ISOLATION AND ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES FROM CHAUNG-THA AREA AND BIOCHEMICAL CHARACTERIZATION OF SELECTED *STREPTOMYCES* (TR-2)

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Abstract

A total of 13 actinomycetes were isolated from two samples sea-water and sludge, Chaung-Tha area, Ayeyarwady region. Among them, 8 strains were obtained from sludge sample and 5 strains from sea water. In the elevation, the isolated strains were circular, raised, convex, umbonate and flat. In the margin and textures, all strains were entire and rough. The colony size of isolated strains were large, small and moderate. The aerial mass colour of all strains was greenish blue, white and centre greenish blue, white in periphery. Their substrate colour was yellow. The antimicrobial activity of all strains were screened by agar well diffusion method on ten test organisms. Of these 13 strains, eight strains showed the antimicrobial activity. Among them, TR-2 showed the highest antifungal activity on *Candida albicans.* Therefore, TR-2 was selected for biochemical characterization. Positive results were found in methyl red test, citrate utilization test, casein test, mannitol salt broth test, potato plug test, nitrate reduction, catalase and urease test. But, Voges proskauer test, hydrogen sulfide test, gelatin hydrolysis test, esterase activity test, oxidase test and motility test were negative. According to the results, TR-2 was classified as the possible genus *Streptomyces* sp.

Keywords: Sea water, sludge, streptomyces, antimicrobial activity

Introduction

Marine ecosystems represent 95% of the biosphere and coastal regions are particularly promising, because of the rightly adapted species found in these harsh environments (Ireland *et al.*, 1993). The oceans represent a virtually untapped resource for discovery of even more novels compounds with useful activity. So far, more than 10000 bioactive molecular have been discovered from marine sources with hundreds of new compound still being discovered every year (Proksch *et al.*, 1997).

It is also reported that marine actinomycetes are useful and suitable source of new bioactive natural product (Nevine *et al.*, 2002).

Mangrove forests are highly productive ecosystems which comprise of unique woody plant communities and located in tropical and subtropical coastal area (Hong, 2009 and Hunadanamra, 2013). According to Ara, *et al.*, 2013, mangroves form unique saline environments under the influence of tidal flow, hence the muddy alluvial soil due to the intermittent flooding. Mangrove ecosystems are nutritionally versatile as they are highly rich in organic matter, nitrogen and sulfur content which can be used by living microorganisms. Thus, it is believed that mangrove ecosystems have the potential of becoming new reservoir for highly diverse actinomycetes as demonstrated by the isolation of *Micromonospora rifamycinica* (Huang, *et al.*, 2003) and *Verrucosispora wenchangensis* (Xie, *et al.*, 2012).

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae (Kampfer *et al.*, 1991). Over 500 species of *Streptomyces* bacteria have been described by Euzeby (Euzeby 2008). Streptomycetes have genomes with high GC content and these are gram-positive (Madigan and Martinko 2003).

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Many species belonging to the genus *Streptomyces* are well known as biocontrol agents that inhibit or lyase several soil borne and air borne plant pathogenic fungi (Sousa *et al.,* 2008). The genus Streptomyces comprises a large group of microorganisms with some characteristic features compared to most other bacteria such as their complex fungi-like life cycle and earthy odor. Furthermore, they are ubiquitous in nature and show a higher diversity in colour of colonies secreted pigments, etc. compared to other bacteria (Good fellow *et al.,* 1983).

The genus *Streptomyces* is morphologically highly diverse. Colour of substrate and aerial mycelium, configuration of spore chains and spore ornamentation are used as taxonomic markers. All of them are determined using cultivation on standard media and fixed incubation times (Shirling and Gottlieb, 1966). The aim of this study is to isolate the actinomycetes from Chaung-Tha area, to screen the antimicrobial activity of actinoymcetes and to study biochemcial characterization of selected Streptomyces (TR-2).

Materials and Methods

Study area and collection of plant samples

Two different samples such as sea water and sludge were collected from Chaung-Tha area, Ayeyawady Region in the month of June, 2016. The sludge sample was taken from top 6 cm soil profile and sea water sample was collected in depth 2 feet. The isolation of actinomycetes were carried out by serial dilution method and utilized on six different media.

Isolation of mangrove microorganisms (Salle, 1948)

In order to isolate, an appropriate amount (1 gm) of soil was put into a conical flask containing 99 mL of distilled water to make a soil water dilution ratio of 1:100. The flask was then shaken for about 30 minutes in order to make the soil particles free from each other. This dilution solution was then serially diluted into 10^{-1} to 10^{-5} dilution in separate test tube and 1 mL each of the above dilution was separately transferred into sterile petridishes under aseptic conditions. The sterilized medium in conical flask was cooled down to about 45ºC and separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anitclock-wise direction to make uniform distribution of the inoculums.

Isolation of pure culture from Plate to Slants (Atlas, 1993)

For pure culture from plate to test tube, about 100 mL of culture media were put into test tube. These test tube were plugged with cotton wool and sterilized by autoclaving. The sterilized media were cooled down. Each of the separate colonies on petridish was taken out to streak on the slant medium on the slant medium to obtain pure cultures.

Media used for the isolation of Actinomycetes

Kuster's agar medium (Balagurunathan and Subramanian, 1992) (Glycerol 10 g, Casein 0.3 g, KNO_3 3 g, NaCl 2 g, $MgSO_4$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4$ 0.01 g, Agar 16 g, DW 1000 mL, Sea water 50%/L), Actinomycetes isolation agar medium (Sodium caseinate 2 g, L-asparagine 0.1 g, Sodium propionate 4 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.1 g, Ferrous sulphate 0.001 g, Agar 15 g, DW 1000 mL, Sea water 50%/L), Yeast extract malt extract agar (ISP-2) (Shirling and Gottlieb, 1966) (Yeast extract 4 g, Malt extract 10 g, Dextrose 4 g, Agar 20 g, DW 1000 mL, Sea water 50 %/ L), Potato dextrose agar medium (Potato 200 g, Dextrose 20 g, Agar

20 g, DW 1000 mL), Modified nutrient agar medium (Glucose 5 g, Peptone 5 g, Beef extract 3 g, NaCl 5 g, Agar 15 g, DW 1000 mL) and Starch casein agar medium (Wellington and Cross, 1983) (Starch 10 g, Casein powder 1 g, Agar 15 g, DW 1000 mL, Sea water 50 %/ L).

Morphological Characteristics and Staining Reactions of isolated actinomycetes

Gram staining

A drop of sterile distilled water was place on clear grease-free slide and a small loop of isolated actinomycetes was smeared on the slide and allows it to dry. The smear was forced by passing dried slide 3 or 4 times rapidly over a flame. The slide was covered with crystal violet strain and allow it to act for 30-60 seconds. Then, the slide was rinsed with distilled for a few seconds. The slide was covered with fresh iodine solution and allowed it to act for about 30 -60 seconds. The alcohol drop was added until no more color flows out from the smear for 10 -20 seconds and washed with distilled water. Then the slide was air dry. The stained slide was examined under the oil immersion objective of the microscope.

Screening for antimicrobial activities (NITE, 2005)

The isolated actinomycetes were grown on ISP-2 medium at room temperature for 7 days. After incubation period, these strains inoculated into the fermentation medium (glycerol 2 g, peptone 5 g, yeast extract 3 g, malt extract 3 g, $CaCO_3$ 2.5 g, DW 1000 mL) the seed medium (glucose 1 g, starch 1 g, peptone 0.75 g, meet extract 0.75 g, NaCl 0.3 g, DW 1000 mL) for 3 days at room temperature. After three days, the seed medium (3%) was transferred into the fermentation medium (glycerol 2 g, peptone 5 g, yeast extract 3 g, malt extract 3 g, $CaCO_3$ 2.5 g, DW 1000 mL) and carried out for 3-10 days and evaluated the antimicrobial activity by agar well diffusion method.

Screening of antimicrobial activity by agar well method (Collins, 1965)

One day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 10 g, peptone 3 g, KNO_3 1 g, DW 1000 mL, agar 18 g) and thoroughly mixed and poured into plate. After solidification, the agar was left to set. Cork borer was used to make the wells (8 mm in diameter). Then, the fermented broth (20 µL) was carefully added into the well and incubated at room temperature for 24-48 hours. The diameter of the zones of inhibition around each well was measured and recorded after 24-48 hours incubation.

Test organisms

Agrobacterium tumefaciens NITE 09678, *Aspergillus paraciticus* IFO 5123, *Bacillus subtilis* IFO 90571, *Candida albicans* NITE 09542, *Micrococcus luteus* NITE 83297, *Salmonella typhi* AHU 7943, *Escherichia coli* AHU 5436, *Pseudomonas fluorescens* IFO 94307, *Staphylococcus aureus* AHU 8465 and *Saccharomyces cerevisiae* NITE 52847 were obtained from NITE (National Institute of Technology and Evaluation, Kisarazu, Japan).

Scope for isolation and identification of marine actinomycetes

Morphological and biochemical characteristics of selected strain TR-2

Morphological

- Gram staining
- Cell morphology

Biochemical tests

- Sugar fermentation of carbohydrate test (Cowan, 1975)
- Catalase test (Salle, 1948)
- Oxidase test (Dubey, 2002)
- Hanging slied test (Dubey and Maheshwari, 2002)
- Urea hydrolysis test (Christenson, 1946)
- Nitrate reduction test (Harrigan and Mc Cance, 1966)
- Mannitol sal**t** broth test (Prescott 2002)
- Methyl red test (Bisen and Verma, 1998)
- Citrate test (Atlas, 1993)
- Hydrogen sulfide Test (Cowan, 1975)
- Voges proskaucer test (Cruickshank, 1963)
- Soluble starch hydrolysis (Pelezar and Chan, 1972)
- Rice powder hydrolysis (Pelezar and Chan, 1972)
- Wheat powder hydrolysis (Pelezar and Chan, 1972)
- Tapioca powder (Pelezar and Chan, 1972)
- Sticky rice powder (Pelezar and Chan, 1972)
- Arginine hydrolysis (Dubey and Maheshwari, 2002)
- Gelatin hydrolysis test (Dubey and Maheshwari, 2002)
- Casein hydrolysis test (Aneja, 1996)
- Esterace hydrolysis test (Prescott, 2002)
- Melanin production test (Shirling and Gottlieb, 1966)
- Potato slice test (Atlas, 1993)
- Salt tolerance test (Atlas, 1993)

Cover slip insertion method (William *et al.*, 1989)

Adequate magnification used to establish the presence or absence of spore chains and to observe with the magnification. By the standard protocol of cover slip culture technique, the plates were prepared and after the incubation of 7 to 10 days it was observed. During this method of spore morphological study, ISP 2 medium plates were prepared. After solidification, by a sharp scalpel from the central portion of the plate, medium should be scooped out making a rectangular area. Then, three sterile cover slips were placed on the hollow rectangular space. Slowly Actinomycetes spores have to be inoculated at the edge of the cover slips touching the medium. The plates must be incubated at 28 ± 2ºC for 5 days and examined periodiacally taking out the cover slips.

Results

A total of 13 strains such as TR-1 to 13, were isolated from sea water and sludge samples collected from Chaung-Tha. All 13 strains had shown white, greenish blue and brownish green color. The form, elevation and margin of these strains were circular, raised and entire, rough in texture, small, moderate and large in colony size. The aerial mass colour of all strains was greenish blue, white and centre greenish blue, white in periphery. After six days, the aerial mass colour of strains TR-4 and TR-8 white that turned into brown and white in colour of TR-12 turned into blue after five days. The spore chain of all strains were straight, flexous, rectiflexibiles, single conidia, ovoid, open spiral, spirals and fragmenting branched aerial hyphae. The spores of isolated strains were globose, ovoid and polytrichous.

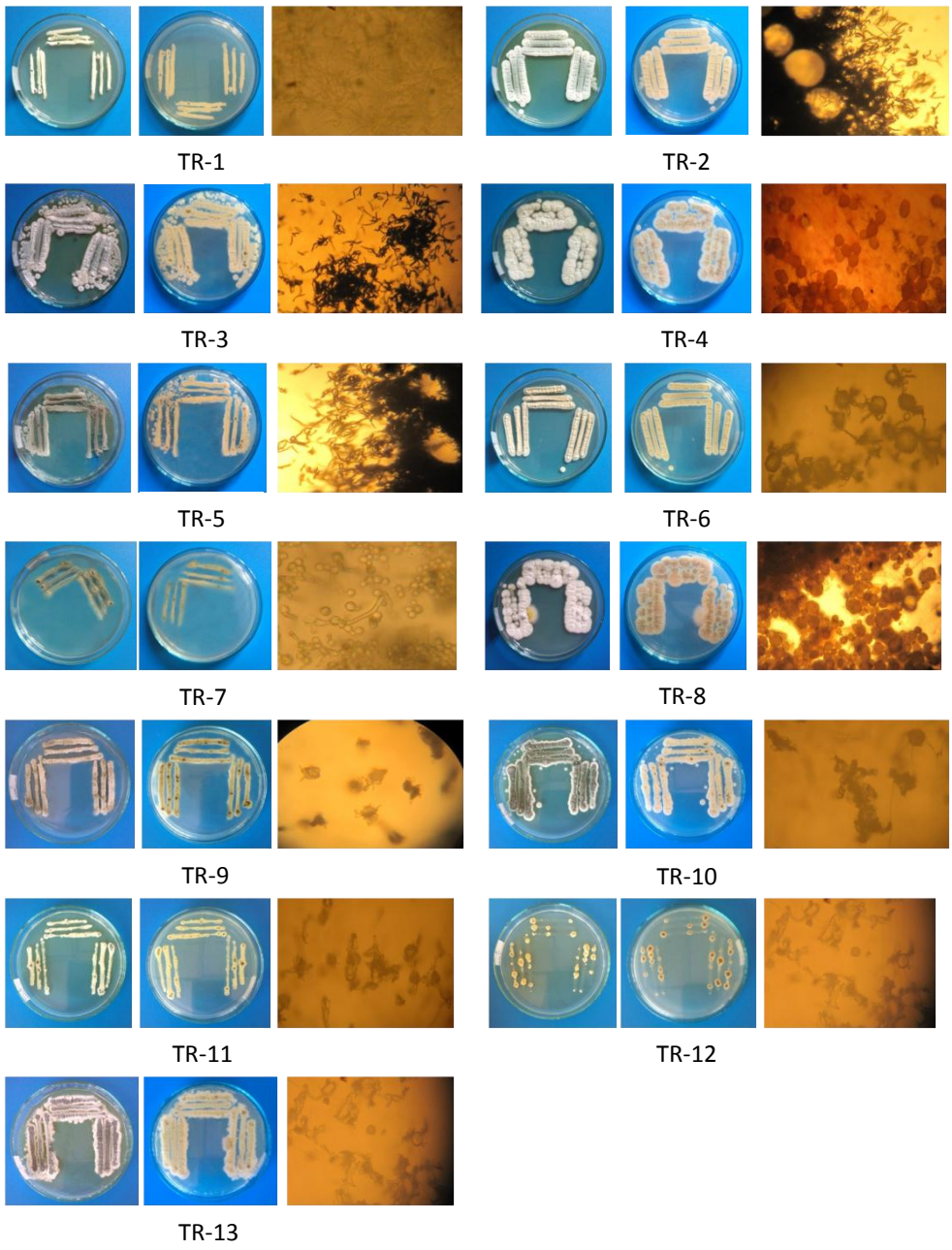


Figure 1 Colony morphology and Cell shape of isolated strains TR 1-13

Colony morphology

All 13 strains had shown white, greenish blue and brownish green color. In these strains, TR-2, 5 and 10 had water drop. In elevation, TR-1-3, 6-11 were raised, flat in TR-12 and 13, TR-4 was convex and TR-5 was umbonate. TR-1, 2, 4 and 8 were large, TR-3, 5, 9-13 were small and TR-6 and 7 were moderate in colony size. In form and texture, all strains were circular and rough as shown in Table 1.

Table 1 Morphological characters of isolated actinomycetes

No	Isolated strains	Form	Elevation	Magin	Pigment		Colony size	Texture
					Front colour	Reverse colour		
1.	TR-1	Circular	Raised	Entire	Greenish blue	Yellow	Large	Rough
2.	TR-2	Circular	Raised	Entire	Centre greenish blue, white in periphery (Water drop present)	Yellow	Large	Rough
3.	TR-3	Circular	Raised	Entire	Centre greenish blue, white in periphery	Yellow	Small	Rough
4.	TR-4	Circular	Raised	Entire	White- Brown After 6 days	Yellow-Red	Large	Rough
5.	TR-5	Circular	Raised	Entire	Greenish blue (Water drop present)	Yellow	Small	Rough
6.	TR-6	Circular	Raised	Entire	Centre brownish green, white in periphery	Brown	Moderate	Rough
7.	TR-7	Circular	Raised	Entire	Greenish blue	Orange	Moderate	Rough
8.	TR-8	Circular	Raised	Entire	White- Brown After 6 days	Green-Red After 5 days	Large	Rough
9.	TR-9	Circular	Raised	Entire	Centre greenish blue, white in periphery	Brown	Small	Rough
10.	TR-10	Circular	Raised	Entire	Centre white, dark green in periphery	Yellow	Small	Rough
11.	TR-11	Circular	Raised	Entire	Centre greenish blue, white in periphery	Yellow-Orange After 5 days	Small	Rough
12.	TR-12	Circular	Raised	Entire	White- Blue After 5 days	Orange	Small	Rough
13.	TR-13	Circular	Raised	Entire	Centre greenish blue, white in periphery	Yellow	Small	Rough

Small < 2 mm (diameter)
Medium between 2 mm & 5 mm (diameter)
Large > 5 mm (diameter)

Spore chain morphology

In the spore surface morphology, TR- 1, 2, 3, 7 & TR-11-13 were globose, ovoid in TR-4, 5, & 8, polytrichous in two strains (TR- 6 & 9) and peritrichous in TR-10. TR-2, 3, 5 & 11 were flexous and TR- 10, 12 & 13 were open spiral and spirals in the spore chain. Another isolates were straight (TR-1), fragmenting branched aerial hyphae (TR-4), rectiflexibiles (TR-6 & 9), single conidia (TR-7) and ovoid (spore production with sporangia) in TR- 8 as shown in Table 2.

Table 2 Morphologies of Spores chains and spores features of isolated actinomycetes

No	Isolated strains	Spore Chain	Morphological feature of spores
1.	TR- 1	Straight	Globose
2.	TR- 2	Flexous	Globose
3.	TR- 3	Flexous	Globose
4.	TR- 4	Fragmentating branched aerial hyphae	Ovoid
5.	TR-5	Flexous	Ovoid
6.	TR- 6	Rectiflexibiles	Ovoid
7.	TR- 7	Single conidia	Globose
8.	TR- 8	Ovoid (Spore production with sporangia)	Ovoid
9.	TR- 9	Rectiflexibiles	Polytrichous
10.	TR- 10	Open spiral	Peritrichous
11.	TR- 11	Flexous	Globose
12.	TR- 12	Spirals	Globose
13.	TR- 13	Spirals	Globose

Antimicrobial activities of isolated actinomycetes strains

All strains were tested for antimicrobial activities with ten test organisms. Among them, the strain TR-2 was selected for further investigation according to the results of maximum inhibition against *Candida albicans*,NITE 09542 than the other.

Table 3 Antifungal activities of selected strain TR-2 on *C. albicans*

Fermentation period (day)	Antifungal activitiy (mm) and Test organisms									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
3	-	14.02	18.35	21.01	-	-	-	21.08	20.10	18.32
4	-	17.45	19.23	**24.52**	-	-	-	23.05	21.54	20.56
5	-	16.08	19.18	21.43	-	-	-	20.87	20.01	20.31
6	-	16.00	18.56	21.14	-	-	-	20.35	19.21	19.21
7	-	15.32	17.42	18.27	-	-	-	20.22	19.05	18.05

1. *Agrobacterium tumefaciens*
2. *Aspergillus paraciticus*
3. *Bacillus subtilis*
4. *Candida albicans*
5. *Micrococcus luteus*
6. *Salmonella typhi*,
7. *Escherichia coli*,
8. *Pseudomonas fluorescens*,
9. *Staphylococcus aureus*
10. *Saccharomyces cerevisiae*

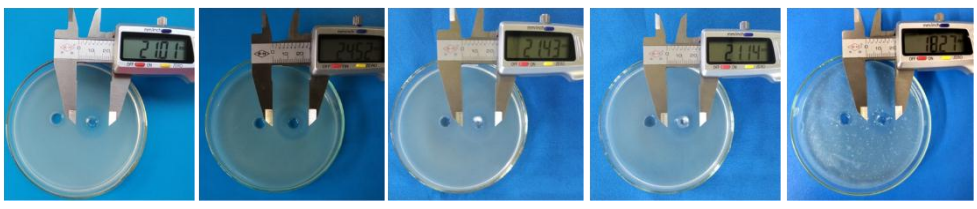


Figure 2 Antifungal activity of isolated strain TR-2 against *Candida albicans*

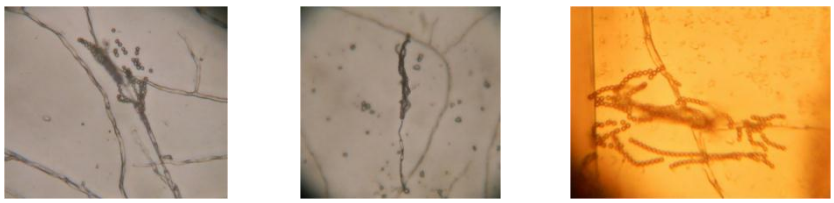
Table 4 Biochemical tests of selected strain TR-2

No.	Test	Result
1	Cell morphology	Slender hyphae spore chain, straight and long
2	Gram staining reaction	Gram positive
3	Catalase test	+
4	Oxidase test	-
5	Motility test	-

(+) positive (-) negative

Front view Reverse view Single colony of selected strain TR-2

Figure 3 Colony morphology and cell shape of selected strain TR-2



Glabrous spore Rectiflexibiles type Gram staining (+)

Figure 4 Microscopical characters of selected strain TR-2

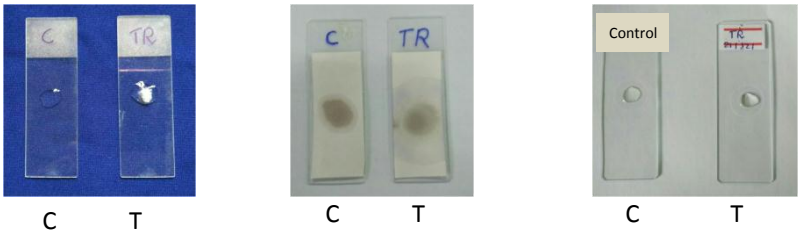


Figure 5 Biochemical tests for selected strain TR-2 **(A)** Catalase test **(B)** Oxidase test **(C)** Motility test (Hanging slide)

Table 5 Colony morphology of TR-2 on three different culture media

Cultural media	Surface color (Aerial mycelium)	Reverse color (Substrate
ISP-2 (yeast-malt extract agar)	White changed into Blue	Yellow
ISP-5 (Glycerol Asparagine	Greenish	Yellow
ISP-6 (Peptone Iron medium)	White	Yellowish

Figure 6 Colony morphology of TR-2

Biochemical characterization of selected strain TR-2

Acid was produced in glucose, galactose, maltose, sucrose, fructose, xylose, lactose and destrose and gas not produced.

Positive results were found in methyl red test, citrate utilization test, casein test, mannitol salt broth test, potato plug test, nitrate reduction, catalase and urease test. But, VP test, hydrogen sulfide test, gelatin hydrolysis test, arginine test, esterase activity test, oxidase test and motility test were negative. In salt tolerance test, the optimum growth of the strain TR-2 was observed at 6% NaCl.

Table 6 Sugar fermentation of selected strain TR-2

No.	Various sugar	Response	Acid
1	Glucose	Yellow colour change in	+
2	Galactose	Yellow colour change in	+
3	Maltose	Yellow colour change in	+
4	Sucrose	Yellow colour change in	+
5	Fructose	Yellow colour change in	+
6	Xylose	Yellow colour change in	+
7	Arabinose	No change in colour	-
8	Lactose	Yellow colour change in	+

+ = positive - = negative

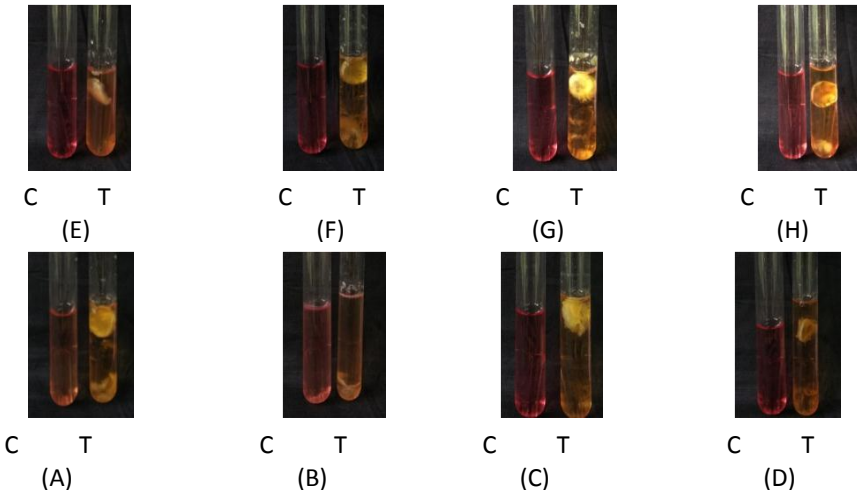


Figure 7 Sugar fermentation of selected strain TR-2 **(A)** Glucose **(B)** Fructose **(C)** Maltose **(D)** Galactose **(E)** Lactose **(F)** Dextrose **(G)** Sucrose **(H)** Xylose

Table 7 Biochemical tests for selected strain (TR-2)

No.	Reaction	Response	Result
1	Citrate utilization test	The colour of medium changes from green to blue	Positive
2	Methyl red test	No colour change from methyl red to yellow	Positive
3	Voges Proskaucer	Test no colour change medium	Negative

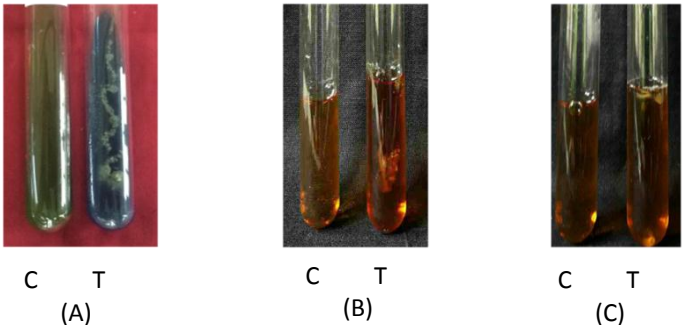


Figure 8 Biochemical test for selected strain TR-2 **(A)** Citrate utilization test **(B)** Methyl red (MR) test **(C)** Voges proskaucer (VP) test

Sodium chloride tolerance test

The selected strain TR-2 can grow well in the sodium chloride (2%, 4%, 6% and 8%) except 10% NaCl.

Table 8 Sodium chloride tolerance test of TR-2

Sodium chloride (%)	Result
NaCl 2%	++
NaCl 4%	++
NaCl 6%	++
NaCl 8%	++
NaCl 10%	+

\+ poor growth ++ moderate growth

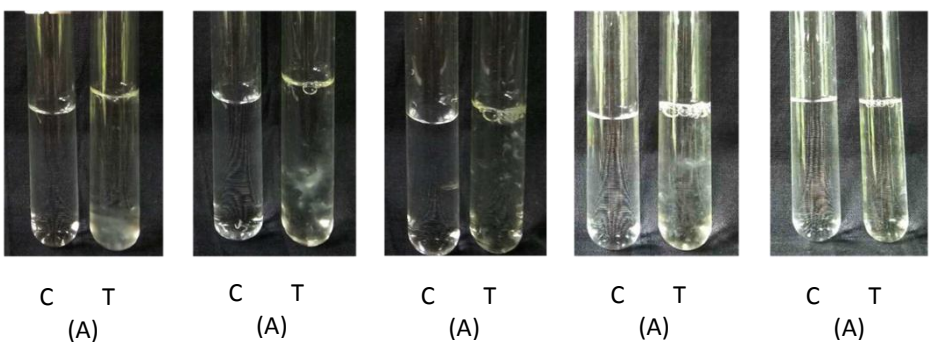


Figure 9 NaCl tolerance test of selected strain TR-2 **(A)** 2% **(B)** 4% **(C)** 6% **(D)** 8% **(E)** 10%

Table 9 Biochemical tests for selected strain TR-2

Sr. No.	Reaction	Response	Result
1	Mannitol salt broth	The colour of medium changed from yellow to pink	Positive
2	Urea hydrolysis	Colour changed from yellow to deep pink	Positive
3	H_2S production	No black colour change in the medium	Negative

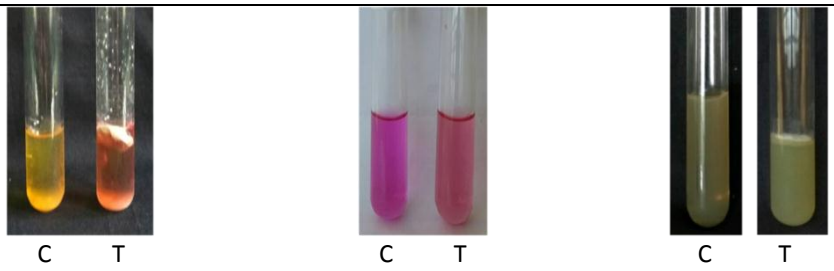


Figure 10 Biochemical test for selected strain TR-2 **(A)** Mannitol salt broth **(B)** Urea hydrolysis **(C)** H_2S production

Table 10 Starch hydrolysis test for selected strain TR-2

No.	Sources of strach	Result
1	Soluble starch	++
2	Wheat flour	++
3	Tapioca powder	+
4	Sticky rice	++
5	Rice powder	++

++ maximum hydrolysis
+ minimum hydrolysis

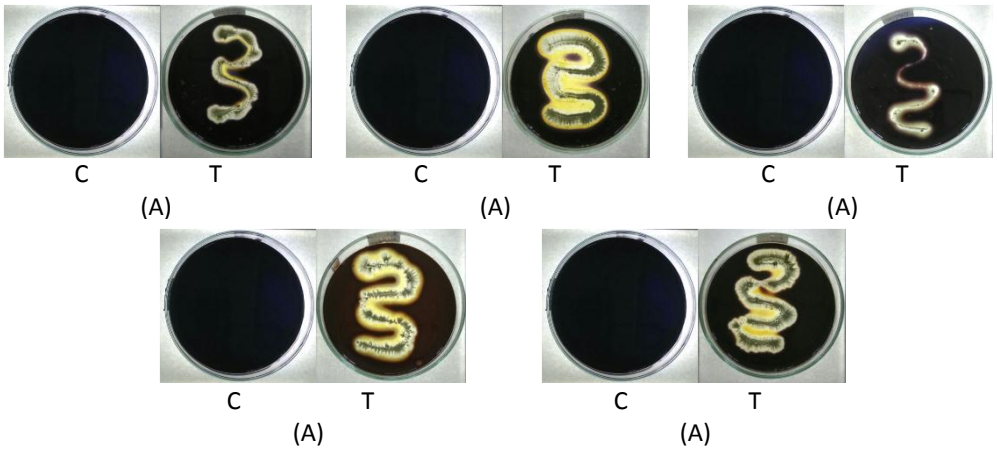


Figure 11 Starch hydrolysis test for selected strain TR-2 **(A)** Soluble starch **(B)** Wheat flour **(C)** Tapioca **(D)** Sticky rice **(E)** Rice powder

Table 11 Biochemical tests for selected strain TR-2

Sr. No.	Reaction	Response	Result
1	Casein test	Clear zone is found around the growth zone	Positive
2	Potato slice test	Growth on the streak line of potato	Positive

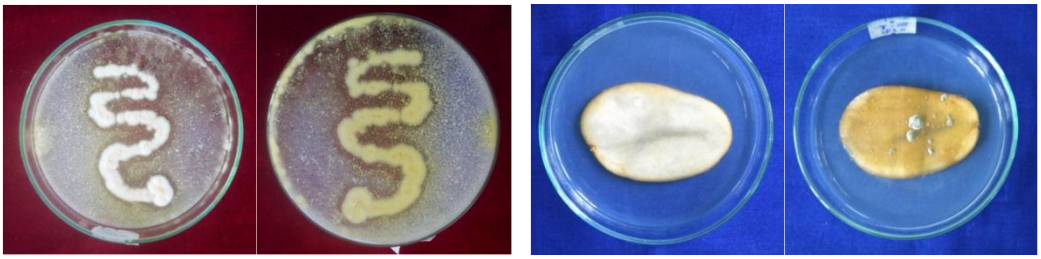


Figure 12 Biochemical test for selected strain TR-2 **(A)** Casein test **(B)** Potato slice test

Discussion and Conclusion

During the study of the isolation of actinomycetes, two samples were carried out by serial dilution method. Of these 13 strains, five strains were got from sea water sample and eight strains from sludge sample. Six different media were utilized only one strain was got from Kuster's agar medium and twelve strains were collected from (ISP-2) medium. Gil, *et al.*, 2009 suggested that nutrient availability is one of the main factors that determine the growth of actinomycetes. Most actinomycetes can use wide variety of compounds such as glucose, starch, proteins and amino acids as their energy source; unlike other bacterial groups that only favour simple carbon and nitrogen compound. Therefore, ISP 2 medium was effective for the isolation of actinomycetes among the other media.

In the identification of TR-2, colony morphology, spore chain and shape, cultural characters and biochemical characteristics were studied. Spore chains were straight and long, rectiflexbiles type, spore wall glabrous. Waksman and Henrici, 1943 described that the streptomyces were spores chain straight and long, rectiflexibiles type, retiaculiaperti type, spira type, spore wall glabrous, hairy. These results were the same of TR-2. Carbohydrate utilization properties are one of the important biochemical activities of microorganisms to identify and classify them (Dielz, 1988 and Holt, *et al.*, 2000). The selected strain TR-2 produced acid from carbohydrate such as glucose, galactose, maltose, sucrose, fructose, xylose, lactose and dextrose. Al-saadi, *et al.*, 2013, suggested that the actinomycetes were positive for citrate and starch hydrolysis test. Similarly, TR-2 was also positive for citrate and starch hydrolysis test. Methyl red (MR) test were positive and Voges Proskaucer (VP) test negative, casein and urea hydrolysis positive, mannitol salt broth positive. TR-2 can grow in NaCl salt (2% to 10%) and potato slice.

These results were similar to the previous research of Waksman and Henrici 1943, in the Bergey's Manual of Determinative Bacteriology and Selman and Waksman (Volume I and II of the Actinomycetes). Based on the obtained results, selected strain TR-2 was classified as the

possible genus *Streptomyces* sp. Streptomycetes has been exploited to produce a wide range of antibiotics. But many Streptomyces species also produce pigments. Actinotiodin is a biological pigment produced by *Streptomyces*. It can be applied as an antibiotic compound against Gram-positive bacteria and also as an indicator compound in laboratory agents.

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