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RESEARCH & KNOWLEDGE

Research Article

Novel enzymes produced by actinobacteria growing on seaweed

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Abstract - Actinobacteria were screened for their ability to produce seaweed polysaccharide-degrading enzymes. *Streptomyces* strains FA24 and FA56 grown on seaweed which could both produce alginate lyase. The polysaccharide-degrading enzymes were precipitated with ammonium sulphate and then purified using dialysis and anion exchange chromatography. Polysaccharide-degrading enzymes from *Streptomyces* sp. FA24 had a molecular mass range of 15 – 37 kDa while enzymes from *Streptomyces* sp. FA56 had an apparent molecular mass of 23 kDa. The optimum pH for activity of polysaccharide-degrading enzymes from *Streptomyces* spp. FA24 and FA56 against sodium alginate were 8.0 and 7.5, respectively. These enzymes of both strains also showed maximum activity at 30°C. *Streptomyces* spp. FA24 and FA56 were observed as having a 95% and 99% 16S rRNA gene sequence similarity, respectively, to previously recorded sequences of type strains of actinobacteria isolates, indicating that Streptomyces sp. FA24 could be novel species. The enzymes purified from *Streptomyces* spp. FA24 and FA56 grown on Bull Kelp powder may be novel alginate lyases.

Keywords: Actinobacteria, polysaccharide-degrading enzymes, seaweed

1. Introduction

Actinobacteria is a phylum of high G+C content bacteria containing over 2,400 species that are present in various ecological habitats and marine environments (Rosenberg *et al*., 2014). In this work the common term actinobacteria will be used to refer to exospore forming filamentous bacteria, predominantly in the order Actinomycetales. They are being exploited for various commercial applications in environmental, biomedical and industrial sectors (Berdy, 2005). Actinobacteria have been proven as producers of antibiotics and of other bioactive compounds and of industrially important enzymes. They are the most economically and biotechnologically valuable bacteria (Jemimah *et al*., 2011). Different studies have shown that actinobacteria are one of the largest bacterial groups which have the ability to produce enzymes due to their rapid doubling time and range of substrates utilised when compared with plants or animals to meet the existing market demand for industrial enzymes (Kumar and Takagi, 1999).

Seaweeds are excellent sources of polysaccharides but have rarely been used as substrates for growth and enzymes. The unique polysaccharides synthesised by seaweeds including alginate, agar, carrageenan, laminarin, fucoidan and cellulose (Sakatoku *et al*., 2012). These polysaccharides have a variety of biological activities such as antitumor (Fukahori *et al*., 2008), antioxidants (Li and Kim, 2011), anticoagulant (Li *et al*., 2008), antiviral and antibacterial properties (Chojnacka *et al*., 2012). They could be used as functional ingredients in many commercial applications in the food, pharmaceutical and in the cosmetic industries (Wijesinghe and Jeon, 2012). In brown seaweed, the carbohydrate content is 30 - 50%, consisting of mostly alginate, fucoidan, cellulose and laminarin (Sakatoku *et al*., 2012). Therefore, seaweed is a rich carbon source that can be used for culturing actinobacteria.

Polysaccharide-degrading enzymes are increasingly becoming industrially important enzymes that could be safely used for human therapeutic purposes (Selvam, 2011) as well as used in a wide range of applications for seaweed bioprocessing. A number of research papers have shown that polysaccharide-degrading enzymes could be found in marine invertebrates and marine microorganisms (bacteria and fungi) (Burtseva *et al*., 2000; Descamps *et al*., 2006, Kusaykin *et al*., 2007; Sawabe *et al*., 1997; Schaumann and Weide, 1990; Silchenko *et al*., 2014). However, enzyme activities produced by these microorganisms are low (Kusaykin *et al*., 2008). Currently, no studies have reported enzymes extracted from actinobacteria that can degrade polysaccharides from seaweed.

This present study was carried out to extract, purify and characterise enzymes produced by actinobacteria grown on seaweed to confirm that actinobacteria can produce polysaccharide-degrading enzymes such as alginate lyase. It is possible that growth of actinobacteria on seaweed can be used to identify new sources of potentially novel polysaccharide-degrading enzymes.

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2. Materials and methods

The seaweed used in this study was *Durvillaea potatorum* (Bull Kelp), which belongs to the brown seaweed group. Bull Kelp was collected at Rivoli Bay, Beachport, South Australia.

Six actinobacteria were used in this study. Three of them were isolated from surface-sterilised wheat roots, namely *Streptomyces* spp. FA24, FA23 and FA27. The other three were isolated from marine sponges, namely *Streptomyces* spp. FA11, FA16 and FA56.

2.1 Cultivation of actinobacteria

The spore mass of each strain of actinobacteria was inoculated into separate 50 ml of IM22 inoculum medium (15 g L^{-1} glucose, 2 g L^{-1} calcium carbonate, 5 g L^{-1} sodium chloride, 15 g L^{-1} soyatone, 5 g L^{-1} pharmamedia) in 250 ml Erlenmeyer flasks and cultured at 27°C on a rotary shaker at 150 rpm. After 3 days, 10 ml of the inoculum was transferred to 500 ml production medium to induce synthesis of carbohydrases. The production media consisted of 2% Bull Kelp powder in 5 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$ and 5 g L^{-1} NH_4Cl. The cultures were incubated at 27°C with shaking at 150 rpm for seven days.

2.2 Concentration of crude enzymes

The crude extracellular enzymes were concentrated by ammonium sulphate precipitation using ammonium sulphate at concentrations 35% and then 45% at pH 8.0 and kept overnight at 4°C. The solutions were then centrifuged at 10,000 g at 4°C for 15 min. The precipitates, dissolved in 0.02M Tris-HCl buffer, were dialyzed against the same buffer overnight at 4°C for desalting. The buffer was changed every two hours. The dialyzed supernatants were used as partially purified enzymes for testing polysaccharide-degrading enzyme activity.

2.3 Purification of enzymes by anion-exchange chromatography (Mono-Q)

The dialysed enzymes were put into a Mono-Q™ 5/50 GL column in an FPLC machine (Amersham Biosciences). The mobile phase included buffer A (50mM Tris-HCl pH 7.6, 5mM EDTA and 1mM DDT) and buffer B (1M NaCl, 50mM Tris-HCl pH 7.6, 5mM EDTA and 1mM DDT). The pump was run at a flow rate of 1 ml min^{-1}. The proteins were eluted with a linear gradient of 0 to 1 M of NaCl in the buffer B and fractionated into 1 ml portions.

2.4 Determination of the enzyme activity

The activities of polysaccharide-degrading enzymes were determined by the amount of reducing sugar produced by each enzyme, following the method of Nelson (1994). Sodium alginate (0.5%) was used as substrate. A reaction mixture containing 0.5 ml partially purified enzyme and 0.5 ml substrate in 0.02M Tris-HCl buffer (pH 7.5) was incubated at 37°C for 2 hours in a water bath. The reaction was stopped by adding 1 ml of 3-5-dinitrosalicylic acid reagent (Miller, 1959) and then the mixture was boiled for 5 minutes. Absorbance resulting from the reaction with the released sugars was measured at 540 nm in an UV Spectrophotometer. The concentration of reducing sugars released into the medium was determined by using D-glucose as standard. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per min. The results were analysed statistically by One-way ANOVA in SPSS

2.5 Characterisation of purified enzymes

Enzyme characterisation was carried out by SDS-polyacrylamide Gel Electrophoresis. The purified enzymes were subjected to SDS-PAGE to determine the molecular weight. SDS-PAGE was performed on an Any kD™ precast polyacrylamide gel (BioRad).

2.6 Determination of the optimum temperature and pH of the enzymes

To determine the optimal temperature, the activities of *Streptomyces* spp. toward sodium alginate were assayed at pH 7.5 for 2 hours in the range of 25 – 50°C. The effect of pH on the enzyme activity was examined for 2 hours at 37°C in a pH range 6 – 9 with 0.5% Tris-HCl buffer. The enzyme activity was measured by the Nelson method as described in section 2.4.

2.7 Identification actinobacteria based on 16S rRNA gene sequencing

DNA of actinobacteria was extracted using the method of Kieser and Foundation (2000). DNA was amplified by PCR with the universal 16S primers designed to amplify the region between positions 27 and 1492r of the 16S rRNA gene in actinobacteria. The primers were designated 27f (5′-AGAGTTTGATCMTGGCTCAG) and 1492r (5′- CGGTTACCTTGTTACGACTT). PCR products were purified by UltraClean ® PCR Clean-Up Kit (MoBio). PCR products were sequenced by Macrogen Inc., Korea. The results of the DNA sequencing were compared against all other 16S rRNA gene sequences in the GenBank database by using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). The 16S rRNA gene sequences of *Streptomyces* spp. were aligned with reference sequences obtained from Genbank using ClustalW program. The phylogenetic tree was generated using the Maximum Likelihood method with MEGA 7.0.25.

3. Results and discussion

Among the six strains of actinobacteria, *Streptomyces* sp. FA24 isolated from surface sterilized wheat roots and *Streptomyces* sp. FA56 isolated from a marine sponge grew best on 2% Bull Kelp in the production medium (data was not shown). On the basis of the growth and production of the enzymes, these two *Streptomyces* strains were selected for enzyme production and characterization.

3.1 Purification of polysaccharide-degrading enzymes from Bull Kelp powder

Polysaccharide-degrading enzymes from *Streptomyces* spp.

FA24 and FA56 were partially purified from the culture broth of 2% Bull Kelp powder. There are two steps of enzymes purification: precipitation with ammonium sulfate and purification by anion-exchange chromatography. The specific activity of alginate degrading enzyme(s) from *Streptomyces* sp. FA24 after purifying by Mono-Q chromatography are approximately thirteen times and two times higher than that of crude enzymes and enzymes after dialysis, respectively (Table 1a). The enzymes from *Streptomyces* sp. FA56 after purifing by Mono-Q chromatography also show higher specific activity in comparision with crude enzymes and enzymes after dialysis (Table 1b). Polysaccharide-degrading enzymes from both strains have a low yield but are more purified. The low yield might be caused by the loss of protein during purification using Mono-Q column chromatography. In order to improve the yield during purification, the proteins should be run over several purification steps like gel filtration chromatography (FPLC, Sephadex 200) and hydrophobic interaction chromatography (HIC). These steps could be significant in enriching the purity and yield of enzymes.

Table 1. Summary of purification of enzymes. (a) Enzyme extracted from *Streptomyces* sp. FA24. (b) Enzyme extracted from *Streptomyces* sp. FA56. Values the mean of two replicates.

	V (ml)	**Protein (mg/ml)**	**Enzyme activity (U/ml)**	**Specific activity (U/mg)**	**Yield (%)**	**Purification** (Fold)
Crude [a]	200	0.043	21.9	625.7	100	1
35% Ammonium sulphate [b] (after dialysis)	4	0.035	152.3	3541.9	13.9	566.1
Mono-Q column [c]	1	0.021	161.0	7666.7	3.7	1225.3

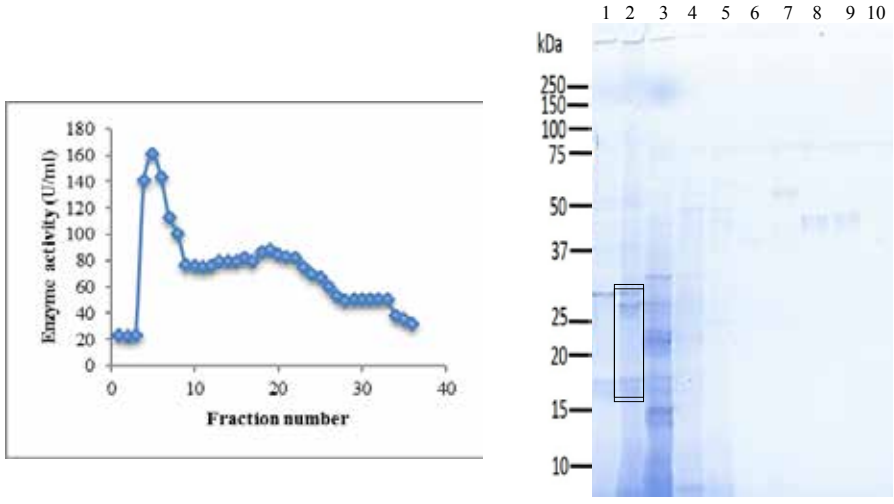
(a)

	V (ml)	**Protein (mg/ml)**	**Enzyme activity (U/ml)**	**Specific activity (U/mg)**	**Yield (%)**	**Purification** (Fold)
Crude [a]	200	0.041	2.5	60.6	100	1
45% Ammonium sulphate [b] (after dialysis)	4	0.166	32.2	194.0	25.8	320.2
Mono-Q column [c]	1	0.067	34.6	516.4	6.9	852.5

(b)

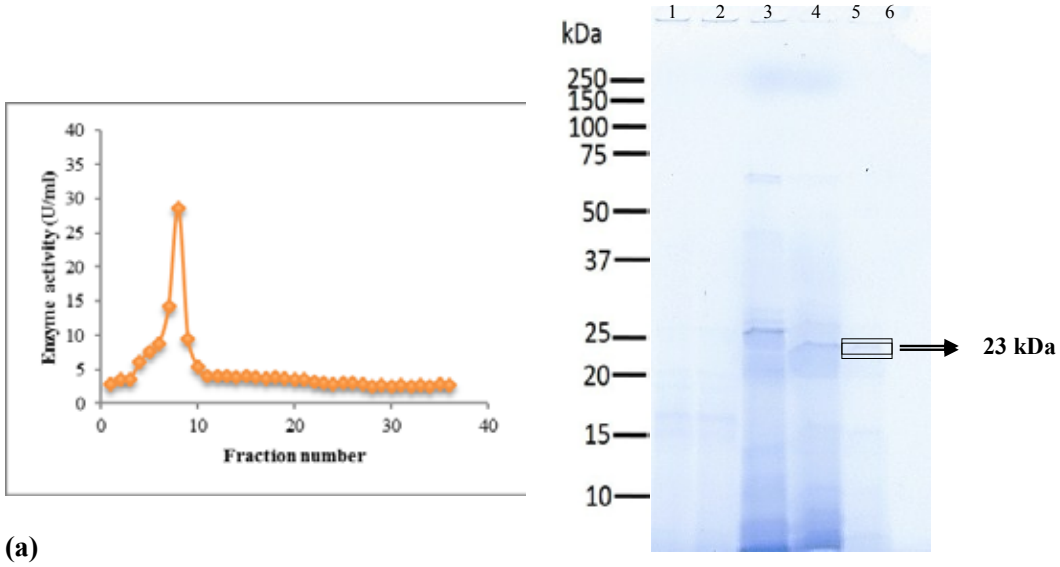
[a] Crude enzyme after centrifuge culture solution
[b] Fraction precipitated by ammonium sulphate and dialysis against 0.02 M Tris-HCl (pH 7.5)
[c] Active fraction obtained by Mono-Q column chromatography

Anion exchange chromatography is known as an effective method to purify proteins based on their charge (Aguilar *et al*., 2006). Figures 1 and 2 show the enzyme activity of different fractions obtained from *Streptomyces* sp. FA24 and *Streptomyces* sp. FA56 against alginate. The peak fraction from *Streptomyces* sp. FA24 (fraction 5) which showed the highest enzyme activity of 161.0 U/ml, consisted of four major proteins between 15 to 37 kDa on SDS-PAGE (Fig. 1a, b), while the peak fraction from *Streptomyces* sp. FA56 (fraction 8) with the enzyme activity of 28.6 U ml^{-1} showed a distinct protein bands of around 23 kDa (Fig. 2a, b). Therefore, in the present study, we used these peak fractions for further characterisation of enzymes extracted from *Streptomyces* spp. FA24 and FA56.



(a) **(b)**

Figure 1. (a) Enzyme activity of different fractions from *Streptomyces* sp. FA24 grown on 2% Bull Kelp against 0.5% sodium alginate after purification by anion exchange chromatography on a Mono-Q column. (b) SDS–PAGE analysis of purified enzyme obtained from *Streptomyces* sp. FA24 by 35% ammonium sulphate fractionation and Mono-Q column chromatography. Lane 1, fraction 4; lane 2, fraction 5; lane 3, fraction 6; lane 4, fraction 7; lane 5, fraction 8; lane 6, fraction 17; lane 7, fraction 18; lane 8, fraction 19; lane 9, fraction 20; lane 10, fraction 21.



(a) **(b)**

Figure 2. (a) Enzyme activity of different fractions. Enzyme activity extracted from *Streptomyces* sp. FA56 grown on 2% Bull Kelp against 0.5% sodium alginate after purification by anion exchange chromatography on a Mono-Q column. (b) SDS – PAGE analysis of purified enzyme extracted from *Streptomyces* sp. FA56 by 45% ammonium sulphate fractionation and Mono-Q column chromatography. Lane 1, fraction 5; lane 2, fraction 6; lane 3, fraction 7; lane 4, fraction 8;lane 5, fraction 9; lane 6, fraction10.

3.2 Effects of pH and temperature on enzyme activity

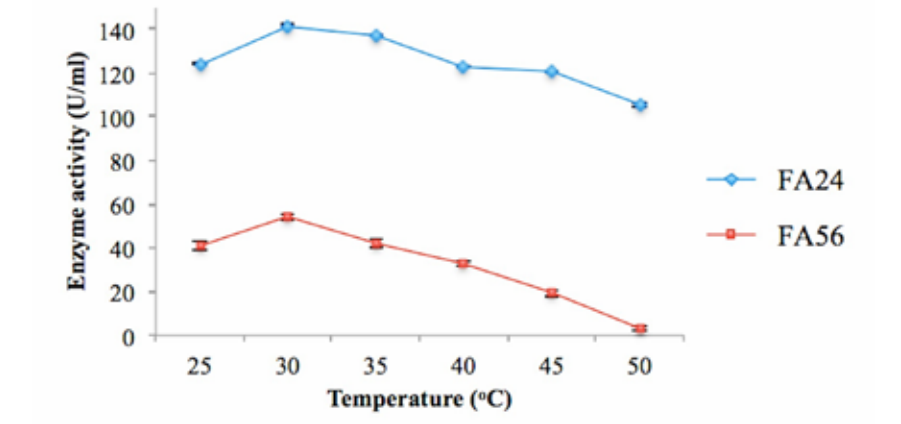
Since the optimum conditions of temperature and pH play an important role in the activity of polysaccharide-degrading enzymes, we optimised the conditions designed in the range of pH 6 – 9 and temperature in the range of 25 – 55°C. In the literature, alginate lyase isolated from marine bacteria are mostly active in the neutral or slightly alkaline pH range (Bakunina *et al*., 2002; Hu *et al*.,2006; Silchenko *et al*., 2013). In this present study, the optimum pH of polysaccharide-degrading enzymes from *Streptomyces* spp. FA24 and FA56 against sodium alginate are 8.0 and 7.5, respectively (Fig. 3b). These enzymes of both strains also show maximum activity at a temperature of 30°C (Fig. 3a). These results are similar with the alginate lyase from

Alteromonas strain 272 that was most active at pH 7.5 - 8 (Iwamoto *et al*., 2001). Additionally, in the study done by Sawabe *et al*. (1992), the optimum pH and temperature of enzyme activity from marine *Alteromonas* sp. H-4 were 7.5 and 30°C, respectively.

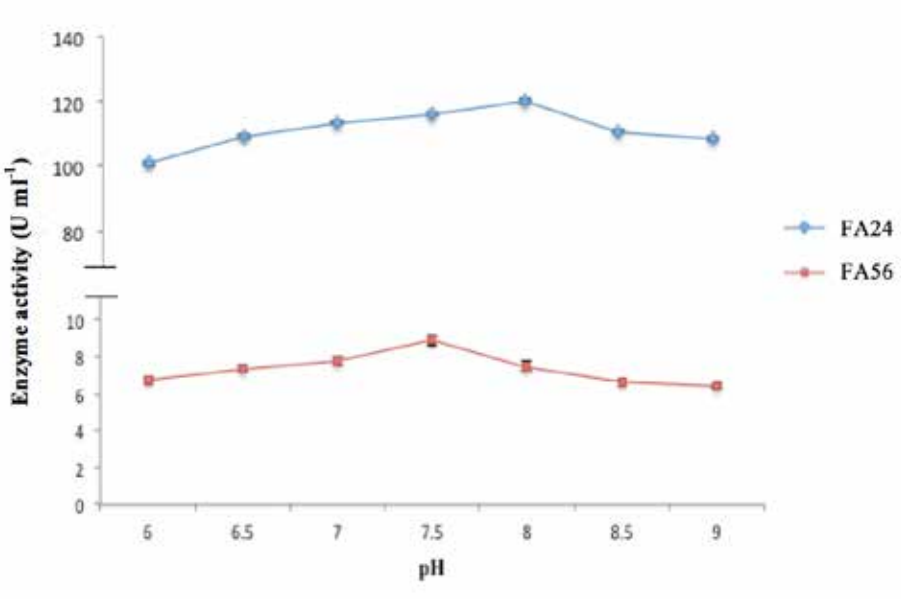
3.3 Identification of actinobacteria based on 16S rRNA Gene sequence

The full-length 16S rRNA gene sequencing was used initially to identify FA24 and FA56 to genus level. The results of the BLAST search comparing *Streptomyces* sp. FA24 and *Streptomyces* sp. FA56 with the NCBI online gene database are showed in Table 2.

In this study, the full-length 16S rRNA gene sequencing was employed to identify the actinobacteria that can degrade polysaccharides from Bull Kelp. Using the primers 27f and 1492r, the sequencing analysis of the 1500 base pair region was carried out. The phylogenetic tree of both *Streptomyces* sp. FA24 and *Streptomyces* sp. FA56 was categorized into two major clads on the basic of their evolutionary distances calculated through maximum likelihood method (Fig. 4), gene sequence of *Streptomyces* sp. FA24 shows distant relationship with *Streptomyces* sp. FA56 sequence. *Streptomyces* sp. FA24 shows a close match of 95% to *Streptomyces microflavus* strain NBRC 13062^T (NR112354). *Streptomyces* sp. FA56 has a 99% similarity to the closest match *Streptomyces carpaticus* strain NBRC 15390^T (AB184641). Vandamme *et al*. (1996) indicated that a genus could be defined by species with at least 96% sequence similarity. Therefore, *Streptomyces* sp. FA24 show a match of only 95%, suggesting that it could be a novel actinobacteria. Confirmation of novelty will require a full polyphasic taxonomy study (Goodfellow *et al*., 1990).



(a)



(b)

Figure 3. (a) Effect of temperature on enzyme activity (after dialysis) of *Streptomyces* spp. FA24 and FA56 against sodium alginate. (b) Effect of pH on enzyme activity (after dialysis) of *Streptomyces* spp. FA24 and FA56 against sodium alginate. Error bars show standard error of mean, n=3.

Table 2. BLAST matches of full-length 16S rRNA gene sequencing.

Isolates	Genera/Species ID (nearest type strain)	Identity	Accession number
FA24	*Streptomyces microflavus* NBRC 13062^T	95%	NR112354
FA56	*Streptomyces carpaticus* NBRC 15390^T	99%	AB184641

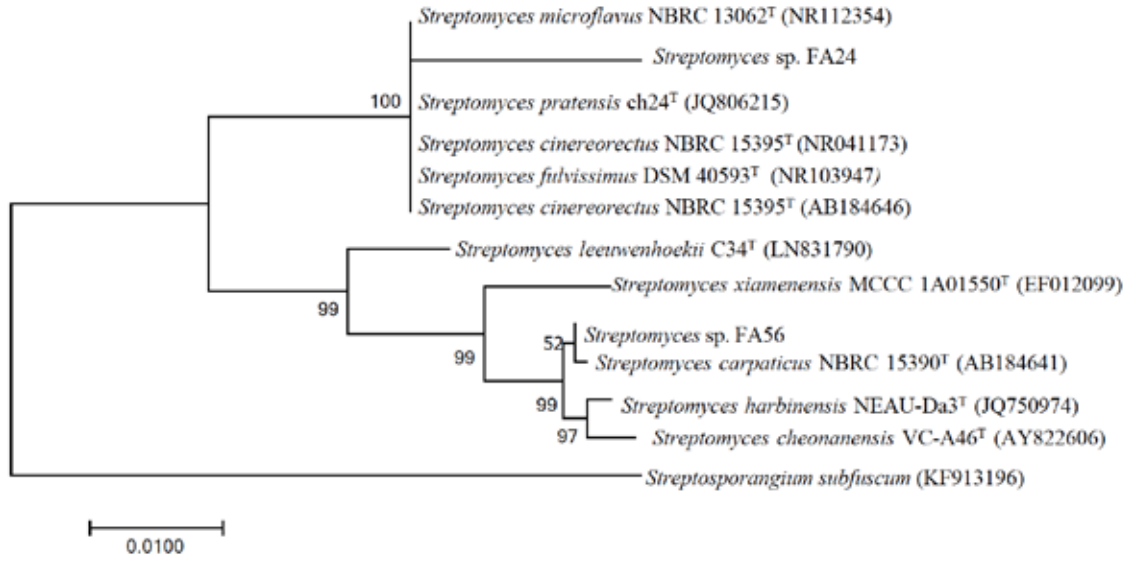


Figure 4. Phylogenetic tree of 12 strains of related *Streptomyces* sp. FA24 and FA56 based on the 16S rDNA gene sequence. The number at the branching points are the percentages of occurrence in 1000 bootstrapped trees. The bar indicates a distance of 0.01 substitutions per site.

4. Conclusion

Streptomyces sp. FA24 from wheat roots showed high activity for alginate degrading enzymes. *Streptomyces* sp. FA56 from marine sponge showed activity of enzymes versus alginate. It is seems that *Streptomyces* strains FA24 and FA56 are good producers of alginate - degrading enzymes. It is expected that these enzyme are able to effectively degrade alginate in the cell walls of brown algae and may be likewise applied to make bioactive compounds that can be used in many biological and pharmaceutical applications.

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