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Isolation and screening of glucose oxidase producing *Aspergillus oryzae* strain MF13 from marine source

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Abstract: Glucose Oxidase (GOx) received numerous applications in the food industry and clinical fields. Marine fungi extensively recognized for their ability to harvest enzyme source. In this study, we have made an effort to collect the sediment samples were collected from the coastal marine source of Karaikal, Puducherry, India. The isolated fungi features were characterized by morphological, physiological and molecular level. A total of 41 fungi isolated from marine soil, from that 8 isolates showed GOx activity. The active GOx producing isolates were selected through screening experiments. The particular isolate (MF13) genomic DNA was extracted. The extracted DNA was applied to be appropriate for Polymerase Chain Reaction (PCR), the amplification of target sequences in the 5.8S rRNA gene complex of internal transcribed spacer region (ITS1-ITS2). The ITS region of fungal rRNA is high importance for species-level identification of *A. oryzae* strain MF13 was found to be more reliable by comparative Genbank sequences. The potent *A. oryzae* was exhibited maximum cell biomass and GOx activity 3.50 g/L, 0.95 U/mg respectively. The present finding reveled GOx producing *A. oryzae* strain MF13 were found in marine source and beneficial for industrial applications.

Key words: Glucose oxidase, marine source, *A. oryzae* strain MF13, screening, Sequence

Introduction

Glucose oxidase (EC: 1.1.3.4) catalyzes the oxidation of β-D glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor through simultaneous production of hydrogen peroxide (1). It has wide range of application. The current world's market value of biosensors to be about 5 billion dollars and 85% is attributed to glucose biosensor. Numerous glucose sensors available in the market are based on immobilized glucose oxidase (2). Glucose oxidase is commonly used to construct amperometric biosensors for medical (3) and food industry (4). GOx has received great commercial significant due to its high specificity for glucose, high turnover and stability (5). In the laboratory, glucose oxidase also has diverse uses, immunoassays, staining procedures as well as removal of excess glucose (6). GOx is used in food industries, as an oxygen scavenger from the foods and beverages (7). One of these, GOx typically oxidizes polyphenols both in grapes and in wine making (8).

The origin of GOx was first discovered (9) in *A. niger* extract. GOx has been comprehensively studied by numerous microorganisms, notably *Penicillium notatum, P. chrysosporium, A. niger, Botrytis cinerea* (10). GOx from *A. niger*, producing homodimeric protein intracellular enzyme present in the mycelium of the organism (11). GOx production was enhanced by screening, mutagenesis and protoplast fusion of various strains of *Aspergillus* species (12). The present work is to investigate isolation and screening of active GOx producing marine *Aspergillus* species for industrial application.

Materials and Methods

Sampling procedure:

Surface sediment soil samples were aseptically collected from the coastal marine source of Karaikal using Petersen grab sampler. Four samplings were done at random at an equal distance of 200 meters seashore differences (Lat.10˚56′ N, Long.79˚51′ E). The collected samples were transferred to pre-sterilized bottle containers, and the precautionary measures were taken to minimize the contamination while handling the samples. The soil physicochemical parameters, such as temperature, pH and salinity were determined by Delux water and soil analysis kit (Model 191E).

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Isolation of marine Fungi

Isolation of fungi was performed by soil dilution plate technique using potato dextrose agar (PDA) medium (13). The medium was prepared 50% sea water and sterilized at 121°C in 15 Lbs pressure for 15 min. The medium was supplemented with streptomycin at 2µg/ml to prevent the bacterial contamination. The medium was poured into the sterile petriplates. The collected soil samples were prepared serial dilution 10^{-3} to10^{-5} with sterilized water. The 0.1 ml of the diluted sample was spread over the agar plates. The inoculated plates were incubated at 28 ± 2°C for 48-72 h. After incubation, the colonies were observed on the plates as mycelium color, spore arrangement and the appearance of colonies. The isolated colonies were preserved on potato dextrose agar at 4°C until further use.

Identification of Fungi

The isolated were identified genus level based on the morphological, physiological and biochemical characteristics. The colonies were examined macroscopically like colour, shape, texture, smooth, rough and pigmentation. The physiological characteristics were determined using different growth media, pH, temperature, carbon and nitrogen sources and NaCl tolerance. Microscopic characters were observed by Lactophenol cotton blue staining experiment. The semi- permanent microscopic preparation of fungi using Lactophenol. The structure of hyphae, conidia, chlamydospores and other structure were observed through (40 X) objective by light microscope (Olympus, CH20i).

5.8S rRNA gene sequencing and phylogenetic analysis

The potent GOx producing *Aspergillus* species were screened for molecular identification. The genomic DNA was isolated as described by the method (14). Polymerase Chain Reaction (PCR) reaction was carried out with Taq DNA polymerase using ITS1-Forward (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS2-Reverse (5' GCTGCGTTCTTCATCGATGC 3'). The PCR product was purified using the Qiagen PCR purification kit and then 5.8S rRNA gene sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). Neighbor-joining phylogenetic tree was constructed for the potential strain *A. oryzae* with their closest NCBI (BLASTn) strains based on the 5.8S rRNA gene sequences (15). This analysis was done based on Maximum-likelihood method using MEGA 7 (16).

Screening of GOx

GOx screening was performed by morphologically distinct colonies. The purified fungi cultures were inoculated on the Cjapek dox agar medium containing 100 µl of O-dianisidine and 100 µl of horseradish peroxidase. After incubation, the GOx production medium was developed brown coloured zone around the colonies, which indicates a positive result for GOx activity (12).

Cell Biomass determination

All the axenic fungal strains were individually cultured in potato dextrose broth (the above- described broth content without agar). After 5 days of incubation, the fermented cell biomass in each case was filtered and then blended intracellular enzyme using Ultra-sonicator (Hielscher, USA) at 20 KHz for 45 sec and centrifuged the lysed broth at 12000rpm for 15 min (17).

Glucose oxidase activity

Glucose oxidase activity was determined by spectrophotometer method. The crude enzyme activity was measured at OD 460 nm wavelength using glucose as a substrate and o-dianisidine buffer as coupling reagent (18). The GOx activity was examined by coupled peroxide O-dianisidine system. The H_2O_2 liberates split into H_2O, and the oxygen is directly coupled to the dye O-dianisidine which turns to a brownish red colour. One unit of enzyme activity was defined as the amount that produced one micromole of H_2O_2 per minute at 30°C under the assay condition.

Estimation of protein

The protein content of the crude intracellular enzyme was determined by the method (19) described using bovine serum albumin as a standard.

Results

The soil physicochemical parameters are shown in the table (1). The temperature studied in the range of 27-30 °C, pH of sample 6.0 ± 6.5. The salinity verified at the

time of sample collection in the range of 31-35 ppt. The determination for the production of GOx was confirmed by agar plate. The positive result indicates the GOx enzyme reacts with O-dianisidine and horseradish peroxidase. The reaction agar plates containing the present enzyme produced brown colour zone around the colonies. The test isolates were exhibited in various diameters zone ranging from 3-10 mm respectively. The highest GOx activity was observed only in *A. oryzae* strain MF13.

The identification of fungal strain all isolates are aerobic. The morphological features of fungal strain are shown in the table (2). The isolates showed ash, black, yellow, green, brown colored colonies with golden and light-yellow reverse pigmentation. The isolates were exhibited well-developed hyphae and conidiophores.

Table 1. Physico-chemical properties of marine soil

Site of sampling	Sample	Depth (cm)	Temperature (°C)	pH	Salinity (g/L)
I	1	2-3	29	6.2	35 ppt
II	1	2-3	30	6.1	34 ppt
III	1	2-3	28	6.7	32 ppt
IV	1	2-3	27	6.4	31 ppt

Table 2: Morphological characterization of Glucose Oxidase producing fungi isolates on PDA medium.

Characteristics	MF1	MF5	MF8	MF9	MF10	MF13	MF14	MF18
Appearance	Light Green	Ash	Light Brown	Black	Yellow Brown	Dark Ash	Green Gray	Black
Mycelium	Hyphae	Dematiaceous Hyphae	Hyphae	Hyphae	Hyphae	Hyphae	Cube Shaped	Hyphae
Spore surface	Conidiophore	Conidiophore-Non Distoseptate	Conidio Phore	Conidio Phore	Conidiophore Are Long	Conidiophore	Conidiophore	Conidiophore
Reverse colour	Yellow	Light Yellow	Light Yellow	Yellow	Golden To Yellow Brown	Light Brown	White	Yellow Gray
Oxygen relationship	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic

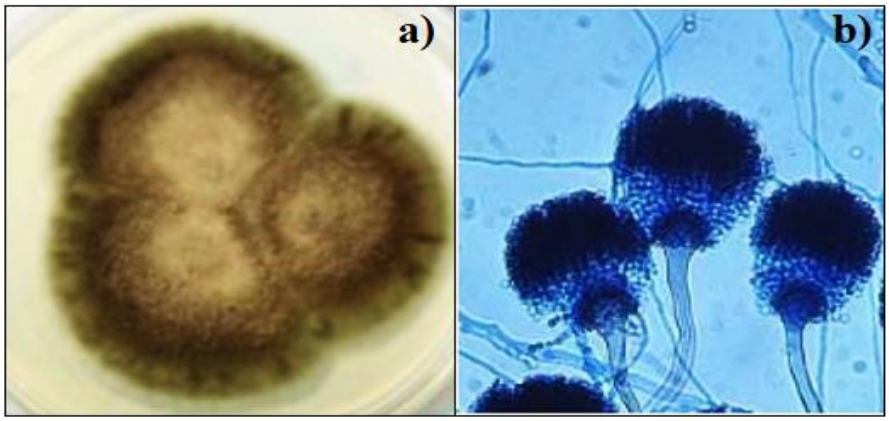

Figure 1: Structural morphology of *A. oryzae* strain MF13 a) Macroscopic observation on PDA medium, b) Microscopic visualization under phase contrast microscope.

The standard fungi identification by Lactophenol cotton blue staining. The microscopic observation of fungi was examined under a phase contrast microscope as shown in figure (1).

The physiological features of the isolates are given in the table (3). The effects of different media were influencing the growth of fungi isolates. The effects of different pH, temperature, carbon, nitrogen source and NaCl concentration were determined. The optimum pH (6.0) and temperature (30° C), carbon (glucose), nitrogen source (peptone) and NaCl tolerance (6%) were studied. Molecular determination of representative GOx producing isolated fungus *Aspergillus oryzae* strain MF13 small subunit rRNA gene,

partial sequence; internal transcribed spacer 1 (ITS1) 5.8S rRNA gene and internal transcribed spacer 2 (ITS2), complete sequence; and large subunit of rRNA gene, partial sequence. The obtained sequences were evaluated with BLASTN 2.2.28+ search, the available averaging nucleotides of the sequences were compared with the other nucleotides of fungi strains from GenBank. The sequence reports revealed the GOx producing strain homologous with the related member of *Aspergillus oryzae* ~99% similarity. The evolutionary relationship between the strains and distinct branch with most closely related fungi as shown in figure (2).

Table 3. Physiological Characteristics of Glucose Oxidase producing fungal isolates

Experiments	MF1	MF5	MF8	MF9	MF10	MF13	MF14	MF18
Growth of different media								
RBA	+++	+	++	+	++	+	+++	+++
PDA	+	+	+++	+	++	++	0++	++
MYA	++	+	++	+	+	+	+	++
SAM	+	+++	++	+	+	+	+++	++
Optimum temp(°C)								
30°C	++	+	+++	+	++	++	++	+++
Optimum pH								
6	+++	+	+++	+++	+++	++	++	++
Carbon source								
Glucose	+++	+	+++	+	+	+	+	+++
Lactose	+	+	++	+	+	+	+	++
Cellulose	+	+	+	+	+	+++	+	++
Nitrogen source								
Peptone	+	+	++	++	+++	+++	++	+++
Yeast extract	+++	+	+++	+	+	++	+	+
Beef extract	+	+++	++	++	+++	+	+	++
Effect of Nacl								
2%	+++	+	++	+	+	+ +	+	++
4%	+	+	+++	++	++	+++	++	++
6%	+++	++	++	++	++	+++	+	+ ++
8%	++	+	++	+	+	+	+	+ +

+, Moderate Growth; ++, Good growth; +++, Excellent growth

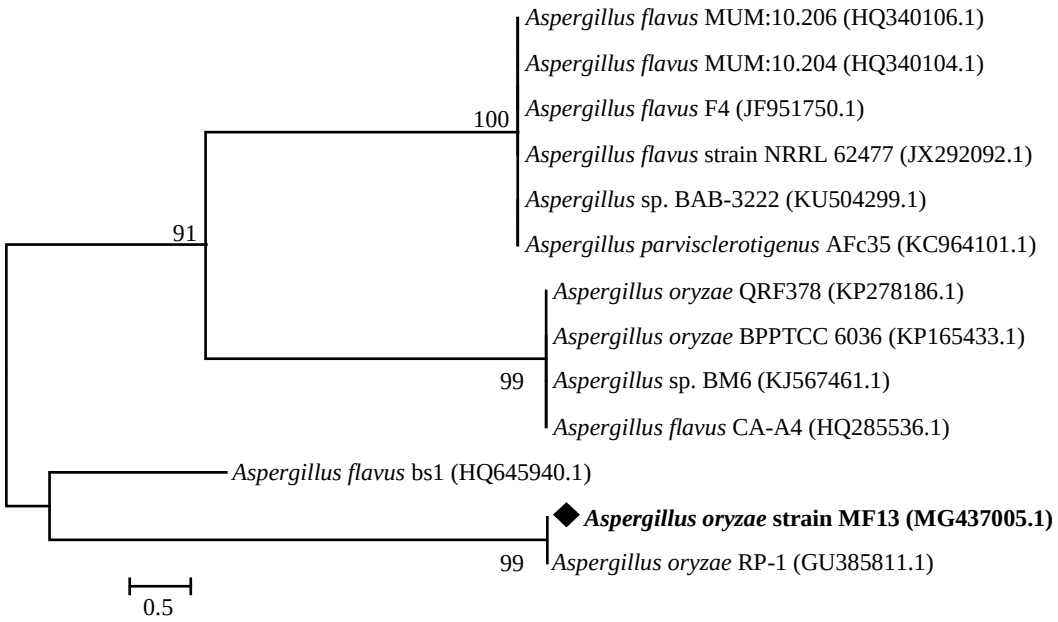

Figure 2: Phylogenetic analysis of 5.8S rRNA gene sequence of GOx producing *Aspergillus oryzae*.

GOx activity and biomass

The quantitative screening of GOx activity are given in table (4). The GOx production was recorded under suitable fermentation. The cell concentration and crude GOx activity were recorded representative fungi isolates. The maximum cell biomass production was observed in the range of (3.07-3.50 g/L), and the GOx activity was observed in the range of 0.03-0.95, U/mg. The highest cell biomass 3.50 (g/L) and GOx activity 0.95 (U/mg) was observed only in *Aspergillus oryzae*. Based on the GOx screening one of the potent *A. oryzae* MF13 selected for further experiments.

Table 4: Quantification of Glucose Oxidase producing fungal isolates

Sources	Cell Biomass (g/L)	GOx activity (U/mg)
MF1	2.51 ± 0.01	0.04 ± 0.06
MF5	2.75 ± 0.04	0.03 ± 0.04
MF8	2.78 ± 0.03	0.3 ± 0.03
MF9	2.98 ± 0.05	0.3 ± 0.05
MF10	2.86 ± 0.02	0.45 ± 0.01
MF13*	3.50 ± 0.01	0.95 ± 0.01
MF14	3.10 ± 0.01	0.51 ± 0.02
MF18	3.07 ± 0.05	0.78 ± 0.01

GOx assay experiments were performed triplicates and the mean values along with standard error mean (n=3) are presented. *indicates highest GOx activity expressed by MF13 strain.

Discussion

Totally 41 marine fungi were isolated from the marine soil. The population of fungi was depended on physicochemical properties of the sample. Similar isolation techniques were used by isolation of fungi from nature (20). The development of fungal colonies quickly in desirable potato dextrose agar medium than other conventional media. This report coincides with previous work on potato dextrose was most favorable for fungi isolation (21). The present reports on isolation and identification coincide with the work of numerous group of soil fungi isolated, identified and fungal diversity population studies (22). The Gox primary screening experiment reveals *A. oryzae* MF13 exhibited better results than the other isolates. The results were similar to the previous reports on GOx screening experiments (23, 24).

The DNA sequence evaluated by percentage of homology for 5.8S rRNA. The taxonomic position of the isolated fungi sequences, and their closest NCBI relatives based on nearly complete 5.8S rRNA gene sequences comparable with earlier findings (25) (26). Molecular and morphological identification of fungal species has been studied in previous reports (27).

Glucose oxidase producing fungi were isolated from marine source. The fungi were screened on the GOx producing ability. The result indicates that *A. oryzae* MF13 is an intracellular glucose oxidase enzyme. The *A. oryzae* MF13 strain composed of a desirable quantity of GOx. This observation is comparable with earlier reports from different *Aspergillus* species (28) (5). Previous report has been studied glucose oxidase from *A. niger* (29) (30). A number of GOx producing group of extremophilic *Aspergillus* species were isolated and characterized (31). Sediment soil and endophytic fungi were isolated and screened for GOx activity (32).

This work clearly describes the isolated fungi exhibited GOx activities. It has been suggested numerous GOx producers are available in fungi species, but very little evidence in the area of a marine source from the coast of Karaikal. This study encouraging GOx producing fungi were present in the marine environment even though *A. oryzae* are considered as one of the best producer.

Conclusion

The present study gives a positive impact of isolation and screening of GOx producers. The *A. oryzae* strain MF13 produce intracellular GOx, the desired enzyme present in the mycelium of the organism. Hence, the investigation creating new platform for the marine fungi depot of GOx from wild type producers. This work motivating marine *A. oryzae* strain MF13 producing GOx can be applicable for industrial application.

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