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Research Article

SCREENING OF ACTINOMYCETES FROM *Cystoseira barbata* (Stackhouse) C. Agardh COMPOST FOR THEIR ENZYME AND ANTIBACTERIAL ACTIVITIES

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Abstract: Bacterial secondary metabolites play an essential role in biotechnological and biomedical applications. Actinomycetes are important bacterial sources of antibiotics and enzymes. Most of the antimicrobials known today have been isolated from actinomycetes, especially from the genus *Streptomyces*. In this study, actinomycete isolation was performed from *Cystoseira barbata* (Stackhouse) C. Agardh compost, collected from the Black Sea coast, by serial dilution method. A total of 73 actinomycetes isolates (BSC) were obtained from the compost samples. The ability of the isolates to produce different extracellular enzymes was investigated qualitatively. It was determined that 68.5% of the isolates have amylase, 100% cellulase, 47.9% chitinase, 94.5% pectinase, 98.6% protease and 96.3% lipase/esterase activity. Antibacterial activities of the isolates were investigated primarily using cross-streak method. Isolates showed high antibacterial activities, with 98.6 and 84.9 % against *Staphylococcus aureus* Rosenbach and *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Bäl, respectively. Three out of six isolates (BSC-13, BSC-17, BSC-37, BSC-38, BSC-45, BSC-49) with high antibacterial activity, were screened secondarily for their antibacterial activities using double-layer method. At day 7, BSC-37 isolate showed a high inhibition (57 mm) against *S. aureus*. Furthermore, these six isolates were identified according to their morphological and physiological characteristics and 16S rDNA sequence analysis. 16S rDNA sequence analysis showed that the isolates with high antibacterial activity belong to *Streptomyces* genus. Results indicated that these isolates have great potential and may serve as a good source for the studies on bioactive natural products.

Özet: Bakteriyel sekonder metabolitler, tıbbi ve biyoteknolojik uygulamalarda önemli bir rol oynamaktadır. Aktinomisetler önemli bakteriyel antibiyotik ve enzim kaynaklarıdır. Bugün bilinen antimikrobiyallerin çoğu, aktinomisetlerden özellikle *Streptomyces* cinsinden izole edilmiştir. Bu çalışmada, Karadeniz kıyılarından toplanan *Cystoseira barbata* (Stackhouse) C. Agardh kompostundan seri seyreltme yöntemi ile aktinomiset izolasyonu yapılmıştır. Kompost örneklerinden toplam 73 aktinomiset izolatı (BSC) elde edilmiştir. İzolatların farklı hücre dışı enzimler üretme yetenekleri kalitatif olarak araştırıldı. İzolatların %68,5'inin amilaz, %100'ünün selülaz, %47,9'unun kitinaz, %94,5'inin pektinaz, %98,6'sının proteaz ve %96,3'ünün lipaz/esteraz aktivitesine sahip olduğu belirlendi. İzolatların antibakteriyel aktiviteleri öncelikle çapraz çizgi yöntemi kullanılarak araştırıldı. İzolatlar, *Staphylococcus aureus* Rosenbach ve *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Bäl'e karşı sırasıyla %98,6 ve %84,9 ile oldukça yüksek antibakteriyel aktivite göstermektedir. Yüksek antibakteriyel aktiviteli altı izolattan (BSC-13, BSC-17, BSC-37, BSC-38, BSC-45, BSC-49) üçü, çift tabakalı yöntemle antibakteriyel aktiviteleri açısından sekonder olarak taranmıştır. 7. günde, BSC-37 izolatı, *S. aureus*'a karşı yüksek bir inhibisyon (57 mm) gösterdi. Ayrıca bu altı izolat, morfolojik ve fizyolojik özelliklerine ve 16S rDNA dizi analizine göre tanımlandı. 16S rDNA dizi analizi yüksek antibakteriyel aktiviteye sahip izolatların *Streptomyces* cinsine ait olduğunu göstermiştir. Sonuçlar, bu izolatların büyük potansiyele sahip olduğunu ve biyoaktif doğal ürünler üzerine yapılan çalışmalar için iyi bir kaynak olarak hizmet edebileceğini göstermiştir.

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Introduction

Actinomycetes are Gram-positive filamentous bacteria in the order Actinomycetales. They include both aerobic and anaerobic species (Adamu *et al.* 2017, Bi *et al.* 2017). These bacteria have been isolated in great numbers from soil and compost samples (Goodfellow & Williams 1983). They are potential producers of many

antibiotics and enzymes (Ventura *et al.* 2007). Among all microorganisms, more than 50% of known antibiotics are produced by Actinomycetes, especially by the genus *Streptomyces* (Passari *et al.* 2015).

Actinomycetes produce valuable extracellular enzymes that have applications in various industries such as food, detergent, and agriculture (Mukhtar *et al.* 2017). Extracellular enzymes produced by Actinomycetes include amylases, proteases, lipases, cellulases and chitinases (Praveen Kumar & Suneetha 2015, Janaki 2017). Amylases are known as important enzymes that hydrolyze starch into high fructose, glucose and maltose syrups (Mukhtar *et al.* 2017). Amylases are of great importance in many biotechnological applications ranging from food industry, fermentation and textile to paper industry (Pandey *et al.* 2000). Proteases, also known as peptide hydrolases, are used in different industrial areas such as textile, leather, detergent, meat tenderizing, cheese making, hair removal, baking, organic synthesis, beer and wastewater treatment (Kalisz 1988). Lipases and esterases are various groups of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides to glycerol and fatty acids. Lipases have wide applications in detergent industries, foodstuffs, oleochemicals, diagnostic environments and pharmaceuticals (Kulkarni & Gadre 2002, Janaki 2017). Cellulases are important industrial enzymes as they digest cellulose and convert it into sugars. These enzymes are mostly used as additives in detergents, textiles, animal additives, and in paper and pulp industry (Jang & Chang 2005, Azzeddine *et al.* 2013). Chitinases are important enzymes capable of hydrolyzing chitin. These enzymes produced by some actinomycetes are preferred for industrial applications because they are thermostable and active in a wide pH range (Kulkarni & Gadre 2002). Pectinase is the most important enzyme group used in vegetable and fruit processing industries. Pectin, which is an important part of the fruit structure, is converted into pectinase and pectinic acid and eventually pectic acid. During this chemical breakdown, the fruit softens as its cell walls break down (Praveen Kumar & Suneetha 2015).

Composting is a process that depends on the breakdown of organic wastes by microbial activity (Recer *et al.* 2001). This process is usually carried out by complex microbial communities (Ryckeboer *et al.* 2003). Compost is a significant source for microorganisms because various microorganisms occur at different times during the composting process (Lima Junior *et al.* 2016). Fungi, bacteria and actinomycetes have important roles in the breakdown of compost (Zeng *et al.* 2011).

Marine microorganisms are becoming an increasingly significant resource in the production of important enzymes that have applications in different industries (Bernan *et al.* 2004). Among these microorganisms, marine actinomycetes isolated from sediments are source of new compounds (Piel 2004). Nowadays, studies on actinomycetes, which adapt to different conditions and have the ability to produce new compounds, have increased (Veyisoglu & Sahin 2014, 2015, Özcan 2017). To meet the demand for new compounds in different areas of industry and to combat antibiotic-resistant pathogens, researchers are researching new microorganisms in different and less studied environments. Algal compost is therefore a tremendous resource for the discovery of new bioactive compounds and the research of new marine actinomycetes. In this study, 73 actinomycetes isolates, obtained from *Cystoseira barbata* (Stackhouse) C. Agardh compost were screened for their enzyme and antibacterial activities. Additionally, isolates showing high antibacterial activity were characterized.

Materials and Methods

Compost samples and actinomycetes isolation

Compost samples were prepared from brown seaweed (*Cystoseira barbata*) collected from the Black Sea coast in Giresun, Turkey (Türkmen & Duran 2021). The samples were collected in sterile tubes, and were taken to the laboratory. Samples from different parts of the compost were combined and homogenized in sterile distilled water. The homogenate was serially diluted, and cultured on medium (1 g KNO_3, 0.5 g K_2HPO_4, 0.4 g $MgSO_4.7H_2O$ and 10 g glucose in 1000 mL distilled water) (Tan *et al.* 2009) with or without 25 mg/L potassium dichromate. Plates were incubated at 30°C for 14-21 days until colonies were observed. Selected colonies were then inoculated into Starch-Casein Agar medium (SCA: 10 g Soluble starch, 0.3 g Casein hydrolysate, 2 g KNO_3, 2 g NaCl, 2 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 18 g Agar in 1000 mL distilled water) (Jeffrey 2008) and stored at -20°C in 20% sterile glycerol.

Test microorganisms and preparation of inoculum

7 Gram-negative (*Proteus vulgaris* ATCC 13315, *Enterobacter cloacae* ATCC 13047, *Escherichia coli* ATCC 35218, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumonia* ATCC 1383, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 27853) and 2 Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) bacteria were used to determine the antibacterial activities of the actinomycetes. Test bacterial inoculums were prepared on nutrient broth medium and incubated at 30°C overnight.

Enzyme activity screening

Actinomycete isolates were screened qualitatively for their lipase/esterase, amylase, chitinase, protease, cellulase and pectinase enzyme productions. Isolates were examined by adding relevant substrates instead of starch in SCA medium that is used for actinomycetes growth. The substrates in SCA medium for enzyme screening are; 1% v/v Tween 20, Tween 80 and tributyrin for lipase/esterase, 0.1% w/v colloidal chitin for chitinase, 1% w/v CMC (Carboxymethyl cellulose) for cellulase, 1.5% w/v gelatin for protease, 0.5% w/v pectin for pectinase. Isolates were incubated at 30°C for 3-7 days. After incubation, the clear zone, formed by the enzyme as a result of the substrate hydrolysis, was evaluated. The

crystal structures formed around the isolates in Tween 20 and Tween 80 medium were examined for lypolytic activity (Haba *et al.* 2000). The lypolytic activity in tributyrin medium was noted by measuring the clear zone around the isolates. For amylase activity, iodine solution (1g I_2, 2g KI in 300 mL distilled water) was dropped and the zone formation around the actinomycete was examined (Bragger *et al.* 1989). The clear zone around the isolates was examined for chitinase activity. For cellulase activity, 0.1% w/v congo red dye was added to the petri dishes including bacteria, and 15 minutes later, the dye was removed and replaced with 1 M NaCl solution. The solution was left for 15 minutes and then the zones around the actinomycetes were examined (Kasana *et al.* 2008). For protease activity, mercury chloride solution (15 g $HgCl_2$ in 20 mL HCl and 80 mL distilled water) was dropped and the zones around the actinomycete were examined (Mitra & Chakrabartty 2005). For pectinase activity, 0.1% w/v CTAB was dropped and the zones around the actinomycetes were examined (Kobayashi *et al.* 1999).

Antibacterial activity screening

The cross-streak method was used to investigate the antibacterial properties of the isolates (Oskay 2009). Plates, containing Mueller Hinton Agar (MHA) medium, were inoculated by the actinomycetes isolates and incubated at 30°C for 4-7 days. After incubation, all test bacteria were streaked perpendicular to the actinomycetes isolates. Plates were further incubated at 30°C for 12-16 h. Antibacterial activities were observed by the inhibition of growing around the tested isolate. The results are presented qualitatively as: - no activity, + weak activity, ++ moderate activity, +++ high activity.

Second screening for antibacterial activity was performed for three isolates that showed high antibacterial activity by spot inoculation and the double-layer method (Westerdahl *et al.* 1991). MHA plates were spot inoculated with isolates and incubated at 30°C for 7 days and then covered with soft MHA agar (0.7% agar w/v) seeded with 100 µL of the overnight culture of test bacteria. Plates were incubated at 30°C for 24 h. Antibacterial activities represented in zones of inhibitions were examined starting from 3rd to 7th days.

Identification of the isolates

Actinomycete isolates which showed the highest antibacterial activity were selected for their identifications according to their morphological, physiological and molecular properties.

Gram staining was performed from cultures of isolates grown on SCA medium for 16-24 h (Williams *et al.* 1993). To determine the colony morphology and colors, the isolates were incubated for 3 days at 30°C on SCA medium. The cover slip method, modified according to Sahilah (1991), was used to define the spore chain morphologies. Spore chain structures of the isolates were defined according to Holt *et al.* (2000).

The physiological properties of the isolates were examined in detail. The effects of different temperatures (10°C, 20°C, 25°C, 30°C, 37°C, 45°C and 50°C), NaCl concentrations (0%, 2.5%, 5%,7.5% and 10%) and pH's (5.0, 6.0, 7.0, 8.0 and 9.0) were tested. Plates were incubated at 30°C for 3-7 days.

Genomic DNA isolation was performed according to the procedure described by Liu *et al.* (2000). The isolated DNA was subjected to polymerase chain reaction (PCR) using the primers, UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTTCA-3′) and UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTTGTA-3′) (Brosius *et al.* 1978). The total 50 µL PCR mixture included the template DNA, 1 µL each primer (10 mM), 1 µL dNTP mix (10 mM), 5 µL $MgSO_4$ (20 mM), 5 µL 10xPCR buffer and 0.2 U GoTaq polymerase. Amplification was performed using the following conditions: 2 min at 95°C for initial denaturation, followed by 36 amplification cycles (1 min at 94°C, 1 min at 56°C, 2 min at 72°C) and 10 min at 72°C for final primer extension. Sequencing of the PCR products was performed at Macrogen Inc. (Amsterdam, The Netherlands). The 16S rDNA sequences of the isolates were compared with the database in the Gene Bank using the BLAST (Basic Local Alignment Search Tool) program.

Results

Actinomycetes isolates

A total of 73 actinomycetes were isolated from *Cystoseira barbata* compost samples collected from the Black Sea coast in Giresun, Turkey. The isolates were named with the abbreviation BSC (Black Sea *Cystoseira*).

Enzymatic production by Actinomycetes

The actinomycete isolates were analyzed for their ability to produce various hydrolytic enzymes. The results showed that 68.5% of the isolates have amylase, 100% cellulase, 47.9% chitinase, 94.5% pectinase, 98.6% protease and 96.3% lipase/esterase enzyme activities (Table 1). Particularly, isolates showed intense lipase/eterase, cellulase, pectinase and protease activities. Tween 20 was the most effectively hydrolyzed substrate in lipase/esterase enzyme assays. Most of the isolates showed no or low activity for chitinase. Enzyme activity images are given in Fig. 1 for some isolates.

Screening of Actinomycetes for their Antimicrobial Activities

According to the cross-streak method, most isolates showed high activity against the test bacteria *E. faecalis* and *S. aureus*. However, none of the isolates showed activity against *Y. pseudotuberculosis*. Of the 73 isolates, 17 showed antibacterial activity against *Klebsiella pneumonia*. Among the isolates, those showing activity against three or more test bacteria are listed in Table 2. At the secondary screening of antibacterial activity, the corresponding zone of inhibition (mm), obtained between 3rd to 7th days, for three isolates (BSC-13, BSC-37 and BSC-38) were given in Table 3. Isolates BSC-13 and

BSC-38 showed maximum activity against *E. faecalis* and produced 30 mm and 26 mm inhibition zones, respectively. Besides, BSC-37 produced a large zone of inhibition as 57 mm against *S. aureus* at the 7th day of inoculation (Fig. 2).

Identification of isolates with high antibacterial activity

BSC-13, BSC-17, BSC-37, BSC-38, BSC-45 and BSC-49 isolates which showed high antibacterial activity against *E. faecalis* and *S. aureus* were selected and characterized by morphologically, physiologically and 16S rDNA sequencing (Table 4, 5). Colony colors were observed as grey in BSC-13 and BSC-45, light grey in BSC-37, cream in BSC-17 and BSC-38, and white in BSC-49 in SCA medium (Fig. 3). All six isolates were determined as Gram-positive. Furthermore, as a result of 72 hours growth, filamentous structures of the isolates were observed under light microscopy as in actinomycetes. The spore chain structures of the isolates are given in Fig. 4. Temperatures between 10°C and 50°C were investigated for the optimum growth. While no growth was observed for any isolate at 10°C, only weak growth was seen at 50°C for the isolates BSC-45 and BSC-49. All isolates had optimum growth between the temperatures values of 20°C and 45°C. The pH requirements of all isolates are found between 6 and 9. The NaCl tolerance for growth of the isolates was up to 7.5%. 16S rDNA sequencing results identified the isolates as *Streptomyces* (Table 5).

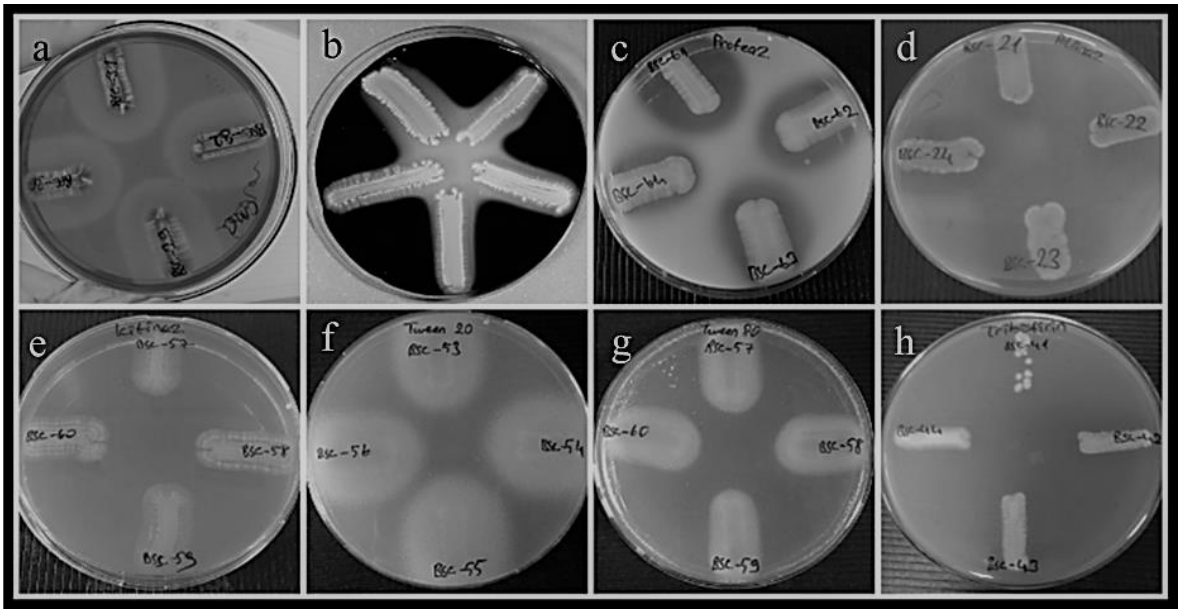

Fig. 1. Qualitative plate assay for different extracellular enzymes of some actinomycetes isolates, **a.** cellulase, **b.** amylase, **c.** protease, **d**. pectinase, **e**. chitinase, **f.** tween 20, **g.** tween 80, **h.** Tributyrin.

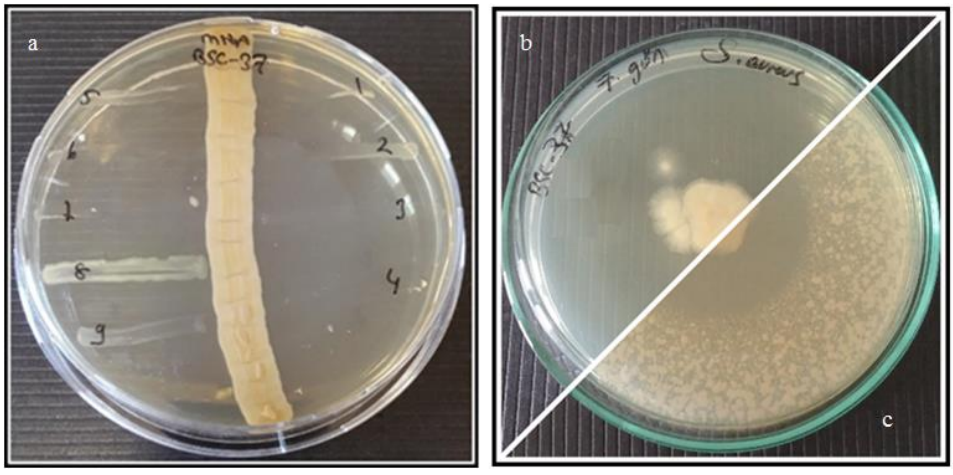

Fig. 2. Antibacterial screening of BSC-37 isolate against test bacteria, **a.** Test bacteria used in cross-streak method; 1. *Klebsiella pneumonia*; 2. *Escherichia coli*; 3. *Enterococcus faecalis*; 4. *Staphylococcus aureus;* 5. *Yersinia pseudotuberculosis*; 6. *Proteus vulgaris*; 7. *Enterobacter cloacae*; 8. *Pseudomonas aeruginosa*; 9. *Salmonella typhimurium*. For second antibacterial activity of BSC-37 isolate, it was spot inoculated by a toothpick against *S. aureus*, **b.** at 7th day, **c.** at 6th day.

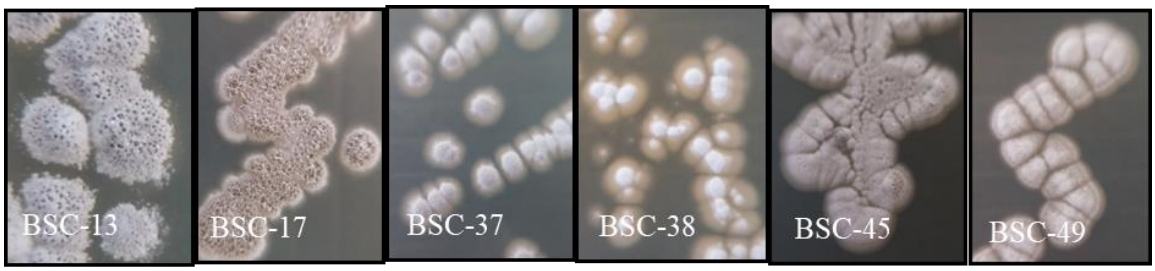

Fig. 3. Colony morphology of some actinomycetes isolates. (From left to right; BSC-13, BSC-17, BSC-37, BSC-38, BSC-45, BSC-49). (Colony colors from left to right; grey, cream, light grey, cream, grey, white).

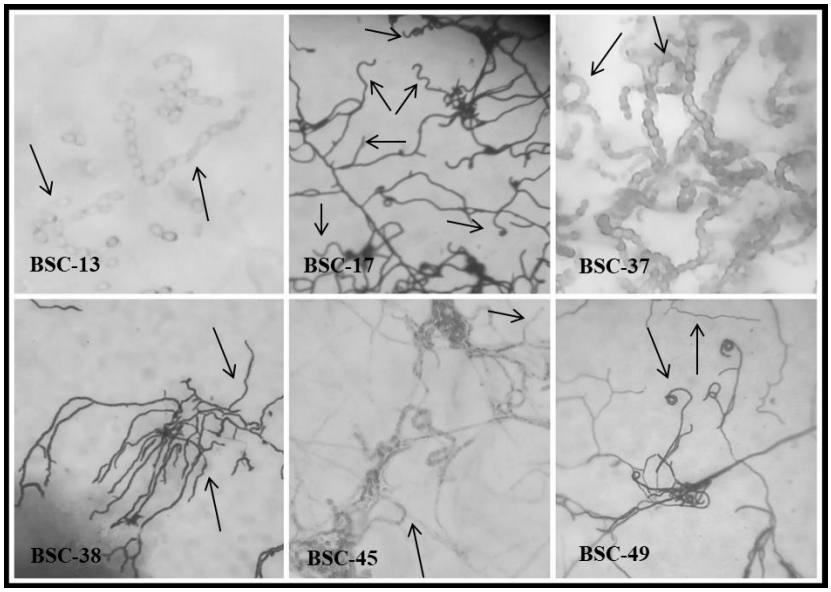

Fig. 4. Spore arrangement of some actinomycetes isolates (BSC-13; Hooks-Rectus, BSC-17; Retinaculum apertum-Hooks-Rectus-Spira, BSC-37; Retinaculum apertum, BSC-38; Rectus, BSC-45; Hooks-Rectus, BSC-49; Retinaculum apertum- Rectus).

Discussion

Organisms living at marine environments are metabolically and physiologically different from terrestrial organisms. This is the case also for Actinomycetes (Jensen & Lauro 2008, Imada *et al.* 2010). *Streptomyces* species, which have a large habitat in terrestrial environments, are also frequently found in marine systems, and it has been shown that the species diversity is higher in marine environments (Williams 2009, Dharmaraj 2010, Goodfellow & Fiedler 2010). *Cystoseira barbata* used in this study was also obtained from marine environments.

We obtained a total of 73 isolates from different parts of the compost and all isolates had grown well in the starch casein agar medium. Isolation of actinomycetes from compost and marine-derived environments is a well documented field (Jurado *et al*. 2019, Özcan *et al*. 2013, Mohseni *et al*. 2013, Amore *et al*. 2012, Groth *et al*. 1997). Özcan *et al*. (2013) isolated a total of 261 actinomycete strains from 30 sediment samples using four different isolation media. Another research group isolated 49 actinomycete strains from composts, soil and modified soil samples using different media (Cuesta *et al.* 2012).

Isolates, named with the abbreviation BSC from 1 to 73, were screened for the presence of extracellular hydrolytic enzymes. All the isolates showed cellulase activity, most with a strong activity exhibition. In accordance with our results, a previous study reported that cellulase producing actinomycetes isolates are very common in marine samples (Lekshmi *et al.* 2014). Previous studies also revealed that actinomycetes are one of the known cellulose producers (Jang & Chen 2003). Considering that the amount of cellulose in the composting process is higher than in soil samples, *Streptomyces* is important in composting processes in terms of breaking down cellulose (Dornelas *et al.* 2017). Furthermore, most isolates in this study showed strong protease, pectinase and lipase (Tween 20 as substrate) activities. Previous studies on the enzymatic activity of actinomycetes indicated that most isolates have ability to produce one or more enzyme (Lekshmi *et al.* 2014). Additionally, other studies have shown that marine bacteria, including marine actinomycetes, present various patterns in secreting extracellular enzymes (Jayaprakashvel *et al.* 2008, Ramesh & Mathivanan 2009). Gulve & Deshmukh (2012) reported that *Streptomyces* strains isolated from sea sediments produced several enzymes including catalase, oxidase, gelatinase, caseinase, amylase, cellulase and lectinase. Another study examined fifty six marine actinomycetes producing amylase, cellulase and lipase enzymes on starch agar plates (Selvam *et al.* 2011).

Table 1. Enzymatic screening results of actinomycetes isolates.

Isolate No	Amylase	Cellulase	Chitinase	Pectinase	Protease	Lipase/Esterase		
						Tween 20	Tween 80	Tributyrin
BSC-1	-	++++	+	++++	+++	++++	++	+
BSC-2	+	++++	+	++++	+++	++++	++	+
BSC-3	-	++++	-	++++	+++	++	+	+
BSC-4	-	++++	+	++++	+++	++++	++	+
BSC-5	-	++++	-	++++	++	+++	++	+
BSC-6	+++	++++	-	++++	+++	+	+	++
BSC-7	++	++++	+	++	+	+++	+	+
BSC-8	+	++++	++	++	++	++	+	+
BSC-9	++	+++	-	+	+++	++	+	+
BSC-10	+	++++	+	++	++	+	+	++
BSC-11	-	++++	-	++++	+++	++	+	+++
BSC-12	+++	+++	++	++	++++	++++	+	+
BSC-13	+++	+++	++	+++	++++	+++	+	+
BSC-14	++	+++	-	+++	++	+++	+	++
BSC-15	-	++	++	++++	+++	++	+	+
BSC-16	-	++++	-	++++	+++	+++	++	+
BSC-17	-	++++	-	++++	+	+++	+	++
BSC-18	-	++++	-	++++	+++	+++	+	++
BSC-19	-	++++	+	++++	++	+++	+	++
BSC-20	-	++	+	++++	+++	+++	+	+
BSC-21	+	+++	+	+++	+	++++	++	+
BSC-22	++	++++	+	++++	++++	+++	+	+++
BSC-23	+	++++	-	++++	+	++++	+	++
BSC-24	++	++++	+	++	+++	++++	+	++
BSC-25	+	++++	++	++	++++	++++	+	+
BSC-26	-	++	-	+++	++++	-	+	+
BSC-27	+	++++	-	++++	++	++++	+	+
BSC-28	-	++++	-	++++	++	++++	+	+
BSC-29	+++	+++	++	++++	++	++++	+	+
BSC-30	+++	++++	++	++++	++	++++	+	+
BSC-31	+++	++++	-	++++	++	++++	+	+
BSC-32	+	+++	-	++++	++	++++	+	+
BSC-33	-	++++	-	++++	+++	++	+	-
BSC-34	+	++++	-	++++	+++	++++	+	+
BSC-35	+	++	-	++++	++++	++++	+	-
BSC-36	++	+++	+	++++	++++	++++	+	-
BSC-37	++	+++	+	++++	++++	++++	+	+
BSC-38	+	++++	+	++++	+++	++++	+	+
BSC-39	++	+++	-	++++	+++	++++	+	+
BSC-40	++	++++	-	++++	++++	++++	+	+
BSC-41	++	+	-	++++	++++	-	-	+
BSC-42	+	+++	++	++++	+	++++	+	+
BSC-43	+	+++	+	++++	+	++++	+	+
BSC-44	-	++++	-	++++	++++	++++	+	+
BSC-45	-	+++	-	++++	+++	++++	++	+
BSC-46	+	++++	-	++++	++++	++	+	+
BSC-47	-	+++	-	++++	+	++++	+	+
BSC-48	-	+++	-	++++	+	++++	+	+
BSC-49	-	+++	-	++++	++	++++	+	+
BSC-50	++	++++	-	++++	+++	++++	+	++
BSC-51	+	+++	-	++++	+++	++++	+	+
BSC-52	+	++	+	++++	++++	+++	+	++
BSC-53	+	+++	+	++++	++	+++	+	+
BSC-54	-	+++	-	++++	++	++++	+	+
BSC-55	-	++++	-	++++	+++	++++	+	+
BSC-56	++	++	++	++++	+++	++++	+	+

Table 1. Continued.

BSC-57	++	+++	-	++++	++	++++	+	+
BSC-58	+	++++	++	++++	+++	++++	+	+
BSC-59	-	+++	-	++++	+	++++	+	+
BSC-60	+	+++	-	++++	+++	++++	+	+
BSC-61	+	+++	-	++++	++++	++++	+	+
BSC-67	+++	+++	+	-	++++	++++	++	+
BSC-68	++	+++	+	-	++++	++++	+	+
BSC-69	+	+++	++	++	+++	+++	+	+
BSC-70	+	+++	+	+	++++	++++	+	+
BSC-71	+	+++	++	++	++++	+++	+	+
BSC-72	++	+++	+	++++	+++	++++	+	+
BSC-73	-	++	-	-	-	-	-	+

- : no activity, +: low activity, ++ : good activity , +++ : very good activity, ++++ : excellent activity.

Table 2. Antibacterial activity screening results of some actinomycetes isolates.

Isolate No	Yp	Pv	Ec	Pa	St	Kp	Eco	Ef	Sa
BSC-11	-	-	-	-	-	+	-	+++	+++
BSC-12	-	-	-	++	-	-	-	++	++
BSC-13	-	-	-	-	-	++	-	+++	+++
BSC-14	-	-	-	-	-	++	-	+++	+++
BSC-16	-	-	-	-	-	+	-	+++	+++
BSC-17	-	-	++	++	-	-	-	+++	+++
BSC-18	-	-	-	-	-	++	-	+++	+++
BSC-19	-	-	-	-	-	+	-	+++	+++
BSC-21	-	-	-	-	-	+	-	+++	+++
BSC-29	-	-	++	++	-	-	-	+++	++
BSC-30	-	-	-	-	-	++	-	+++	++
BSC-31	-	-	-	-	-	+	-	+++	+++
BSC-37	-	++	++	-	-	++	++	+++	+++
BSC-38	-	-	-	++	-	-	-	+++	+++
BSC-45	-	-	-	-	-	++	-	+++	+++
BSC-46	-	-	-	++	-	-	-	+++	++
BSC-49	-	+	+	-	+	-	-	+++	+++
BSC-51	-	-	-	-	-	+	-	+++	+++
BSC-66	-	-	-	-	-	++	-	+++	++
BSC-67	-	+	-	-	-	+	+	+++	++
BSC-68	-	+	-	-	-	++	-	+++	++
BSC-70	-	-	-	-	-	+	-	+++	++
BSC-71	-	-	++	++	-	-	-	+++	++

Pv: *Proteus vulgaris*; Ec: *Enterobacter cloacae*; Eco: *Escherichia coli*; St: *Salmonella typhimurium*; Kp: *Klebsiella pneumonia*; Yp: *Yersinia pseudotuberculosis*; Ef: *Enterococcus faecalis*; Pa: *Pseudomonas aeruginosa;* Sa: *Staphylococcus aureus*

-: no activity, +: weak activity, ++: moderate activity , +++ : high activity.

Table 3. Second antibacterial activity results of some actinomycetes isolates.

Isolate No	BSC-13	BSC-37	BSC-38
Test bacteria / Day	***Enterococcus faecalis***	***Staphylococcus aureus***	***Enterococcus faecalis***
3.	-	15 mm	-
4.	10 mm	21 mm	10 mm
5.	11 mm	20 mm	11 mm
6.	17 mm	38 mm	17 mm
7.	30 mm	57 mm	26 mm

The mm value is the inhibition zone diameter.

-: no activity.

Table 4. Temperature, pH and NaCI requirements of some actinomycetes isolates.

Tests	Isolate No	BSC-13	BSC-17	BSC-37	BSC-38	BSC-45	BSC-49
Temperature (°C)	10	-	-	-	-	-	-
	20	++	++	++	++	++	++
	25	++	++	++	++	++	++
	30	++	++	++	++	++	++
	37	++	++	++	++	++	++
	45	++	++	++	++	++	++
	50	-	-	-	-	+	+
pH	5	+	+	+	+	+	+
	6	++	++	++	++	++	++
	7	++	++	++	++	++	++
	8	++	++	++	++	++	++
	9	++	++	++	++	++	++
NaCl (%)	Control	++	++	++	++	++	++
	2.5	++	++	++	++	++	++
	5	++	++	++	++	++	++
	7.5	++	++	++	++	++	++
	10	-	-	-	-	-	-

++: good growth, + : weak growth, - : no growth.

Table 5. Similarity of the some actinomycetes isolates according to their 16S rDNA sequence analysis.

Isolate No	Access Number	Name of Bacteria	Percent Similarity
BSC-13	KX352792	*Streptomyces griseorubens* strain JJ67	99
	KF742497	*Streptomyces coelicolor* strain 140	99
	FJ775012	*Streptomyces pseudogriseolus* isolate SA-5	99
	KF029644	*Streptomyces albogriseolus* strain ASC842	99
	MF352004	*Streptomyces viridodiastaticus* strain sh15	99
BSC-17	JN627185	*Streptomyces variabilis* strain A4-3	99
	KU500360	*Streptomyces labedae* strain NHF7	99
	KJ571055	*Streptomyces erythrogriseus* strain 7-6	99
	HQ848083	*Streptomyces coelicolor* strain MLA-21	99
	NR_112312	*Streptomyces griseoincarnatus* strain NBRC 12871	99
BSC-37	KU535562	*Streptomyces griseorubens* strain G19	98
	KF742497	*Streptomyces coelicolor* strain 140	98
	KF029644	*Streptomyces albogriseolus* strain ASC842	98
BSC-38	KX352792	*Streptomyces griseorubens* strain JJ67	98
	KF742497	*Streptomyces coelicolor* strain 140	98
	MF352004	*Streptomyces viridodiastaticus* strain sh15	98
	KF029644	*Streptomyces albogriseolusstrain* ASC842	98
BSC-45	EU593727	*Streptomyces thermocarboxydus* strain 173998	96
	KJ789389	*Streptomyces matensis* strain SUZ96	96
	EU684319	*Streptomyces xylophagus* strain kx6	96
	KY120283	*Streptomyces althioticus* strain PST5	96
	AY999782	*Streptomyces almquistii* strain NRRL B-1685	96
BSC-49	KU535562	*Streptomyces griseorubens* strain G19	97
	KF742497	*Streptomyces coelicolor* strain 140	97

Studies on actinomycetes are thought to be important for the discovery of new species that produce bioactive compounds. All isolates in this study were tested against nine test bacteria using cross-streak method for antibacterial activity screening. All isolates exhibited antibacterial activities against at least one of the test bacteria. Twenty-three out of seventy-three isolates produced higher activity against tested bacteria than the other isolates. These twenty-three actinomycetes isolates showed moderate to high antibacterial activity against *E.*

faecalis and *S. aureus.* BSC-37 and BSC-49 isolates showed activity against five or more different test bacteria. A similar result was obtained by Özcan *et al.* (2013). They studied with marine actinomycetes isolates and found similar antibacterial activities against the same test bacteria. Furthermore, isolates in this study exhibited strong activity against Gram-positive bacteria compared to Gram-negative bacteria. This result is in accordance with the previous findings (Kokare *et al.* 2004, Mohseni *et al.* 2013). In the second antibacterial activity screening test, the highest antibacterial activity (57 mm) was produced by BSC-37 against *S. aureus*. Additionally, BSC-13 and BSC-38 produced inhibition zones of 30 and 26 mm against *E. faecalis*, respectively. In the time-dependent experiment, BSC-37, at 7th day, exhibited strong inhibition compared to the inhibition at 6th day. The antibacterial activity produced by *Streptomyces* sp. H-KF8 strain against *S. aureus* increased over time. It has been emphasized that *Streptomyces* sp. H-KF8 has a relationship between growth and antimicrobial activity (Undabarrena *et al.* 2017).

The isolates BSC-13, BSC-17, BSC-37, BSC-38, BSC-45 and BSC-49, which exhibited high antibacterial activity against the tested pathogens, were identified further according to their morphological, physiological and molecular properties. All the isolates grow well in the starch casein agar medium. Gram staining technique classified these isolates as Gram-positive. Filamentous structures were also observed under light microscope. Kokare *et al.* (2004) expressed the filamentous structure of Gram-positive actinomycetes. In the cover slip method, the spore chain structures of the isolates were defined as rectus, hooks, spira and open loops-retinaculum apertum. Gautham *et al.* (2012) showed that 20 out of 42 actinomycetes have different spore morphology such as rectus, flexibilis, retinaculum aperatum–open loops, hooks and spira-simple spirals, short and compact spirals. They stated that according to these spore chain arrangements, the isolates belonged to the genus *Streptomyces*. None of the isolates grew at 10°C. The isolates BSC-45 and BSC-49 grew at 50°C. Stackebrandt & Schuman (2006) reported that thermophilic actinomycetes are found in plant wastes, fertilizers and composts where high temperature occurs. In another study, actinomycetes growing at 45°C were reported as thermotolerant and actinomycetes growing at 55°C were reported as thermophilic (Korn-Wendisch & Kutzner 1992). According to our results, six isolates may be thermotolerant as they showed optimum growth at 45°C. When the pH tolerances were examined, isolates showed intense growth at pH 6-9 and little growth at pH 5. According to their salt tolerance, the isolates are not halotolerant because they do not grow in 10% salt. Halotolerants do not need absolute salt to grow and can tolerate at least 100 g/l salt (Oren 2002). In a study on protease-producing actinomycetes from marine saltern, 18 of 189 strains were found to grow in media containing 15 to 21% NaCl concentration, 23 strains in media containing 9% to 15% NaCl concentration, 57 strains in media containing 3 to 9% NaCl concentration and 91 strains in media containing 1 to 3% NaCl concentration (Suthindhiran *et al.* 2014). 16S rDNA sequence analysis placed these six isolates in the genus *Streptomyces.* Most extensively studied actinomycetes members belong to *Streptomyces* (Jagannathan *et al.* 2021). The isolates BSC-45 and BSC-49 are thought to be new species since they have about 97% 16S rRNA gene sequence similarity to the strains of the genus *Streptomyces*. These bacteria are very difficult to identify at species level because they have many similar properties. Additional studies including protein-coding gene sequences with higher phylogenetic resolution and genome-based studies are needed to identify the species in *Streptomycetes* genus (Kämpfer *et al.* 2014).

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

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