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ORIGINAL ARTICLE

Studies on Enzymes Extracted From Microbes Isolated From Mangrove Sediments

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ABSTRACT
The present work was aimed at isolating some unique bacteria from the local sediment sample from a mangrove habitat and screening these isolates for capability to produce different enzymes. The microbial isolate that showed promising enzyme productivity was identified using molecular marker as Bacillus spp. Attempt was also made to partially purify the extracted amylase and to study the factors affecting the activity of this extracted amylase.
Key Words: *Bacillus, Mangroves, Enzyme, Amylase, 16S rRNA*

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INTRODUCTION

Enzymes are biocatalysts produced by living cells that are proteinaceous in nature and act on their specific substrate to bring about specific biochemical reactions [1]. More than 300 enzymes are now used commercially. Though enzymes are found in all living organisms, most of the commercial enzymes come from bacteria, fungi and yeast.

Microbial enzymes are widely used in industrial processes due to their low cost, large productivity, chemical stability, environmental protection, plasticity and vast availability. Uses of microbial enzymes in food, pharmaceutical, textile, paper, leather, and other industries are numerous and are increasing rapidly [2].

Although many potent strains are on market for enzyme production, scientists prefer studying the new isolates because they could be alternative for commercial use. Therefore research will continue to isolate the alternative strains for the production of enzymes.

The present study was undertaken to isolate some microbial isolates from mangrove sediments and evaluate their potential to produce a few enzymes. Attempt was also made to investigate the factors affecting the activity of the extracted enzyme. Finally, amplified gene sequence of the 16S rRNA was used to identify the microbial isolate that showed promising enzyme production.

MATERIALS AND METHODS

Microbial Isolation

Mangrove sediment samples were collected from Ratnagairi, West Coast of India and were stored at 30°C before inoculation. 1g of soil sample was dried at 40ºC for 5 hours was suspended in 9 ml of sterile distilled water. Sediment samples were diluted and spread plated on two media namely Glycerol yeast extract agar containing 5ml glycerol, 2g yeast extract, 0.1g K_2HPO_4, 25g peptone, 15g agar, 1L distilled water and ISP2 containing 4g glucose, 4g yeast extract, 10g malt extract, 15g agar in 1L distilled water. Antibiotics were added to above media in order to eliminate fungal growth. Plates were incubated at 30°C for 48 hours.

Morphological characteristics

Colony characteristics of a 48 hour grown colony were noted for all the isolated cultures. The smears prepared were observed under oil immersion and cell type was noted.

Screening of microbial isolates for enzyme production

The isolated bacteria were screened for the detection of Pullulanase, cellulase and amylase enzyme production. The microbial cultures were spot inoculated on pullulan, Carboxy-methyl cellulose and starch

in order to detect pullulanase, cellulose and amylase production respectively. The plates were incubated for 48 hours at 30°C. Zone of clearance was checked by flooding the plates with Gram's Iodine.

pH variation study on enzyme production

Three sets of media were prepared by adjusting pH for all the three substrate media to 6, 7 and 8 each (3).

Temperature variation study on enzyme production

Enzyme production was checked for the isolates on substrate media in temperature range of 30°C and 40°C [3].

Studies on Amylase enzyme

The sediment derived bacterial culture no. 10 was taken further for the production of amylase enzyme on suitable medium and quantification of amylase activity. The effect of temperature and pH on enzyme activity was also estimated. Finally, method described by Chandra et al. (1980) was adopted for measuring the enzyme activity [4].

Phylogenetic analysis of culture 10 (Amylase positive) strain by 16s rRNA sequencing

Amplification reactions were performed as given in the 5X qarta.Taq master mix protocol using 16s rRNA primers. Sequences for primers used are as follows: 519F (5'- CAG CAG CCG CGG TAA TAC -3') and 1385R (5'- CGG TGT GTA CAA GGC CC -3'). 50µl PCR reaction was prepared containing 10µl of 5X PCR master mix, 2µl each of 10pM 519F and 1385R primers, 1µl of DNA (80µg) and nuclease free water made up to 50µl. The conditions for thermal cycling were 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 52°C for 45s and primer extension at 72 °C for 1 min 30s. All the PCR products were sequenced at MWG Biotech, Bangalore, India and chromatograms were obtained. The sequences of PCR products were analyzed by using Basic Local Alignment Search Tool (BLAST). Nucleotide- nucleotide BLAST was carried with facility of National Centre for Biotechnology Information (NCBI) [5].

RESULTS AND DISCUSSION

From the mangrove sediment samples, a total 11 bacteria were isolated. The morphological characters of the bacterial isolates are given in Table 1.

Table 1 Colony characteristics of bacterial isolates obtained from sediments

Culture no.	Pigment	Surface	Shape	Edge	Size	Consistency	Media
1.	Off white	Raised	round	smooth	Large	Mucoid, opaque	ISP2
2.	Light brown	Raised, folded	rough	uneven	Large	Opaque, pellet	ISP2
3.	Light brown	Smooth, flat	round	smooth	4mm	translucent	GYEA
4.	Light brown	Dry, raised	round	rough	4mm	opaque	GYEA
5.	Cream	Slimy, flat, metallic sheen	round	uneven	2-3mm	opaque	ISP2
6.	Light brown	Dry, wrinkled, flat, metallic sheen	round	uneven	2-3mm	opaque	GYEA
7.	Off white	Slimy, flat	uneven	uneven	2-3mm	Translucent	ISP2
8.	Light brown	Slimy, flat	round	smooth	3-4mm	transparent	GYEA
9.	Cream	Dry, slightly raised	round	uneven	Large	opaque	GYEA
10.	Light brown	Partially dry, rough, slightly raised	round	smooth	4-5mm	translucent	GYEA
11.	Off white	Partially dry, slightly raised	rough	uneven	3mm	opaque	GYEA

Out of these total 11 bacterial isolates, six showed enzyme production. The bacterial isolates showed 7 mm to 23 mm zone of clearance within 48 hrs as shown in Table 2. Culture no. 10 showed a promising production of all the three enzymes studies and hence, this isolate was further studied and explored.

pH 7 was found to be the most suitable among the positive strains in case of cellulase and amylase whereas pH 6 was most suitable for pullulanase producers. The zone of clearance in mm for pH variation is shown along with the colony size in Table 2.

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Table 2 Zone of clearance in mm for enzyme producers at different pH

Culture no.	Substrate	pH 6		pH 7		pH 8	
		Zone of clearance	Colony size	Zone of clearance	Colony size	Zone of clearance	Colony size
10	Carboxy-methyl cellulose	21	14	22	04	18	05
6		-	04	08	02	10	03
4		-	05	17	03	19	02
3		-	03	09	02	10	02
2		-	03	07	03	07	03
1		-	-	-	-	-	-
10	Pullulanase	21	08	18	03	18	04
4		20	07	21	04	18	03
3		22	10	13	02	-	03
2		11	03	-	04	-	02
6		15	03	-	03	-	03
11		23	07	-	02	-	03
10	Amylase	16	03	18	03	16	02
4		11	06	11	03	10	03
3		18	04	-	02	-	03

Table 3 Zone of clearance in mm for enzyme producers at pH 7 with varying Temperature

Sr. no.	Culture no.	Substrate	30°C pH 7		40°C pH 7.5	
			Zone of clearance	Colony size	Zone of clearance	Colony size
1.	10	Carboxy-methyl cellulose	20	04	16	01
2.	6		08	02	08	01
3.	4		17	03	12	01
4.	3		09	02	09	02
5.	2		07	03	10	01
6.	1		06	02	08	01
7.	10	Pullulanase	18	03	15	01
8.	4		21	04	13	01
9.	3		13	02	-	-
10.	10	Amylase	18	03	No positive isolate seen	
11.	4		11	03		

The zone of clearance for temperature variation is shown along with the colony size as shown in Table 3. For cellulases, pH 7 and temperature of 40°C was found to be optimum. For pullulanase, pH of 6 and temperature of 40°C was found to be optimum whereas in case of amylase, pH 7 and temperature of 30°C was found to give the best results.

Quantitative assay of amylase production

In quantitative assays, crude enzyme showed 13.40 U/ml amylase activity.

Amylase enzyme activity at different pH

Optimum activity of 13.1 U/ml was observed at a pH of 6 (Table 4).

Table 4 Effect of pH on the amylase activity

pH	Enzyme activity in U/ml
3	2.83
4	6.22
5	9.45
6	13.10
7	12.30
8	11.00
9	6.25

Amylase enzyme activity at different temperature for 30 minutes

Optimum activity of 11.88 U/ml was observed at a temperature of 50°C (Table 5).

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Table 5 Effect of Temperature on the amylase activity

Temperature (°C)	Enzyme activity in U/ml
30	2.34
40	8.55
50	11.88
60	9.21
70	3.68

Amylase enzyme stability assays

As seen in Table 6, 7 and 8, the enzyme activity and stability was found to vary with fluctuations in the temperature. Highest stability of amylase was observed at a temperature of 50°C for 15 to 30 mins.

Table 6 Amylase enzyme stability at 20°C

Time (mins)	Enzyme Activity in U/ml
0	2.10
15	1.98
30	1.06
45	0.89
60	0.38

Table 7 Amylase enzyme stability at 50°C

Time (mins)	Enzyme Activity in U/ml
0	13.02
15	12.95
30	11.65
45	8.97
60	8.23

Table 8 Amylase enzyme stability at 80°C

Time (mins)	Enzyme Activity in U/ml
0	3.23
15	3.10
30	2.65
45	1.29
60	0.82

Phylogenetic analysis by 16S rRNA gene sequences

Phylogenetic analysis of culture 10 by BLAST sequence alignment, the bacterial strain was found to belong to genus *Bacillus* as it showed 99% sequence similarity with the available nucleotide database (Table 9).

Table 9 Phylogenetic neighbors of culture 10 based on partial 16S rRNA gene sequence through NCBI

Description	Max score	Query cover	E value	Ident	Accession
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	1513	94%	0.0	99%	NR_074453.1
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	1502	94%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	1502	94%	0.0	99%	NR_102506.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	1502	94%	0.0	99%	NR_074540.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence	1502	94%	0.0	99%	NR_113266.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	1502	94%	0.0	99%	NR_112780.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	1502	94%	0.0	99%	NR_115714.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence	1502	94%	0.0	99%	NR_112630.1

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So, the present study clearly shows that the mangrove habitat continues to remain a cradle for novel biochemicals such as enzymes. The most promising enzyme producer was identified as *Bacillus* spp. There are previous reports which support this observation that bacteria from *Bacillus* genus have been proved to be excellent source of industrial enzymes such as amylase [6, 7] protease, [8], pullulanase [9] and cellulase [10, 11]. Usefulness of molecular markers and gene sequences in reliable species identification has also been indicated.

CONCLUSION

The bacteria derived from mangrove sediments were found to produce enzymes having industrial applications. Out of 11 bacteria, culture No. 10 produced potential amylase enzyme having thermostability. Optimum pH for amylase was found to be 6.0 whereas optimum temperature was 50°C. Amylase enzyme was found to be stable at 50°C for 30-60 minutes.

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