Int J Pharm Bio Sci 2013 July; 4(3): (B) 813 - 824

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ISOLATION, CHARACTERIZATION AND SCREENING OF ENZYME PRODUCING BACTERIA FROM DIFFERENT SOIL SAMPLES

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ABSTRACT

The present study was aimed to isolate, identify and screening of enzyme producing bacteria from soil samples. For this purpose the soil samples were collected from agriculture fields at sangam dairy, Guntur district, fish culture ponds, timber depot soil at rural areas of Bhimavaram, West Godavari district and oil mills at Vijayawada, Krishna district, Andhra Pradesh. A total of seventeen strains of bacteria were isolated from soil samples. Out of them nine isolates have amylase producing activity, eight have protease producing activity, two isolates have cellulose releasing activity and two have lipase producing activity. Among seventeen strains of bacteria 8 isolates belongs to *Micrococcus sp*., six isolates belongs to *Bacillus sp*., two belongs to *Staphylococcus sp.* and one belongs to *Streptococcus sp.* Further optimization studies and gene 16s RNA studies needed to confirm the strains.

KEYWORDS: **Soil, Bacteria, Screening, Microbial Enzymes.**

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INTRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells [1, 2]. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group. The bio-catalytic uses for enzymes have grown immensely in recent years since they are ecologically correct, have a high specificity, present chemo-regioenantio selectivity, and have a wide diversity of reactions. The main industries that apply microbial enzymes are the food, textile, leather, pharmaceutical, cosmetics, fine chemicals, energy, biomaterials, paper, cellulose and detergent industries [3-7]. Enzymes occur in every living cell, hence in all microorganisms. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing, and metabolic in nature individual enzymes produced vary markedly between species and even between [8]. Hence, it is customary to select strains for the commercial production of specific enzymes which have the capacity for producing highest amounts of the particular enzymes desired. Moreover, the conditions to obtain and optimize the production of enzymes in terms of nutrients, pH, temperature, and aeration are easily controlled in bioreactors. Microorganisms can also be manipulated genetically to improve the desirable characteristics of a bio-catalyzer. Additionally, the substrates used in the cultural medium are sustainable and industrial residuals can be used to produce value-added products. All these characteristics together have encouraged the ever-growing search for bio-catalytic processes. Immobilization processes allow the reuse of these enzymes and increase stability. The present investigation was designed to evaluate the presence of amylase producing bacteria in two different soil samples. In this study microorganisms from different sources have been isolated and identified their ability to produce different enzymes. Several microorganisms have been discovered for decades which have capacity to produce enzymes. But the need for newly isolated enzyme producing microorganism still continues [9]. In the present paper efforts have been made to isolate, identify and screening of enzyme producing bacteria from soil samples.

MATERIALS AND METHODS

Collection of Soil Samples

For the purpose of present study soil samples were collected in sterile containers from agriculture fields at Sangam Dairy, Guntur District, Fish culture ponds, and Timber depot soil at rural areas of Bhimavaram, West Godavari District and Oil mills at Vijayawada, Krishna District, Andhra Pradesh.

Isolation and identification

The collected soil samples were serially diluted up to 10^{-1} to 10^{-7} and spread on agar plates followed by incubation at 37°C for 24-48 hrs. The isolated bacteria identified by using morphological and biochemical tests as per procedures described in Bergey's Manual of systematic Bacteriology [10].

Identification of enzyme producing bacteria

For the identification of enzyme producing bacteria, the isolates were observed under the microscope and the bacterial colonies were recorded in respect to their color, size, shape, margins and pigmentation.

Microscopic observation

Attempts have been made to identify the bacterial strains on the basis of Gram staining, endospore staining, capsule staining and motility test.

Biochemical characterization

Biochemical characterization of bacterial isolates were done by performing various biochemical tests like Indole test, MR-VP test, Simmons citrate, Starch hydrolysis, H_2S production, Catalase, Oxidase, Urease , Nitrate reduction test and Gelatin hydrolysis test.

SCREENING FOR ENZYME ACTIVITY

Many distinctive enzyme activities can be demonstrated by observing the products resulting from the action of enzymes on specific substrates within the specific prepared media. For determination of enzyme producing activity (amylase, cellulase, protease and lipase) of the isolates screening tests are conducted. In order to observe enzymatic activities of microorganisms, pure cultures of microorganisms were inoculated in selective media.

Screening of Amylase bacteria

Isolates were taken for enzymatic screening for amylase activity[11-14]. Isolates were grown on starch agar medium (composite of soluble starch-2%, peptone-0.05%, KCL-0.01% (w/v), MgSo4.7h20-0.05% (w/v), (NH4)2 So4-0.01%, NaH2Po4-0.0.1% (w/v)[15]. After seven days of incubation at 28°C the culture plates were tested. Around the fungal inoculums when Grams iodine solution (for amylase) is added the cultures making zone of clearance were selected for further biochemical estimations. Biochemical estimation for amylase was done by starch –iodine method for selected isolates showing high zone of clearance.

Screening of Protease bacteria

The isolate was screened for protease [16-20] producing ability by using Casein Agar Medium Composed with Casein-0.3%, KNO_3-0.3%, Nacl-0.2%, K_2HPO_4-0.2%, $Mgso_4$-0.005%. $CaCl_2$-0.002%, Yeast extract-0.1%, Agar-3% and Skin milk agar Medium composed with Skin milk powder-10%, NaCl-5%, and Agar-2%. The protease activity was detected due to occurrence of a zone of clearance around the colony.

Screening of Cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis [21]. The bacterial colonies having the largest clear zone were selected for identification and cellulase [22-25] production in submerged system.

Screening of Lipase bacteria

The lipolytic activity of the culture was observed, using Rhodamine plate assay and Modified lipase assay media [26-27]. Bacterial isolate was inoculated in the plates with Modified lipase assay medium (15g Peptone, 5g NaCl, 1g CaCl2, 10 ml Tween 20, 15g Agar, and pH 7.0) was used for enzyme assay. The lipase activity was detected due to occurrence of a zone of clearance around the colony and subsequent formation of white precipitate of calcium monolaurate around the colony [28-29]. The lipolytic activity of the culture was observed using Rhodamine plate assay [30] with medium Rhodamine B- Olive oil Agar composite of Olive oil 3 % (v/v), Agar 2 % (w/v) , Rhodamine B I % (v/v) , Tris HCl buffer (pH 7) , 50 mM CaCl 1 %. Based on the screening test results the 9 isolates have amylase producing activity, 8 isolates have protease releasing activity, 2 isolates have cellulose releasing activity and 2 isolates have lipase producing activity which was reflected by wide clear zone formation around the colony on the plates of selective media none of them have lipase producing activity. All the positive isolates from primary screening were subjected to secondary screening to confirm the enzyme activity. Enzymatic assay studies are conducted for positive isolates to estimate the enzyme releasing activity.

DETERMINATION OF ENZYME ACTIVITY

Determination of Amylase activity

Amylase activity was determined as described by Okolo et al., [31]. The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25ml of crude enzyme extract. After 10minutes of incubation at 50^0C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitro salicylic acid (DNS) method of Miller[32]. The blank contained 0.5ml of 0.1M acetate buffer (pH 5.0), 1.25ml 0f 1% starch solution and 0.25 ml of distilled water. One unit (IU) of amylase is defined as the amount of enzyme releasing 1 µmol glucose equivalent per minute under the assay condition.

Determination of Protease activity

Protease activity was assayed using Hayashi et al., modified method[33]. The reaction mixture containing 1ml enzyme, 5ml of 1% casein (50 mM Tris, pH-8) substrate were incubated for 1 hr at 37^0C. To the reaction mixture 5 ml of TCA (110mm) was added to stop the reaction and incubated for 30 min at 37^0C. The mixture solution was filtered. 2 ml of filtered solution was added to 5ml of 500mM sodium carbonate solution and 1ml of folin phenol reagent. This was incubated for 30 min at 37^0C and the colour intensity was observed at 660nm.

Determination of Cellulase activity

Cellulase activity was assayed using dinitrosalisic acid (DNS) reagent[33] by estimation of reducing sugars released from CMC solubilized in 0.05 M phosphate buffer at pH 8. The culture broth was centrifuged at14000 × g for 10 min at 4˚C and the clear supernatant served as crude enzyme source. Crude enzyme was added to 0.5 ml of 1 % carboxymethyl cellulose in 0.05 M phosphate buffer and incubated at 50˚C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100˚C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve. One unit (IU) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions.

Determination of Lipase activity

Lipase assay was carried by using the copper soap method. Quantification of fatty acid released by lipase is determined by reference to a standard curve prepared using oleic acid. 5ml of benzene and 1ml of cupric acetate were taken into screw-cap test tubes and into a 50ml Erlenmeyer flask with a stopper, 25 ml olive oil taken then Pre-incubated for 15 min with magnetic stirring in a water bath at37°C. Sufficient amount of enzyme was added to initiate lipolysis on the emulsion substrate. Immediately vortexes for 2 min. to stop the reaction and form the colored fatty acid cupric soap, centrifuged for 5 min at 1000rpm at room temperature, to obtain the clear benzene upper phase and then measured the absorbance at 715nm for the benzene layer of sample against reagent blank [34].

RESULTS AND DISCUSSION

In the present study 17 strains of bacteria namely *Bacillus sp., Monococcus sp., Staphylococcus sp.,* and *Streptococcus sp.* were isolated from four different soil samples (Table 1). Among the 17 strains, 8 belong to *Monococcus sp.,* 6 belong to *Bacillus sp., 2* belong to *Staphylococcus sp.* and one belongs to *Streptococcus sp.* The isolated bacteria were identified by morphological and biochemical tests (Table 2) as per procedures described by Bergey's Manual of systematic Bacteriology [10].

Table 1
Details of number of isolates isolated from different soil samples

S. No.	Source of Sample	Number of Isolates	Isolates
Sample- 1	Fish culture ponds at Bhimavaram, West Godavari District, A.P.	6	TS13SP
			TS6MCN
			TS10MCN
			TS17BP
			TS15MCN
			TS3BP
Sample -2	Soil near to oil mills at Vijayawada, Krishna District, A.P.	3	TS2MCN
			TS11BP
			TS 1F
Sample- 3	Timber depot soil at rural areas of Bhimavaram, West Godavari District, A.P.	3	TS 26
			TS 24
			TS 27
Sample- 4	Agriculture fields at Sangam Dairy, Guntur District, A. P.,	5	TS7BN
			TS8MCN
			TS9MCN
			TS1MCP
			TS12BN

Table 2
Details of bacterial Strains Isolated from Soil samples

S.No.	Stain Code	Type of Strain	Grams Staining
1	TS1MCP	*Monococcus sp.*	+ve
2	TS2MCN	*Monococcus sp.*	-ve
3	TS3BP	*Bacillus sp.*	+ve
4	TS4BP	*Bacillus sp*	+ve
5	TS5SRP	*Streptococcus sp.*	+ve
6	TS6MCN	*Monococcus sp.*	-ve
7	TS7BN	*Bacillus sp.*	-ve
8	TS8MCN	*Monococcus sp.*	-ve
9	TS9MCN	*Monococcus sp.*	-ve
10	TS10MCN	*Monococcus sp.*	-ve
11	TS11BP	*Bacillus sp*	+ve
12	TS12BN	*Bacillus sp.*	-ve
13	TS13SP	*Staphylococcus sp.*	+ve
14	TS14SN	*Staphylococcus sp.*	-ve
15	TS15MCN	*Monococcus sp.*	+ve
16	TS16MCN	*Monococcus sp.*	-ve
17	TS17BP	*Bacillus sp.*	+ve

**** Note: +ve = Positive, -ve = Negative***

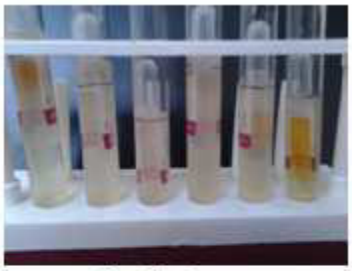
Strain Identification

Morphological and biochemical characteristics were used to identify the genus of the strain. The strain *TS1MCP* is a Gram +ve *Monococcus,* unicellular spherical shaped, motile and gave positive results for Citrate utilization, Oxidation, Methyl red, Catalase and nitrate reductase (Figure 1) tests and negative results for Urease, H_2S, Indole, Vogues-Proskaeur (VP) and fermentation tests (Table 3). Based on the results, the strains *TS2BP* was identified as genus *Monococcus.*

Table 3
Morphological and Biochemical characteristics of Monococcus sp. isolated from soil samples

		Monococcus sp.							
S. No	**Strain Code→ Name of the Test**	**TS1MCP**	**TS2MCN**	**TS6MCN**	**TS8MCN**	**TS9MCN**	**TS10MCN**	**TS15MCN**	**TS16MCN**
1	Gram Staining	+ (GP)	-	-	-	-	-	+(GP)	-
2	Citrate utilization	+	+	+	+	+	+	+	+
3	Urease test	-	-	-	-	-	-	-	+
4	H_2S test	-	-	-	-	-	-	-	+
5	Oxidation	+	+	+	+	+	+	+	+
6	Methyl Red	+	+	+	+	+	+	-	+
7	Indole test	-	+	-	-	+	+	+	-
8	Vogues-Proskaeur(VP) test	-	-	-	-	-	-	-	-
9	Catalase production	+	+	+	+	+	+	+	+
10	Fermentation test	-	-	+	-	+	+	+	+
11	Nitrate reductase	+	+	+	-	+	-	-	-

Citrate Utilization test



Oxidation test

Methyl Red test

Catalase test

Nitrate Reductase test

Figure 1
Photographs of biochemical tests showing positive results for citrate utilization, oxidation, methyl red, catalase and nitrate reductase tests shown by Monococcus sp.

The strain *TS2BP* is a gram positive *Bacillus*, rod shaped, motile and gave positive test for Citrate utilization, Urease, H_2S, Oxidation, Vogues-Proskaeur (VP), Catalase and fermentation tests (figure 2), and negative test for Methyl red, Indole and Nitrate reductase tests. Based on the results, the strains *TS2BP* was identified as genus *Bacillus* (Table 4).

Table 4
Morphological and Biochemical characteristics of Bacillus sp. isolated from soil samples

		Bacillus sp.					
S. No	**Strain Code→ Name of the Test**	**TS3BP**	**TS4BP**	**TS7BN**	**TS11BP**	**TS12BN**	**TS17BP**
1	Cell Shape	Single shaped	Rod Single shaped	Rod Single shaped	Rod Single shaped	Rod Single shaped	Rod Single shaped Rod
2	Gram Staining	+(GP)	+(GP)	-	+(GP)	-	+(GP)
3	Citrate utilization	+	+	+	+	+	+
4	Urease production	+	-	-	+	-	-
5	H_2S test	+	-	+-	-	-	-
6	Oxidation	+	+	+	+	+	+
7	Methyl Red	-	+	+	+	+	+
8	Indole test	-	+	+	-	-	-
9	Vogues-Proskaeur (VP) test	+	-	+	+	+	-
10	Catalase production	+	+	+	+	+	+
11	Fermentation test	+GP	+	+	+GP	+	+
12	Nitrate reductase	-	-	-	-	+	+

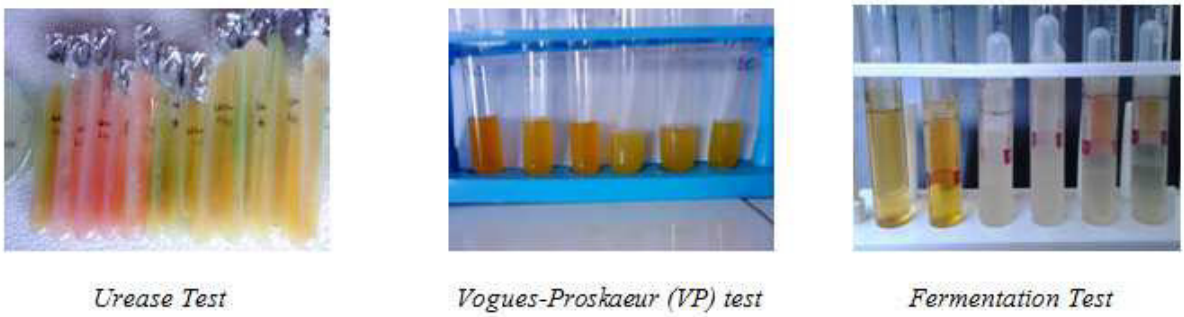


Figure 2
Photographs of Biochemical tests showing Positive results for *Urease test, Vogues-Proskaeur (VP) test, Fermentation tests* shown by *Bacillus sp.*

The strain *TS5SRP* is a gram positive Streptococcus, cells appear in chains, spherical shaped, motile and gave positive tests for Citrate utilization, Urease, Oxidation, Methyl red, Indole, Vogues-Proskaeur (VP), Catalase and fermentation and negative for H_2S, and Nitrate reductase tests. Based on the results, the strains *TS5SRP* was identified as genus *Streptococcus sp.,* (Table 5).

Table 5
Morphological and Biochemical characteristics of Streptococcus sp and Staphylococcus sp isolated from Soil sample

		Streptococcus sp.	*Staphylococcus sp.*	
S. No	**Strain Code→ Name of the Test**	**TS5SRP**	**TS13SP**	**TS14SN**
1	Gram Staining	+	+	-
2	Citrate utilization	+	+	+
3	Urease	+	-	-
4	H_2S test	-	-	-
5	Oxidation	+	+	+
6	Methyl Red	+	+	+
7	Indole test	+	-	-
8	Vogues-Proskaeur (VP) test	+	+	-
9	Catalase:	+	+	+
10	Fermentation test	+	+	+
11	Nitrate reductase	-	+	-

The strain *TS13SP* is a gram positive Staphylococcus, cells appear in bunch ,spherical shaped, motile and gave positive test for Citrate utilization, Oxidation, Methyl red, Vogues-Proskaeur (VP), Catalase, fermentation and Nitrate reductase and negative test for Urease, H_2S, and Indole production. Based on the results, the strains *TS13SP* was identified as genus *Staphylococcus sp.* (Table 5).

Enzyme activity Screening

17 strains were tested for enzyme producing ability, among them 9 isolates have amylase producing activity, 8 isolates have protease releasing activity, two isolates have cellulose and lipase releasing activity. Majority of strains has the amylase producing activity. The starch degradation around the bacterial colony indicated the amylase positive strain which produced clear halos with iodine solution due to hydrolysis of starch (Figure 3). The strains *TS3BP, TS5SRP, TS6MCN, TS15MCN, TS13SP, TS16MCN, TS10MCN, TS14SN and TS17BP* have shown Amylase producing activity (Table 6) among the 17 strains isolated from the four soil samples.

Figure 3
Culture plates showing the white color zone formation when iodine was added to the incubated plates

Eight isolates *TS1MCP, TS3BP, TS8MCN, TS7BN, TS9MCN, TS12BN, TS4BP, TS14SN* were protease producer (Table 6) and clear zone around colonies (Figure 4) was considered as the evidence of protease production. Since there is not necessarily good correlation between zones of clearing around colonies on skim milk agar plates and levels of protease activity.

Figure 3
Culture plates showing the clear zone formation around the colony grown in Casein medium (primary screening) & skimmed milk agar medium (secondary screening).

Two isolates exhibits its cellulolytic activity by zone formation in CMC agar media and two isolates have shown Lipase activity by forming the zone on Rhodamine agar plate. All these positive isolates are subjected to secondary screening and confirm their ability to produce enzyme (Table 6).

Table 6
Production of different enzymes by soil bacteria

S. No	Organism tested	Result			
		Amylase	Protease	Cellulase	Lipase
1	TS1MCP	-ve	+ve	-ve	-ve
2	TS2MCN	-ve	-ve	-ve	+ve
3	TS3BP	+ve	+ve	-ve	-ve
4	TS4BP	-ve	+ve	-ve	-ve
5	TS5SRP	+ve	-ve	+ve	-ve
6	TS6MCN	+ve	-ve	-ve	-ve
7	TS7BN	-ve	+ve	-ve	-ve
8	TS8MCN	-ve	+ve	-ve	-ve
9	TS9MCN	-ve	+ve	-ve	-ve
10	TS10MCN	+ve	-ve	-ve	-ve
11	TS11BP	-ve	-ve	-ve	+ve
12	TS12BN	-ve	+ve	-ve	-ve
13	TS13SP	+ve	-ve	-ve	-ve
14	TS14SN	+ve	+ve	-ve	-ve
15	TS15MCN	+ve	-ve	-ve	-ve
16	Lc24	+ve	-ve	+ve	-ve
17	Lc27wn	+ve	-ve	-ve	-ve

Determination of Enzyme activity

Estimation of enzyme activity for the positive isolates has conducted. Concentration of enzyme in crude sample was calculated by reacting with the enzyme with substrate comparing the resultant O.D with standard graph. The enzyme activity was expressed in International Unit (IU). 1U/ML = Amount of enzyme which releases 1micro mole glucose under assay conditions. The results of amylase assay are shown in Table 7. The results of Determination of Protease, cellulase and lipase activity from respective strains are given in Table 8, 9 and 10 respectively.

Table 7
Determination of amylase from amylase producing strains

S. No	Organism tested	Type of Strain	Amylase activity in the culture supernatant IU/mg
1	TS3BP	*Bacillus sp.*	2.3
2	TS5SRP	*Streptococcus sp.*	0.9
3	TS6MCN	*Monococcus sp.*	1.7
4	TS15MCN	*Monococcus sp.*	1.6
5	TS13SP	*Staphylococcus sp.*	2.6
6	TS16MCN	*Monococcus sp.*	4.5
7	TS10MCN	*Monococcus sp.*	6.2
8	TS14SN	*Staphylococcus sp.*	4.3
9	TS17BP	*Bacillus sp.*	3.2

Table 8
Determination of protease from protease producing strains

S.No.	Organism tested	Type of Strain	Protease activity in the culture supernatant IU/mg
1	TS1MCP	*Monococcus sp.*	2.7
2	TS3BP	*Bacillus sp.*	1.6
3	TS8MCN	*Monococcus sp.*	1.2
4	TS7BN	*Bacillus sp.*	3.5
5	TS9MCN	*Monococcus sp.*	2.3
6	TS12BN	*Bacillus sp.*	4.6
7	TS4BP	*Bacillus sp.*	6.7
8	TS14SN	*Staphylococcus sp.*	8.1

Table 9
Determination of Cellulase from Cellulase producing bacteria

S.NO	Organism tested	Type of Strain	Cellulase activity in the culture supernatant IU/mg
1	TS5SRP	*Streptococcus sp.*	4.2
2	TS16MCN	*Monococcus sp.*	3.6

Table 10
Determination of Lipase from Lipase producing bacteria

S.NO	Organism tested	Type of Strain	Lipase activity in the culture supernatant IU/mg
1	TS2MCN	*Monococcus sp.*	3.9
2	TS11BP	*Bacillus sp.*	1.6

Among 17, seven isolates showed good amylase activity, four isolates showed moderate activity, three isolates showed poor activity and five isolates exhibited very poor activity. Structural, staining and biochemical activity results have revealed that these active enzyme producing bacteria are *Monococcus sp., Bacillus* sp., *Streptococcus sp. and Staphylococcus sp.* (Table 11). All biochemical tests have shown similar results as described in Bergy's manual of determinative Bacteriology[10]. Further characterization has to be done on the basis of 16S r DNA gene sequencing for further conformation of strains.

Table 11
Highest Enzyme producing bacteria (2 for each Enzyme) isolated from different soil samples

S.no	Strain Code	Type	Source	Enzyme produced	Yield
1	TS10MCN	*Monococcus sp.*	Fish culture pond Soil	Amylase	**6.2**
2	TS16MCN	*Monococcus sp*	Timber depot soil	Amylase	**4.6**
3	TS14SN	*Staphylococcus sp*	Agriculture soil	Protease	**8.1**
4	TS4BP	*Bacillus sp.*	Soil near oil mills	Protease	**6.7**
5	TS2MCN	*Monococcus sp.*	Soil near oil mills	Lipase	3.9
6	TS11BP	*Bacillus sp.*	Soil near oil mills	Lipase	1.6
7	TS5SRP	*Streptococcus sp.*	Timber depot soil	Cellulase	4.2
8	TS16MCN	*Monococcus sp.*	Timber depot soil	Cellulase	3.6

CONCLUSION

Seventeen strains of bacteria were isolated from different soil samples, were studied for enzyme producing activity. They were belong to genus namely *Bacillus sp., Monococcus* sp., *Streptococcus sp* and *Staphylococcus sp.* Some of them have both amylase and protease producing activity. Among all the positive isolates, strain TS14SN and TS4BP have shown high enzyme producing activity in compared to other strains. Highest enzyme producing strains can be subject for optimization studies to get high yield.

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