ISSN: 2226-7522(Print) and 2305-3327(Online)
Science, Technology and Arts Research Journal
Oct-Dec 2012, 1(4):27-32
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Original Research

Isolation and Screening of Industrially Important Fungi from the Soils of Western Ghats of Agumbe and Koppa, Karnataka, India

Mukunda S[1*], Onkarappa R[2], Prashith Kekuda T.R[1,2]

[1] Department of Microbiology, SRNM National College of Applied Sciences, NES Campus, Shivamogga-577201, Karnataka, India

[2] Department of Studies and Research in Microbiology, Sahyadri Science College (Autonomous), Shivamogga-577203, Karnataka, India

Abstract

The present study is an attempt to isolate the fungi from the unexplored soils of Western Ghats and screen them for the production of some very important hydrolytic enzymes such as amylase, cellulose, CMCase, protease, lipase and pigment production. In this work, more than 200 fungal isolates were isolated from the forest soil were screened for the production of extracellular enzymes, identification of the screened isolates and secondary screening for amylase production was done. A total of 167 isolates were characterized, in them some up to species level. *Aspergillus*, *Penicillium*, *Trichoderma* and *Cladosporium* were predominated. A total of 14 isolates were selected for the secondary screening for amylase production. *Aspergillus* isolate 199 and *Aspergillus* isolate 43 produced more protein (169±07 and 160±04µg) respectively. Amylase assay also revealed greater activities (4.98±0.06 and 4.93± 0.07µ moles/ ml) in *Aspergillus* isolate 199 and *Aspergillus* isolate 43 respectively.

Article Information

Article History:

Received : 14-10-2012
Revised : 26-12-2012
Accepted : 29-12-2012

Keywords:

Western Ghats
Soil
Agumbe
Hydrolytic Enzymes

***Corresponding Author:**

Mukunda S

E-mail:

mukundas_micro@rediffmail.com

INTRODUCTION

The role of many enzymes has been known for a long time. Their existence was associated with the history of ancient Greece, where they were using enzymes from microorganisms in bakery, brewing, alcohol production, cheese making etc., (Haki and Rakshith, 2003). Nowadays enzymes are used in large scale in the textile (amylase, cellulose, oxidoreductase); detergents (protease, lipase, cellulose, amylase and oxidoreductase), food (pectinase, protease, cellulose and oxidoreductase); paper (xylanase, oxido-reductase and lipase) and leather (protease and lipase) industries. The major classes of enzyme offering immediate application are the hydrolytic enzymes (Kirk *et al*., 2002). Among all industrial enzymes, hydrolytic enzymes account for 85%. Microbial enzymes are preferred to those from plants and animal sources because they are cheaper to produce and their enzyme contents are more predictable, controllable and reliable (Oyeleke and Oduwole, 2009) and also because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Today, the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes have stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Buzzini and Martini, 2002; Gupta *et al*., 2003; Bakri *et al*., 2009). Selection of the right organism plays a key role in high yield of desirable enzymes (Satyaprabha *et al*., 2011). Fungi are microorganisms which are well known for their wide range of novelty of enzymes they produce and enzymes of fungal origin are used in the industrial process for which, amount to billions of dollars of revenue annually (Arunsasi *et al*., 2010). Due to their diversity, fungi have been recognized as a source of new enzymes with useful and/or novel characteristics (Bakri *et al.,* 2009). Filamentous fungi are

particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential (Mishra and Dadhich, 2010).

Soil provides a heterogeneous and complex environment for all soil inhabitants. Soil is also known to harbor different microorganisms including diverse group of fungi. Western Ghats are considered as one of the hot spot locations for biodiversity including microbial diversity. Hence the soils from Western Ghats can be a source of fungi of industrial importance. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta *et al.,* 2003). In the present work, we have isolated, characterized and screened the fungal isolates for the production of industrially important enzymes and pigments from soil samples of Agumbe and Koppa of Karnataka, India.

MATERIALS AND METHODS

Isolation of Fungi

Soil samples from 6 to 10 inch depth were collected aseptically from 18 different zones of deep forests of Agumbe region and near Jayapura, Koppa using sterile zip lock covers. The soil samples were sieved and processed aseptically and used for the isolation of fungi. The soil samples were inoculated to the Potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) Potato carrot agar (PCA) and Czapek Dox agar (CZA) by Spread plate technique following serial dilution, Soil direct plating technique, Warcup method and stress technique and incubated at 25^{o}C for 3 days. The media were amended with Chloramphenicol to prevent the growth of bacteria (Srinivasan, 2004). The fungal species were grown on the Czapek Dox agar plates, identified based on Cultural and morphological characteristics using standard manuals (McGinnis *et al.*, 1982; Barnette and Hunter, 1972; Malloch, 1981).

Screening for Enzymes and Pigment Production

Screening for Amylase Production

Preliminary screening was done by inoculating the fungal isolates on starch agar (containing peptone, 1%; yeast extract, 1%; KH_2PO_4, 0.5%; agar 2% and supplemented with 1% (w/v) starch (HiMedia) as a carbon source and supplemented with antibacterial antibiotic Chloramphenicol) plate with fungal isolates. After incubation, the plates were flooded with Iodine solution (Iodine - 0.2g, Potassium Iodide- 0.4g, Distilled water- 100ml), and observed for the clear zone of hydrolysis surrounding the colony (Aneja,1996; Kathiresan and Manivannan, 2006).

Screening for Cellulase Production

The fungal isolates were inoculated into cellulose media and incubated at 25 ^{o}C for 7 days. The plates were flooded with 0.1% Congo red solution and observed for zone of clearance (Samira *et al*, 2011).

Screening for Carboxymethyl Cellulase Production

The fungal isolates were inoculated in Carboxymethyl cellulose media and incubated at 25^{o}C for 7 days. The plates were flooded with 0.1% congo red solution and observed for zone of clearance (Samira *et al.*, 2011).

Screening for Protease Production

The fungal isolates were inoculated on Casein agar plates and incubated at 25^{o}C for 4 days. After incubation, the plates were observed for the clear zone surrounding the colony (Aneja, 1996).

Screening for Lipase Production

The fungal isolates were inoculated on mineral medium amended with Tween 80. The formation of opalescence surrounding the fungal colony was recorded as positive for lipase production (Rajan *et al.*, 2011).

Pigment Production

The isolates producing diffusible extracellular pigments were selected and preserved.

Secondary Screening for Amylase Production by Submerged State Fermentation

Based on the measurement of zone of clearance around the colony, the 14 fungal isolates belonging to 4 genera were selected for the secondary screening. The cultures were grown in 250 ml Erlenmeyer flask containing 100 ml of production medium (NH_4NO_3, 1%; KH_2PO_4, 0.2%; $MgSO_4.7H_2O$, 0.2%; $FeSO_4.7H_2O$, 0.001% and soluble starch, 2%; pH 6.0) and incubated at 30^{0}C for 5 days. After incubation, the cultures were filtered and the culture filtrate was centrifuged at 4000 rpm for 10 minutes and the supernatant was used as crude enzyme (Kundu and Das, 1970).

Protein Estimation

The protein content of culture filtrate was determined by the Lowry's method, as described by Lowry's (1951) using bovine serum albumin (BSA) as a Standard, absorbance was read at

660 nm using *Systronics* Spectrophotometer Model 104.

Estimation of Reducing Sugars

The glucose concentration was determined by DNS method, as described by Miller (1959) using glucose as a standard. The color developed was measured at 540 nm using *Systronics* Spectrophotometer Model 104.

Determination of Amylase Activity

The amylase activity of the crude enzyme was determined using 1% soluble starch as substrate, prepared in sodium phosphate buffer (0.1 M, pH 6.0). The reaction mixture containing 0.5 ml of enzyme and 0.5 ml of substrate (1% starch) by maintaining a blank containing 0.5 ml of enzyme and 0.5 ml of buffer was incubated at 30˚C for 15 min in a water bath. The DNS method was followed for the incubated samples and optical density was read at 540 nm against blank. A standard curve of glucose (1mg/ml) was developed under identical conditions to determine the reducing sugars formed. The enzymatic activity of filtrate was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme which liberates 1 µmol of reducing sugar per ml per minute under assay conditions.

RESULTS AND DISCUSSION

More than 200 fungal isolate were isolated from soil by different techniques and 167 isolates were characterized based on colony morphology and microscopic mount of the isolates. About 35 isolates were not identified. The isolates belonged to the genera *Aspergillus (A. niger, A. flavus, A. terreus, A. fumegatus, A. nidulans, A. versicolor), Penicillium, Trichoderma, Fusarium, Cladosporium, Pacillomyces, Gliocladium, Scopulariopsis, Verticillium, Curvularia, Alternaria, Rhizopus* and *Mucor*. Some *Aspergillus* and *Penicillium* isolates were characterized only up to Genus level. The members of *Aspergillus*, *Penicillium* and *Trichoderma* were dominant among fungal isolates. Table 1 depicts production of hydrolytic enzymes and pigment by the isolated fungi.

More than 85% of the isolates showed the amylase production, followed by Cellulase (58%), CMCase (64%), protease (36%), lipase (37%) and water soluble pigments (40%). Of these starch hydrolysis positive isolates, based on the extent of zone of hydrolysis formed on starch agar plates, 14 isolates belonging to different genera were selected for secondary screening by submerged fermentation. The amount of extracellular protein synthesized and the specific amylase activity of the isolates is shown in the table 2.

In the secondary screening, the production of extracellular proteins was found to be more in *Aspergillus* isolates compared to the isolates of other genera. In *Aspergillus* isolates, isolate No. 199 and 43 produced highest concentrations of protein. The Specific activities of amylases of respective isolates were also calculated. The *Aspergillus* isolates (Number 199 and 43) showed more specific activity.

Bankar *et al.* (2012) worked on the isolation and screening of forest soil of Bhadra Wild Life Sanctury, for potent amylolytic fungi. The isolated fungi were mainly belonged to *Penicillium chrysogenum*, *Aspergillus candidus*, *Aspergillus fumigatus.* It was found that *Penicillium sp* showed more amylase activity for both 3rd day and 7th day incubation. *Penicillium chrysogenum* was found to produce more soluble crude protein. Friedrich *et al*. (1999) isolated of more than 300 fungal isolates from air and screened for keratinase production. Moallaei *et al.* (2006) isolated a total of 357 fungal colonies including 13 genera with 11 species *Anixiopsis stercoraria* (16.24%), *Arthroderma cuniculi* (12.04%), *Reniospora flavissima* (9.24%), *Fusarium oxysporum* (9.24%), *Aspergillus flavus* (8.68%), *Chrysosporium keratinophilum* (8.40%), *Trichophyton vanbreuseghemii* (7.84%), and other fungi (37.56%), and reported that non-keratinophilic fungi were prevalent in the forest soil. Arunsasi *et al.* (2010), isolated 15 fungal species namely, *A. fumigatus, A. oryzae, A.niger, A. flavus, A. nidulance, A. sulphurus, A. terreus, Trichoderma vessei, T. viridae, Penicillium citrinum, P. oxalicum, Fusarium moniliformis, F.oxalicum, F. oxysporum, Rhizopus oryzae* from the soil samples of the coastal region of Neendakara, along the West cost of Kerala, India and screened them for amylase production and found that *A. flavus* produced maximum zone of hydrolysis on Starch agar media. Sathyaprabha *et al*. (2011) reported the isolation of fungi namely, *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus nidulans* and *Aspergillus niger* from soil samples obtained from crude petroleum oil contaminated soil. They screened the isolates for amylase and cellulose production. Kathiresan and Manivannan (2006) isolated Strains of *Penicillium* sp. from the coastal soil of a mangrove habitat and later identified as *P. fellutanum* and screened for amylase production. Tiwari (2007) isolated a fungus from soil identified as *P. rugulosum* and screened it for amylase

Table 1: Enzyme and pigment production by fungal isolates.

Sl. No.	Isolate	Amylase production	Cellulase production	CMCase production	Protease production	Lipase production	Pigment production
1.	*Aspergillus niger (19)**	+++	+	+	+	+	–
2.	*A. flavus (13)**	+++	–	+	–	+	–
3.	*A. terreus (14)**	+	–	–	–	–	+++
4.	*A. fumigatus(4)**	+	+	+	–	+	–
5.	*A. nidulans(3)*	++	–	–	–	+	–
6.	*Aspergillus isolate* 16**	++++	–	–	++	+	–
7.	*Aspergillus isolate* 25**	++++	++	+++	+	+	–
8.	*Aspergillus isolate 43**	+++++	+	+	+	–	++
9.	*Aspergillus isolate 122**	++++	–	++	+	+	+++
10.	*Aspergillus isolate 160**	+++	–	–	–	–	++
11.	*Aspergillus isolate 199**	+++++	–	–	+	+	–
12.	*Penicillium notatum (6)**	+++	+	+	+	+	+
13.	*Penicillium isolate 73**	+++	+	++	+	–	–
14.	*Penicillium isolate 33**	++	+	++	–	–	++
15.	*Trichoderma sp.(24)**	++	+++	+++	–	–	+++
16.	*Fusarium sp.(12)**	+++	+	++	–	–	++
17.	*Cladosporium sp.(13)**	–	+	+	+	–	–
18.	*Pacillomyces sp.(6)**	+	+	+	–	–	–
19.	*Gliocladium sp.(3)**	–	+	+	–	–	–
20.	*Scopulariopsis sp.(4)**	–	–	–	–	–	–
21.	*Verticillium sp.(4)**	+	+	–	–	–	–
22.	*Curvularia sp.(6)**	+	+	+	–	–	+
23.	*Alternaria sp.(3)**	+	–	–	–	–	–
24.	*Rhizopus sp.(17)**	++	–	–	+	+	–
25.	*Mucor sp.(8)**	+	–	–	–	–	–

*The numbers within the bracket corresponds to number of isolates obtained.

** Fungal isolates characterized up to genus level.

The sign (+) indicates the positive screening test and (–) indicates a negative screening test.

The number of (+) mark ranging from 1+ to 5+ indicates the extent of hydrolysis as indicated by the zone of clearation surrounding colony.

Table 2: Protein content of culture filtrate and specific activity of crude amylase.

Sl. No.	Name of the isolate	Extracellular protein µgs/ml*	Specific Activity (µmol/ml/Min)*
01.	*Aspergillus niger*	139 ± 10	4.08 ± 0.09
02.	*A. terreus*	112 ± 07	2.91 ± 0.10
03.	*A. flavus*	119 ± 09	3.02 ± 0.12
04.	*A. oryzae*	102 ± 08	3.27 ± 0.07
05.	*A. versicolor*	141 ± 09	3.28 ± 0.13
06.	*A. fumigatus*	123 ± 05	3.19 ± 0.16
07.	*Aspergillus isolate 16*	144 ± 12	3.52 ± 0.05
08.	*Aspergillus isolate 25*	132 ± 07	3.30 ± 0.11
09.	*Aspergillus isolate 43*	160 ± 04	4.93 ± 0.07
10.	*Aspergillus isolate 199*	169 ±07	4.98 ± 0.06
11.	*Penicillium notatum*	78 ± 06	2.01 ± 0.03
12.	*Penicillium isolate 73*	86 ± 11	2.95 ± 0.06
13.	*Fusarium sp.*	75 ± 09	2.92 ± 0.10
14.	*Rhizopus sp*	58 ± 04	2.12 ± 0.08

*Mean of three replicates, ± Standard deviation (SD)

production. Pothiraj *et al.* (2006) isolated *Rhizopus stolonifer, Aspergillus niger* and *Aspergillus terreus* by primary selection from a naturally contaminated cassava waste by serial dilution and pour plate technique and reported the production of cellulase by solid state fermentation. Gautam *et al.* (2010) studied on the isolation and screening of cellulolytic fungi from municipal solid waste. Out of 20 fungal culture isolates from environmental sources including 8 different zones, 16 fungi were found to passes cellulose degrading ability. Cellulolytic fungi belonging to *Aspergillus funmigatus*, *Trichoderma* sp.I and *Chaetomium* sp. Results obtained during this study clearly indicate that cellulase activity of *Aspergillus fumigatus* and *Trichoderna* sp.1 were found relatively towards the higher side and *A. niger, A. flavus, A. nidulans*, *Alernaria* sp., *Penicillium* sp. moderate range while *Fusarium* sp., *Humicola* sp. and *Torula* sp. showed low cellulase activity. Damisa *et al.* (2011) reported the isolation of a native *Aspergillus niger* strain from the soil samples taken from six locations in Zaria. The samples were collected from rice growing field, compost soils, street soil, fallow farm land, flower beds and maize farm. They screened the cellulolytic efficacy of the isolate on modified Mondels mineral agar. However, screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications.

CONCLUSION

The present study showed that the forest soils of Western Ghats can be a very good source of industrially important enzymes. The secondary screening for the production of amylase was also done for 14 selected isolates. Among the fungal isolates, *Aspergillus, Penicillium, Trichoderma* and *Cladosporium* predominated. In the present study, the screening for only the main groups of hydrolytic enzyme producers was made. The vast microbial biodiversity of the Western Ghats is yet to be exploited so that the indigenous soils can be screened for the isolation of other novel fungi with the ability of production of some other important enzymes, antibiotics and other bioactive compounds. Since each application of enzymes in industry requires different specificities, the hydrolytic enzymes with different properties have to be isolated. Further studies with regard to the optimization of production parameters and the catalytic properties are required to be done.

ACKNOWLEDGMENTS

The authors are thankful to The Principal, SRNM National College of Applied Sciences, The Management, National Education Society, Shivamogga, Sri. Ramachandra K, Jayapura, Koppa for their support to carry out the work.

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