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# ISOLATION AND CHARACTERIZATION OF THERMOPHILIC ACTINOMYCETES WITH EXTRACELLULAR ENZYME AND BIO-SURFACTANT PRODUCTION POTENTIAL FROM THAR DESERT, INDIA

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**Abstract-** In the present study thermophilic actinomycetes isolates were isolated from Thar desert of India. Identification of four actinomycetes isolates were done by various morphological and biochemical tests. All the isolates were screened for the presence of extracellular enzyme and bio-surfactant production potential. The results showed that the isolates namely TDI-7, TDI-10, TDI-12, TDI-13 showed the presence of extracellular enzymes and bio-surfactant activity. Isolate TDI-7 and TDI-12 showed the presence of protease production whereas in TDI-10, TDI-12, TDI-13 amylase activity was reported. Lipase activity was found absent in all the isolates. Significant bio-surfactant activity was reported against TDI-13. Further molecular identification of these four isolates were done by partial 16S rRNA gene sequence analysis which showed 95-99 % similarities of references sequences TDI-7 with *Streptomyces sp.,* TDI-10 is with *Streptomyces sp.,* TDI-12 with *Streptomyces variabilis strain* and TDI-13 with *Streptomyces rubiginosus* during NCBI blast.

**Keywords**-Thermophilic actinomycetes, extracellular enzymes, bio-surfactant production

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## Introduction

Actinomycete is a phylum of gram-positive, filamentous, aerobic bacteria with high G+C content of genome and well-developed vegetative hyphae system [1]. Initially actinomycetes were thought to be intervening between bacteria and fungi however now they are perceived as prokaryotic organism. Actinomycetes constitute ~50% of the total microbial bacterial population in desert soil [2]. They are numerous and widely distributed in soil and are next to bacteria in abundance. Thar desert covers about 320,000 $km^2$ (120,000 sq mi), of which 60% is in Rajasthan, India [3]. It is characterized by extremes of climate, erratic rainfall, high evapo-transpiration rate, low humidity extremes of diurnal and annual temperature. Desert soils are considered as the main reservoirs of actinomycetes. The thermophilic actinomycetes found in desert soil thrive at temperature between 40 to 56 °C [4]. The adaptation in actinomycetes to survive at high temperature is due to the presence of membrane lipids, which contains more saturated and straight chain of fatty acids [5-7]. Among the thermophilic actinomycetes members of order *Actinomycetales, Streptomyces, Micromonospora, Actinomadura,* and *Streptosporangium* were mainly spread in desert soil [8]. It is found that members of actinomycetes growing in extreme condition produce industrially important compounds like antibiotics, proteins, hormones and enzymes [9]. The under-investigated environment of Thar desert are viewed as bio-assorted hotspots, where it is presume that the impact the thermophilic environment may bring about the advancement of biotechnologically essential thermophilic actinomycetes [10]. As Thar desert harbours a treasure trove of microbial diversity still no literature is found on isolation of thermophilic actinomycetes, possessing extracellular enzymes and bio-surfactant production. Actinomycetes have an un-estimated wealth of microbial diversity in Thar desert, which needs to be tapped properly. Therefore, in the present study an attempt was made to search potentially important thermophilic actinomycetes strain, which posses extracellular enzymes and bio-surfactant activity from under- exploited ecosystem of Thar desert.

## MaterialsandMethods

### Microbial Culture methods

#### 1) Collection of soil samples

Soil samples for isolation of actinomycetes were collected in clean, sterile and dry polythene bags from Jodhpur and Bikaner domain of Rajasthan.

#### 2) Isolation

Soil samples were pre-heated at 50°C for 1 hr. in oven, to reduce the growth of other microbes. Serial dilution of soil samples were prepared by adding 1 g of soil in bottle containing 9 ml of distilled water and mix thoroughly by vortex [11]. For making ten-fold dilution 1.0 ml of the original suspension was transferred by sterile pipette in the bottle containing 9 ml of distilled water. Subsequently, it was further diluted up to $10^{-6}$ dilutions. From each dilution, 0.5 ml of aliquot was spread on surface of Actinomycetes Isolation Agar (AIA) by using spread plate technique. After inoculation, all plates were incubated at 37°C in the BOD incubator for 4 days. The colonies showing typical characteristics different from other bacterial colonies were separated by streaking on AIA perti plates. Isolates were stored in 20% glycerol at -20°C and subculture on the AIA at 37°C for 4 days before use.

#### 3) Screening for hydrolysing extracellular enzymes

All the isolates were screened for extracellular enzyme activity by plate assay method.

##### i. Plate assay of enzyme for amylase activity

Amylase activity was determined by inoculating the isolates on starch nutrient agar plates containing starch 10 gm; peptone 5 gm; yeast extract 3gm; agar 30 g m; NaCl 10 gm; pH 7 in 1liter. Plates were incubated at 37 ºC for 72 hour, the clear zone obtained by flooding the plates with iodine solution showed the production of amylase enzyme [12].

##### ii. Plate assay of enzyme for Protease activity

To determine the protease activity nutrient agar with 2% gelatin at neutral pH 7.0 was prepared. The petri plates were incubated at 37 ºC for 72 h. Protease enzyme activity was visualized by flooding the plates with 0.1% mercuric chloride solution. The formation of a clear zone around the colony was an indication of gelatin hydrolysis [13].

**iii. Plate assay of enzyme for Lipase activity**

Lipase production was assessed by inoculating isolates on Nutrient agar containing 2% Tween 20 with Tween 80 and methyl red indicator. Lipolytic activity was determined by the formation of the zone of intensification of the indicator colour after 24-48 hr [13].

**4) Bio-surfactant production**

Isolates were screened for presence of bio-surfactant activity by inoculating in Luria broth for 7 days at 37°C. After incubation, the broth was centrifuged at 7000 rpm for 20 minutes and was filtered. The obtained cell free supernatant was used in the following preliminary test for identifying the presence of bio-surfactant [14, 15].

**i. Oil spreading method**

A thin oil film was made by adding 10 µl of crude oil over 40 ml distil water in a petri dish. 10 µl of cell free supernatant was added carefully in the middle of the oil film. A clear zone formed in the film showed the presence of bio-surfactants [16].

**ii. Parafilm M test**

2 ml of cell free supernatant was mixed with one drop of bromo-phenol blue indicator. 10 µl of this solution was placed on the Parafilm-M like a drop with the aid of micropipette. Observe the shape of drop after one minute. If bio-surfactants present, the shape of drop becomes flat, in case of absence the shape of drop remains dome shaped.

**iii. Emulsification index**

Emulsification index (E24) was used to determine the bio-surfactant property of the isolates. Emulsification index was obtained by adding 1 ml the cell-free broth with 4 ml water and 6ml kerosene in a test tube. The mixture was subjected to vortex for 2 minutes at high speed. After that it was allowed to stand for 24 h. The emulsification index is given as percentage of the height of emulsified layer divided by the total height of the liquid column. Following equation was used to calculate the percentage of emulsification index [17, 18].

$$E24 = \frac{\text{Height of emulsion formed (cm)}}{\text{Total height of solution (cm)}} \times 100$$

**Characterization Methods**

**Phenotypical**

Isolates were identified by microscopic methods, gram staining, aerial mass colour, spore chain morphology. Macroscopic characterization of isolates was done according to the methodology of the International Streptomyces Project (ISP) [19] on different ISP 4 to 7 media and other media like Starch Casein Agar [20], Actinomycetes Isolation Agar (AIA). Biochemical characterization includes catalase, oxidase, nitrate tests, carbon utilization, $H_2S$ production, mannitol salt agar [21]. Growth of isolates at different temperatures was noticed to select the thermophilic strains.

**Molecular**

Isolate was inoculated separately in McCartney bottle containing 15 ml of autoclaved LB broth. The inoculated bottles were kept on to rotary shaker in incubator for 48 hours. The genomic DNA was isolated following HiPurATM Bacterial and yeast Genomic DNA purification spin kit (MB 505; Hi Media Laboratories, Mumbai, India) following manufacturer's protocols.

Quantity and quality of DNA were estimated by U-V visible spectrophotometer (ELICO SL 164) as well as by Gel electrophoresis (Banglore GeNei) with 0.7 % (w/v) Agarose (Sisco Research Laboratories, India) in 1x TAE buffer to ensure compatibility for PCR amplification. The genomic DNA of isolate was used for PCR reaction and sequencing of 16S rRNA gene. The PCR primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5'CGG TTA CCT TGT TAC GAC TT 3') were used to amplify the 16S ribosomal DNA [22]. Amplification by PCR was performed in a total volume of 25 µl containing: 2.5 ul Taq DNA polymerase (Bangalore Genei), 1.5 ul dNTP mix (Bangalore Genei), 2.5 ul Taq buffer with $MgCl_2$, 0.5 ul 27F and 0.5 ul 1492R primers (Bangalore Genei), 186 ng/ul genomic DNA in distilled water. The reactions were performed in a gradient thermal cycler with following conditions: 1 min denaturation at 95°C, 1 min annealing at 55°C, 2 min elongations at 72°C for 30 cycles with a final elongation step of 72°C for 15 min. The PCR products were visualized on 1.5% Agarose gel in 1X TAE buffer at 60 V. Agarose gels were stained with ethidium bromide and observed under UV light in Gel Doc ESCO (Swift. Max Pro). The PCR products were directly sequenced using 27F (Forward) and 1492R (reverse) universal primers from Macrogen, Korea.

**Results**

The soil of area investigated under study showed occurrence of actinomycetes in most of the samples collected from different region of Jodhpur and Bikaner, Rajasthan and confirmed the soil as rich source of actinomycetes.

**Isolation and characterization of isolates**

The isolation of isolates was done by inoculating the sample on AIA agar as per methodology. Colonies of actinomycetes isolate appeared more at specific dilution i.e. $10^{-2}$ to $10^{-4}$. Colonies showing typical characteristics different from other bacterial colonies were randomly selected primarily on the basis of colony color, colony texture, sporulation and staining behavior i.e. gram staining. Isolates showed gram-positive behavior, non-motile and powdery in texture on AIA. When observed under microscope it showed filamentous and spore bearing structures. Isolates were numbered as TDI-7, TDI-10, TDI-12, TDI-13. The isolates were inoculated on different available media i.e. Starch casein agar (SCA) and International streptomyces project (ISP 4 to 7) media, at 37° C for 4 days to determine and identify the suitable medium, optimal nutritional and cultural conditions for the growth [Table-1]. The effects of different incubation temperatures (36°, 38°, 40° and 42°C) on the growth of isolates were also studied and all the isolates showed significant growth at 42°C, which showed the isolates were thermophilic in nature [23]. Different biochemical tests were performed for physiological identification of isolate [Table-2].

**Table-1** *Growth Characteristic on different media*

| S. No | Isolates | Colony characteristic on different media | | | | | |
|---|---|---|---|---|---|---|---|
| | | AIA | SCA | ISP-4 | ISP-5 | ISP-6 | ISP-7 |
| 1. | TDI-7 | Grey | Grey | Off white | Grey | Colourless | Colourless |
| 2. | TDI-10 | Grey | White | Colourless | Grey | Colourless | Offwhite |
| 3. | TDI-12 | Grey | Grey | Colourless | Grey | Colourless | Colourless |
| 4. | TDI-13 | Grey | Grey | Colourless | Grey | Colourless | Colourless |

AIA: Actinomycetes Isolation Agar, SCA: Starch Casein Agar, ISP- International streptomyces Project

**Screening for hydrolysing extracellular enzymes**

All the isolates obtained from soil after identification were screened for extracellular enzyme activity as per the described method and it is found that isolate TDI-10 ,TDI-12, TDI-13 showed good amylolytic activity where as no activity was recorded by TDI-7. Protease enzyme activity was recorded by the formation of clear zone around the isolates. Among the four isolates only two

showed the protease activity i.e.TDI-7 and TDI-12. All isolates were unable to produce lipase enzyme [Table-3].

**Screening for bio-surfactant production**

Actinomycetes were screened for extra-cellular bio-surfactant production. Out of these 4 isolates, only three isolates, namely, TDI-7, TDI-12 and TDI-13, showed positive results for oil spreading technique.

**Table-2** *Characterization of isolates*

| Isolates | Test | | | | | Carbon source | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gram Staining | PH | Temp. | $H_2S$ Prod. | MSA | Sc | Fr | Ar | Xy | Dx | Ra | In |
| TDI-7 | + | 7.0 | 42°C | - | - | + | + | - | - | + | + | - |
| TDI-10 | + | 7.0 | 42° C | - | + | + | - | - | + | + | - | + |
| TDI-12 | + | 7.0 | 42°C | + | + | + | + | - | - | - | + | + |
| TDI-13 | + | 7.0 | 42°C | - | + | + | + | + | + | + | - | + |

+ Positive, - Negative, MSA Mannitol-Salt Agar, Temp- Temperature, Prod- Production, Sc-Sucrose, Fr-Fructose, Ar- Arabinose, Xy- Xylose, Dx- Dextrose, Ra- Raffinose, In Inositol

**Table-3** *Screening for hydrolysing extracellular enzymes*

| Isolates | Amylase activity | Protease activity | Lipase activity |
|---|---|---|---|
| TDI-7 | - | + | - |
| TDI-10 | + | - | - |
| TDI-12 | + | + | - |
| TDI-13 | + | - | - |

+ Positive , -Negative

From the oil drop spreading test it was found that except TDI-10 all the isolates showed positive results and maximum displaced area with a diameter of 5.4 cm, indicating a good bio-surfactant production was observed by TDI-13 [Fig-1]. Further the isolates were subjected for parafilm-M test, sodium laryl sulphate was taken as a positive control and distilled water as negative control [24]. Formation of flat drop was shown by TDI-7, TDI-12 and TDI-13 isolates on parafilm indicates the production of bio-surfactant. The emulsification index of isolates was detected from first day of incubation period and the results obtained showed the highest emulsion formation after 72 h by isolates TDI-13 i.e. 20.5% in comparison to sodium laryl sulphate. [Table-4]

**Table-4** *Screening for biosurfactant production*

| S. No. | Test | TDI-7 | TDI-10 | TDI-12 | TDI-13 | SLS |
|---|---|---|---|---|---|---|
| 1. | Parafilm- M | + | - | + | + | + |
| 2. | Oil spreading method | 3.6 cm | - | 3.2cm | 5.4 cm | 6.8 cm |
| 3. | Emulsification index | 14.6% | - | 16.6% | 20.5% | 18.7 % |

**SLS-** sodium laryl sulphate

**Fig-1 Bio-surfactant production through oil spreading method by TDI-13**

**Molecular identification**

Identification of actinomycetes using molecular tools proved to be faster and least tedious compared to classical biochemical method. Therefore, in present investigation taxonomic characterization of extra-cellular and bio-surfactant-producing actinomycetes was done by 16S rRNA. The PCR amplified products of the 4 isolates of actinomycetes generated a single prominent band on agarose gel. The amplified products of 16S rRNA gene of four isolates ranged between 1.5 Kbp. The results obtained from the direct sequencing of purified PCR products showed the all isolates belong to the class actionomycetes. Sequenced data of 1492 reverse primer was inverted and complimented using Gene tool software and aligned with sequence data of 27F primer to obtain continuous sequence of amplified products. Result of BLAST N search of 16S rRNA gene sequence of the isolates is given in [Table-5]. All isolates were identified with the available sequences in the NCBI database.

**Table-5** *Molecular characteristics of isolates of actinomycetes*

| S. No. | Isolates | Gen Accession No. (NCBI) | Name of species |
|---|---|---|---|
| 1. | TDI-7 | KT021813 | *Streptomyces sp.* TDI-7 |
| 2. | TDI-10 | KT021817 | *Streptomyces sp.* TDI-10 |
| 3. | TDI-12 | KT021818 | *Streptomyces variabilis strain* TDI-12 |
| 4. | TDI-13 | KT021819 | *Streptomyces rubiginosus strain* TDI-13 |

**Discussion**

In the present study all the isolates were isolated from Jodhpur and Bikaner domain of Thar desert. The isolation of actinomycetes was done by inoculating these samples on agar plates as per methodology described. Identification of isolate was done by microscopic method. Lakshmipathy and Krishnan [25] reported that the appearance of colonies of actinobacteria in culture occurred after incubation of 7 to 10 days, but interestingly in present investigation colonies appeared on AIA and SCA after incubation of 4 days only. SCA was found best medium for the growth of actinomycetes. It was interesting to note that they can survive for a much longer period without sub culturing. Actinomycetes found to produce many kinds of metabolites, including antibiotics, pigments, enzymes and bio-surfactants [26-29].

All the thermophilic actinomycetes isolates were screened for the presence of extracellular enzymes and bio-surfactant production. Isolates were initially screened for extracellular enzyme activity and results obtained showed amylase producing activity by isolates TDI-10, TDI-12 and TDI-13. Earlier extracellular enzyme activity by *streptomycetes sp.* and its importance in antifungal and bioleaching was reported by researchers [30, 31]. Besides this, scientists reported the production of extracellular enzymes involved in lignin solubilisation by *Strepomycetes viridosporous* [32, 33]. It is also found that some antibiotic biosynthetic enzymes are extracellular in nature and also regulate the production of antibiotics [34]. Actinomycetes are good choice as bio-surfactant producers, because of their abundance in soil and their major roles in recycling of material in nature [14, 35]. No study is reported till date on the bio-surfactant activity of thermophilic actinomycetes from Thar desert of India. Therefore, in the present

study first time thermophilic actinomycetes were screened for bio-surfactant activity. The results showed the production of bio-surfactant activity by isolate TDI-7, TDI-12 and TDI-13. Extra-cellular and bio-surfactant-producing actinomycetes were identified on molecular basis. Ninawe [36] reported that many actinobacteria genera show morphological variation at different stages of growth when cultured on various media causing confusion during their taxonomic identification. Therefore, in present investigation molecular characterization by using 16S rRNA gene sequences were used for species identification by the help of reference sequences of NCBI Data base. During the present course of investigation four isolates showed 95-99 % similarities of references sequences during NCBI blast TDI-7 with *Streptomyces sp.*(SHXFF2), TDI-10 is also with *Streptomyces sp.*MD16, TDI-12 with Streptomyces *variabilis strain* and TDI-13 with *Streptomyces rubiginosus,* which is first time isolated from Thar desert. Earlier the strain *Streptomyces rubiginosus* was investigated for bio-surfactant production, from rhizosphere of medicinal plant is differs in physical parameters and growth pattern from TDI-13 thermophilic strain [37]. Although species TDI-7 and TDI-10 are same but polymorphism is noticed in 16S rRNA sequences. Similarly, morphological parameter is also showed different characters in reference to colony colour, sporulation and filamentous nature.

## Conclusion

It is concluded that under-explored habitats of Thar desert are very rich source of thermophilic actinomycetes which have capacity to produce industrially important extracellular enzymes and biosurfactants. Considering the present results, further studies on identification, structure elucidation of biosurfactant and antimicrobial potential activity of thermophilic actinomycetes are required.

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