukey's multiple comparison tests (P<0.05).

Identification

Morphological and microscopic analysis

Morphological study of the fungal isolates was carried out by growing the isolates on PDA growth media. Among the forty different isolates, twenty isolates such as MSF-1 to MSF-20 were found commonly in all the five study areas. The twenty strains were further characterized and screened for the presence of amylase enzyme. Out of the 20 isolates, five strains (MSF-3, MSF-7, MSF-9, MSF-13 and MSF-28) were found to possess more activity after screening and were observed under microscope by using cotton blue stain (Figure 2). The growth patterns of the potent fungal isolate; that is, MSF-9 was carried out in different media such as PDA, CDA (Czapek Dox Agar) and MA (Mycological Agar). It was showing very good growth in CDA medium than other two media (Table 1 and 2). The mycellial structure observed under microscope and identified (Figure 7).

SEM Study

The dried aerial mycelium with spore of the potent fungal strain (MSF-9) was studied by cover slip culture method in the SEM (Zeiss). The spores were attached like woolen balls and the round balls like structures forms a chain which observed at different magnifications from low to high; that is, 4.21K×10, 000, 8.78 K×10,000, 23.98 K×10,000 and 29.32 K×10,000 at 2.00 KV. From the spore structures it was assumed to be the member of the genus *Penicillium* (Figure 8).

Molecular identification of the potent most productive isolate (MSF-9)

The potent most productive fungal isolate, MSF-9, was identified by molecular methodology. Partial 18S rDNA sequence having up to 555 bp was amplified by PCR. The similarity pattern of the target sequence was compared with other sequences in NCBI database. The similarity pattern of the target isolate was compared with other strains and it was showing showed 97% significant homology with *Penicillium citrinum* GZU-BCECYN60-2 by using the universal marker; that is, F-27 and R-1492. The band pattern of the PCR product of MSF-9 resembles 97% with of the strain *Penicillium citrinum* (Table 4).

Phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). From the tree it can be confirmed that the isolate belongs to *Penicillium citrinum* (Figure 10).

Table 1. Morphological and microscopic characteristics of the fungal isolates.

Culture	Morphological characteristics
MSF-3	Black colony with whitish margin, smooth, reverse light yellow
MSF-7	Colonies growing rapidly up to 9cm in four days, whitish, yellowish green
MSF-9	Pale yellowish brown colony, white periphery, reverse light brownish, medium to small in size
MSF-13	White at first becoming pinkish later on, small to medium colony
MSF-28	Greenish, slow growing, reverse pale yellow in colour

Table 2. Identification of fungal Isolates showing maximum amylase enzyme activity.

Given names	ID No.	Identified strains
MSF-3		*Aspergillus niger*
MSF-7	3724.10	*Trichoderma viride*
MSF-9	3728.10	*Penicillium citrinum*
MSF-13	3731.10	*Paecilomyces variotii*
MSF-28	4108.10	*Eurotium amstelodmi*

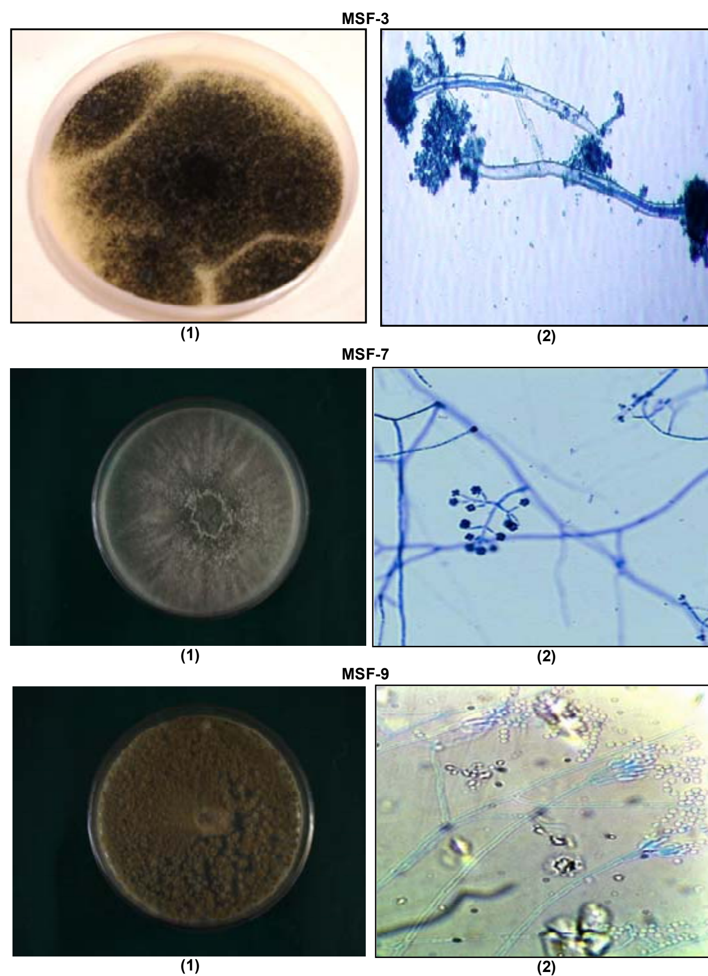

Figure 7. Pure culture ((1)) and respective microscopic photo ((2)) of MSF-3, MSF-7, MSF-9, MSF-13 and MSF-28 fungal isolates

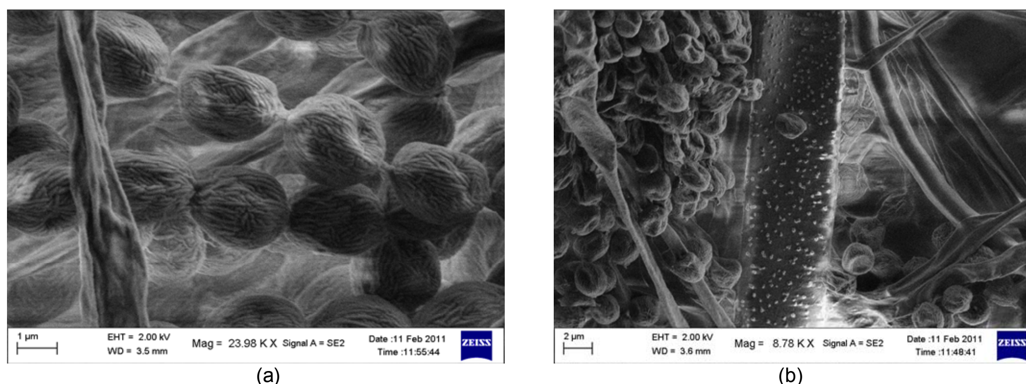

Figure 8. SEM photos of the fungal isolate (MSF-9) having magnification at 23.98 K×10,000 (a) and 8.78 K × 10, 000 (b).

Table 3. Growth characteristics of MSF-9 in different media.

Media Used	Growth characteristics
PDA	Good growth (Upper part- Light brownish, lower part- off white or light orange, pigment- No)
CDA	Very good (Upper part- Light brownish, lower- yellowish black, Pigment- yellow to black soluble)
MA	Slow growth (Upper part- Light brownish, lower- yellowish black, Pigment- No)

PDA; CDA; MA, Mycological agar

DISCUSSION

Distribution of fungal species within the mangrove habitat may reflect the physical conditions and/or habitat preference such as temp., salinity, humidity, organic contents (Das et al., 2008; Ravikumar et al., 2004). The diversity was maximum at Bhitarakanika that is, 74% and least 1% at Budhabalanga mangrove sediments which may be due to the clayey sediment which indicates the presence of more humus content.

Selections of new microorganisms for enzyme production are increasing all around the world. So the study site is mainly focused on mangrove areas of Odisha, India which are very less explored. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000). The selection of potent strains for enzyme production was done by screening and calculating the index values which indicates the utilization of substrate by the mangrove isolates. During screening of amylase enzyme activity, the fungal isolate *Penicillium citrinum* has maximum I_{Famy} value 5.2 and selected for enzyme assay purpose. The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition for fungal strains in amylase production (Quang et al., 2000). The potent fungal strain That is, *Penicillium citrinum* from the present study was found to have better activity in all the different optimized culture conditions. The maximum amylase production for the strain was found to have maximum enzyme activity at pH-5.0, 1% NaCl, 1% of substrate (starch) and Inositol as carbon source. Similar finding has been done by Kathiresan and Manivannan (2006) for fungi. Similar type of study was conducted by Gupta et al. (2010) where maximum enzyme production has been observed for the fungal strain *Aspergillus niger.* The pH change observed during the growth of microbes also affects product stability in the medium (Gupta et al., 2003). Most of the earlier studies revealed the optimum pH range between 5.0 and 7.0 for the growth of some fungal strains such as *P. fellutanum,* however, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 (Yabuki et al., 1977).

Identification of fungi was carried out by the usual methods such as cotton blue staining and conidia or spore structure attachment etc. The two genus such as *Aspergillus* sp., and *Penicillium* sp. were found to be dominant in the present study area, which were expected to furnish optimal conditions for the discovery of new metabolites from mangrove associated fungi. The fungal strains were identified as *A. niger, Trichoderma viride, Penicillium citrinum, Paecilomyces variotii, Eurotium amstelodmi* by microscopic and morphological observation (Table 3 and Figure 7). *Penicillium citrinum*

Table 4. Alignment view and distance matrix of the MSF-9 isolate.

Accession	Description	Max. score	Total score	Query coverage (%)	E value	Max ident (%)
GU565136	*Penicillium citrinum* GZU-BCECYN60-2	795	795	85	0	97
HQ407424	*Eupenicillium brefeldianum* TZ-16	728	728	85	0	94
HM214448	*Penicillium janthinellum* Zh9A	728	728	85	0	94
GU981580	*Eupenicillium brefeldianum* CBS:235.81	728	728	85	0	94
AF033443	*Penicillium fuscum* NRRL 721	728	728	85	0	94
AF033435	*Eupenicillium brefeldianum* NRRL 710	728	728	85	0	94
HM469409	*Penicillium* sp. 6 JJK-2011	725	725	86	0	94
FJ613818	Fungal endophyte sp. ZY-2009	725	725	85	0	94
AM262422	*Eupenicillium* sp. SS-1627	725	725	83	0	94
AF481123	*Penicillium* sp. NRRL 28214	725	725	86	0	94

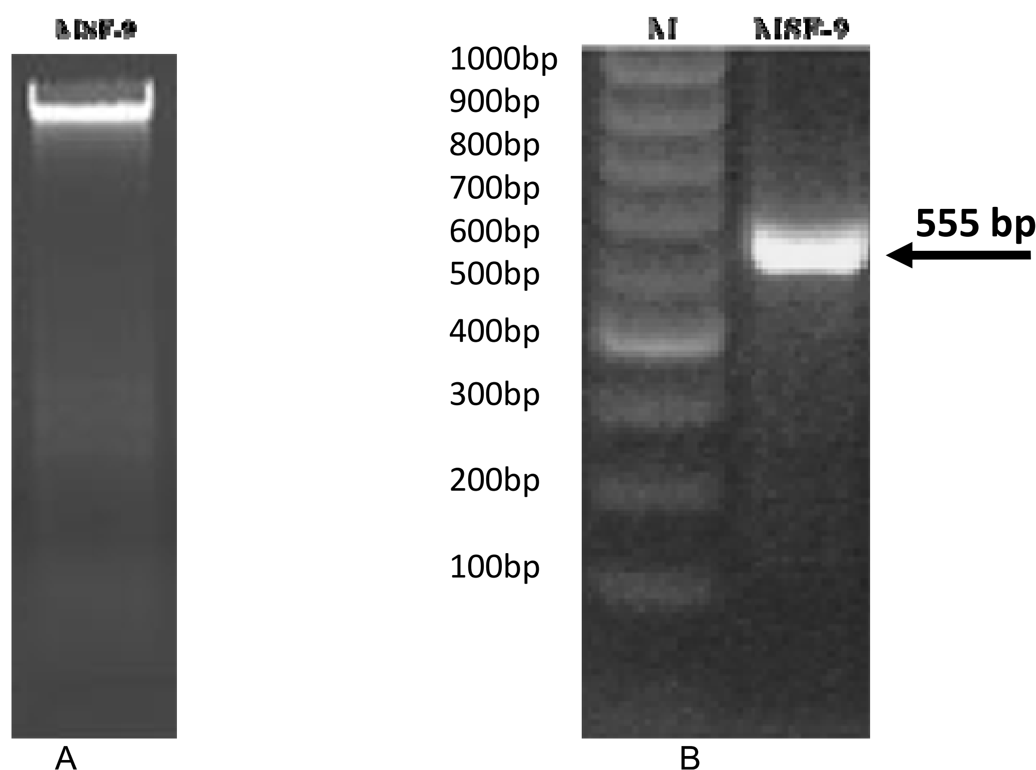

Figure 9. Agarose gel electrophoresis of the genomic DNA (A) and 18S rDNA PCR Product (B) of MSF-9 (M- 100bp Marker).

strain was found to grow luxuriantly in CDA media and the SEM study indicates the general Penicillium. The molecular study that is, 18S rDNA analysis showed that the MSF-9 isolate has 96% similarity with *Penicillium citrinum* GZU-BCECYN60-2 (Table 4, Plate 35). The phylogenetic analysis showed the similarity pattern of *P. citrinum* (MSF-9) with other species (Figures 9 and 10). It can be concluded from the present study that the mangrove isolate *P.citrinum* produced more amylase enzyme. Hence this potent fungal strain can also be used in large scale industry for various useful product formation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

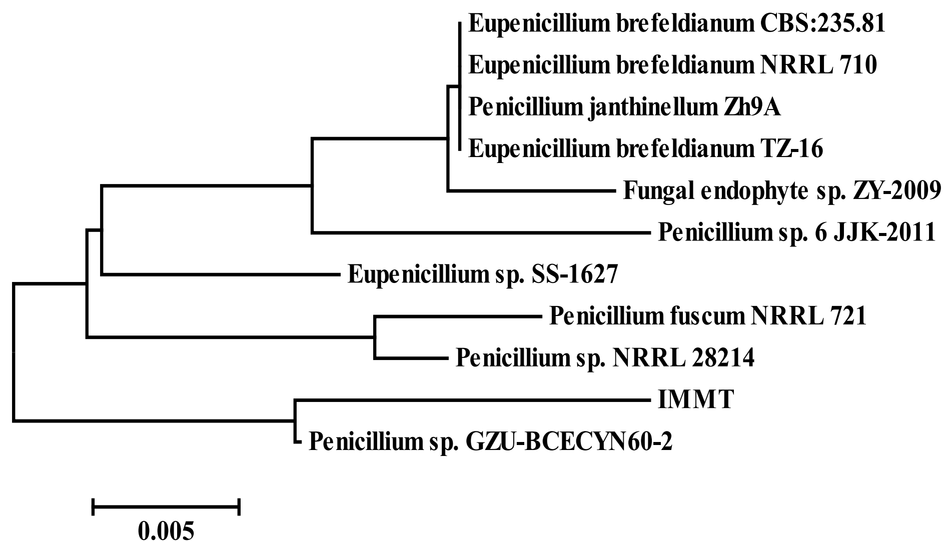

Figure 10. Evolutionary relationships of 11 taxa of MSF-9.

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