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Antifungal Production of a Strain of *Actinomycetes spp* Isolated from the Rhizosphere of Cajuput Plant: Selection and Detection of Exhibiting Activity Against Tested Fungi

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Abstract

Actinomycetes are bacteria known to constitute a large part of the rhizosphere microbiota. Their isolation is an important step for screening of new bioactive compounds. Culturable actinomycetes populations from cajuput plant rhizosphere soils in Wanagama I Forest UGM Yogyakarta were collected to study about their antifungal activity. Among 17 of a total 43 isolates that showed activity were screened for producing antifungi substances. Screening for antifungal activity of isolates were performed with dual culture bioassay *in vitro.* One isolate that was designated as *Streptomyces* sp.GMR-22 was the strongest against all tested fungi and appeared promising for a sources of antifungal. Culture's supernatant and mycelia were extracted with chloroform, ethyl acetate and methanol, respectively. Antifungal activity of crude extracts was tested by diffusion method against tested fungi. The result indicates that isolates of actinomycetes from cajuput plant rhizosphere could be an interesting sources of antifungal bioactive substances.

Keywords : Rhizosphere, Cajuput, Streptomyces, Antifungal

Introduction

Numerous microbia lived in the portion of soil modified or influenced by plant roots, they so called 'rhizosphere'. Among these microbia, some have positive effects on plant growth. Actinomycetes are bacteria known to constitute a large part of the rhizosphere microbiota. Evidence indicates that actinomycetes are quantitatively and qualitatively important in the rhizosphere, where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Sardi *et al*., 1992; Crawford *et al*., 1993; Atalan *et al*., 2000; Basil *et al*., 2004). Member of the genus *Streptomyces* are well known for their ability to suppress growth of a wide variety of fungal pathogens. Among of soil microbia, actinomycetes are source of important therapeutically products (Kavitha *et al*., 2010). It has been found also that some endophytic microbia can produce valuable pharmaceutical substances of biotechnological interest (Strobel & Long, 1998). Therefore, screening of actinomycetes from these habitat is important for identification of useful strains that produce novel bioactive compounds (Matsukawa *et al*., 2007).

Many researches has been reported that actinomycetes produced antifungal compounds isolated from plant rhizosphere such as *Vitis vinifera L* (Loqman *et al*., 2009), *Argania spinosa* (Barakate1 *et al*., 2002), sagebrush (*Artemisia tridentata*)(Basil *et al*., 2004) and *Zingiber officinale* (Taechowisan and Lumyong, 2003).

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Here, we are reporting the isolation of a number of Actinomycetes from cajuput plant rhizosphere, showing activities against tested fungi. Selected isolates were also screened for their *in vitro* antagonism against fungal pathogens and non pathogens. A strong antagonists was selected for studies of the physiological and identified based on 16S rRNA gene sequences for phylogenetic tree analysis.

Materials and Methods

Isolation of actinomycetes

Samples were taken from rhizospherical soil of Cajuput plant (*Melaleuca leucadendron*) at Hutan Wanagama, Yogyakarta Province, Indonesia as described by Lee and Hwang (2002) and Aghighi *et al*., (2005). Samples were obtained from a depth of 5 to 15 cm and placed in sterile 50-ml conical tubes. Samples were kept at ambient temperature during the expedition and at 4°C upon return to the laboratory. Sample (about 10 g) of air-dried soil were mixed with sterile destiled water (100 mL). The soil suspension were heated in waterbath at 70°C for 1 h and then shaking at 200 rpm for 30 min at 30°C. One ml of soil mixtures were transferred to 9 mL of sterile destiled water and subsequently to final dilution 10^{-6}. About 0.1 mL of final dilution was spreaded of the Starch Nitrate agar media [(20 g soluble starch, 0.5 g NaCl, 1 g KNO_3, 0.5 g $K_2HPO_4.3H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 20 g agar, 1000 mL aquadest (pH 7.2-7.4), supplemented by cyclohexamide 50 ppm]. Plate were incubated at 30°C for up 20 days. Actinomycetes that is grown on the media were isolated, purified and identified. Actinomycetes colonies with different morphologies were selected and transferred to the same media slant for taxonomic studies and identification of antifungal activity.

Dual culture bioassay by In vitro

The antifungal activity of the isolates was determined by the dual culture bioassa method (Barakate *et al*., 2002) against tested fungi. Agar disk of actinomycetes (diameter 6mm) were cultured on SNA for 7 days was cutted by using sterile cork borers and then placed on the plate about 30 mm from test fungal inocula (Khamna *et al*, 2009; Lee and Hwang, 2002). The tested fungal were *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus flavus* and *Fusarium oxysporum*. The plate was incubated for 7 days at 28°C and the degree of inhibition of fungal mycelial growth were measured from the dual test plate. The plate with no actinomycetes inocula were used as a control. The inhibition zone were determined as the radius of fungal growth in the direction of an actinomycetes colony and evaluated as percentage of inhibitoy >70 % (+++,strong inhibition), 50-70 % (++, moderate inhibition) and <50% mm (+/-, weak inhibition).

Characterization of isolates by colour grouping

Isolates were determined based on morphological in oatmeal agar media for 7-10 days incubation at 30°C. Purified isolates were identified to genus level according to Bergey's Manual of Determinative Bacteriology (Holt *et al*,.1994). Characterization of colonies were determined by colour grouping (aerial mycelium, substrate mycelium and diffusible pigment) developed as growth on cover slips buried in oatmeal agar medium.

Production, Extraction Antifungal
Production and extraction of antifungal metabolites

All Isolates were grown in submerged culture in 250 ml flasks containing 50 ml of SN broth medium. The flasks were inoculated with 1 ml of isolates culture and incubated at room temperature for 8 days with shaking at 150 rpm. After growth, isolates culture broth was collected and centrifuged at 5000 rpm for 10 min and filtered through Whatman No.1 filter paper. Antifungal compound containing supernatant was extracted using equal volume of solvent (1:1 v/v). The culture filtrates were extracted twice with chloroform, ethyl acetate and the pooled solvent extracts were evaporated to dryness under vacum to yield a

crude residue before using for antifungal assay (Arasu *et al*., 2009).

Determination of the antimicrobial activity

The antifungi activity was determined by diffusion method (Badji *et al*, 2006 and Pandey, 2004). The extract was dissolved in 15 µL DMSO and then paperdisk saturated with extract. The disk was placed on plate surface which seeded test fungi (*Candida albicans, Saccharomyces cerevisiae, Fusarium oxysporum,* and *Aspergillus flavus*). The plates were incubated at 30°C for 18-24 h and examined. The diameter of the zones of complete inhibition was measured.

Genomic DNA extraction

Extraction of genomic DNA were done as described by Song *et al.*, (2004) and Badji *et al.,* (2006). Isolated actinomycetes were grown for 4 days at 30°C with shaking in 250 mL flask containing 70 mL of ISP2 medium. Pellet of isolate was harvested by centrifugation (3.000 rpm 15 min) and washed twice using aquabidest. About 300 mg of mycelia was used for DNA extraction as follows: the sample was dispersed in 400 µL TE and resuspended with 400 µL SET buffer, 50 µL Lysozyme (10mg/mL), 20 µL Proteinase K (20mg/mL) was added and incubated at 37°C for 1 h. RNAase A solution was added, and the suspension incubated at 65°C for 10 min. The lysate was extracted with an equal volume of phenol and centrifuged (13.000 rpm 10 min). The aqueous layer was re-extracted with phenol (1:1v/v), and then by chloroform (1:1v/v). DNA was recovered from the aqueous phase by adding cooled ethanol prior to centrifugation. The precipitate DNA was cleaned with 100 µL ethanol 70% and then supernatan was removed. The purity of DNA solution was resuspended with aquabidest and checked spectrophotometrically at λ_{260} and λ_{280} nm.

Amplification and sequencing 16S rRNA gene

The 16S rRNA genes were amplified from purified DNA of the strain as decribed previously (Sacido and Genilloud, 2004; Petrosyan *et al*, 2003) using a commersial kit (Mega Mix-Blue ®) and primers 27f(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r(5'-GGTTACCTTGTTACGACTT-3'). The condition of amplified gene fragment: pre-denaturation of the target DNA at 98°C for 3 min followed by 30 cycles at 94°C for 1 min, primer annealing at 57°C for 1 min, and primer extension at 72°C for 5 min, the reaction mixture was held at 72°C for 5 min. Detection of gene were analyzed by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide.

The amplified of DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISMA 3100 Genetic Analyzer User's Manual).

Phylogenetic analysis

Sequence similarity search of the 16S rRNA gene sequence from GMR-22 strain was carried out using BLAST (NCBI). An evolutionary tree was inferred by using the neighbour-joining method (Saitou and Nei, 1987). The clustal X program was used for multiple alignments and phylogenetic analysis (Thompson *et al*., 1997)

Results and Discussion

Isolation and characterization of isolates

The five rizospherical type of the sampling site yielded 43 actinomycetes isolates. A total of 17 actinomycetes were isolated from the cajuput, while 9, 10 and 6 were recovered from gliseride, teak and mahogany tree, respectively. However, only one actinomycetes was isolated from bamboo (data not shown). It is indicated that nature of the vegetation occurring at the sampling sites probably influences the actinomycete populations through root exudates and sloughed off root material and might well account for the presence of different groups in the different soils studied.

Among 17 strains were isolated from cajuput rhizosphere and then classified into

streptomycete-like strains (abundant aerial mycelium with powdery spores). The isolates were grouped based on different colour groups of aerial mycelium, substrate mycelium and soluble pigment (Table 1). Most of isolates members were found to represent 53% of the total number of isolates of grey aerial mycelium; however, the lowest occurrence was noted for the brown (5.6%). Many author reported that the highest occurrence of streptomycete-like strain of the grey aerial mycelium (Saadou *et al*., 1999; Ndonde & Semu 2000; Awad *et al*., 2009). However, Masmeh (1992) in his study on distribution of Streptomycete flora in Jordan, reported that the white colour class dominated (43.6%). The differences in characteristic of the aerial mycelia color of the isolates and the soluble pigments they produce, is one of an indication of the diversity of Streptomycete isolates in the sampling sites. It is clear that cultures that were collected from sampling site were potential for screening and identification of active substances.

Actinomycetes isolated from this study belongs to Streptomycete-like strains group in whole population (data not shown). However, 17 isolates were tested by dual culture assay *in vitro* method showing weak and strongest inhibition activity against fungi. Rhizosphere represents a unique biological niche that supports abundant and diverse saprophytic microbia because of a high input of organic materials derived from the plant roots and root exudates (Merckx *et al*., 1987). Futhermore, presences of actinomycetes may play important roles in plant because they affect plant growth either by nutrient assimilation or by secondary metabolite production (Hasegawa *et al*., 2006)

Antagonist activity of isolates

In the dual culture assay, fiften of 17 isolates showed antagonistic property in at least one of the four tested fungi. Meanwhile, 14 of isolates inhibited all test fungi (unicelellar and multicellar fungi). The diameter of zone of inhibition was measured and percent inhibition was recorded and then illustrated in Table 2. Based on percentage of inhibitory, 84.8% isolates were found active against all fungi tested. This result implies that isolates showing higher inhibition that probably due to the presence of some inhibitory substance

Table 1. Cultural characteristics of Actinomycetes strain isolated from cajuput rhizosphere on Oatmeal agar media

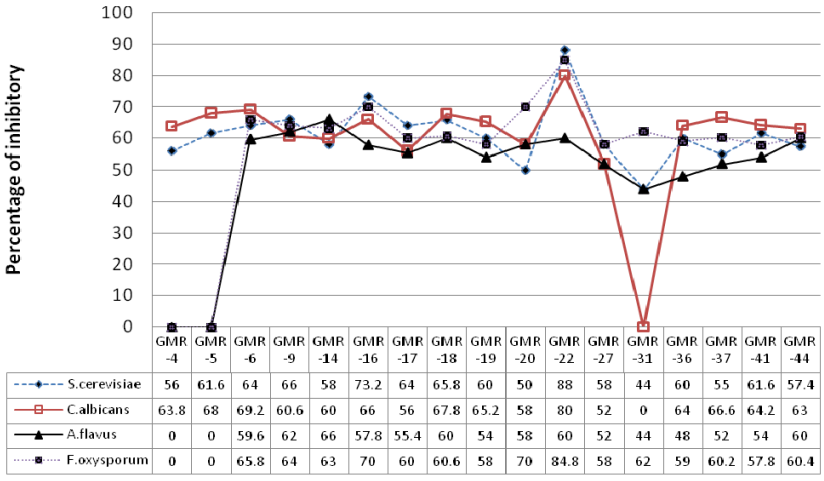
Strain	Spore chains*)	Aerial mycelium	Substrate mycelium	Soluble pigment
GMR-4	RF	Brown	Light Brown	Brown
GMR-5	RA	White-Grayish	Pale Brown	-
GMR-6	S	Gray	Light Brown	-
GMR-9	RF	White	Creamy	Yellow
GMR-14	RA	Gray	Pale Brown	-
GMR-16	RF	Grayish	Pale Brown	Brown
GMR-17	RA	White	Grayish	-
GMR-18	RF	Pale Yellow	Pale Brown	-
GMR-19	RF	Creamy	Light Brown	-
GMR-20	RF	Gray	Pale Brown	Pale Brown
GMR-22	RA	Gray	White	-
GMR-27	RF	Gray	Light Brown	-
GMR-31	RA	White	Pale Brown	-
GMR-36	S	Pale Yellow	Creamy	-
GMR-37	RA	Gray	Brown	-
GMR-41	RA	White	Gray	Yellow
GMR-44	S	Gray	Pale Brown	-

*): RA, *rectiaculiaperti*; RF, *rectiflexibiles*; S, *spiral*

Tabel 2. Average of inhibition zone of ethyl acetate extract of Actinomycetes isolated from cajuput rhizosphere against tested fungi.

No.	Isolate code	Weight of ethylacetate extract (g)/ paper disk	Diameter of inhibition zone (mm)			
			S. cerevisiae	*C. albicans*	*A. flavus*	*F. oxysporum*
1	GMR-4	0.0127	13.88	11.88	-	-
2	GMR-5	0.0124	14.63	10.11	-	-
3	GMR-6	0.0122	15.23	9.99	13.74	15.21
4	GMR-9	0.0114	14.88	11.38	14.02	16.12
5	GMR-14	0.0101	14.63	11.50	11.88	14.12
6	GMR-16	0.0104	15	11.00	7.89	10.11
7	GMR-17	0.0096	12.38	10.21	12.32	12.28
8	GMR-18	0.0088	11.88	12.11	8.65	10.19
9	GMR-19	0.0100	10.25	10.68	7.41	14.25
10	GMR-20	0.0192	15.63	10.88	9.98	16.03
11	GMR-22	0.0098	-	-	-	-
12	GMR-27	0.0107	7.88	-	8.25	9.21
13	GMR-31	0.0192	-	-	7.75	10.87
14	GMR-36	0.0114	17.38	9.98	10.15	11.78
15	GMR-37	0.0072	18.23	10.25	11.45	9.63
16	GMR-41	0.0077	15.22	10.15	10.12	9.35
17	GMR-44	0.0039	12.99	9.25	10.10	9.63

[-] : *negative activity*



	GMR -4	GMR -5	GMR -6	GMR -9	GMR -14	GMR -16	GMR -17	GMR -18	GMR -19	GMR -20	GMR -22	GMR -27	GMR -31	GMR -36	GMR -37	GMR -41	GMR -44
S.cerevisiae	56	61.6	64	66	58	73.2	64	65.8	60	50	88	58	44	60	55	61.6	57.4
C.albicans	63.8	68	69.2	60.6	60	66	56	67.8	65.2	58	80	52	0	64	66.6	64.2	63
A.flavus	0	0	59.6	62	66	57.8	55.4	60	54	58	60	52	44	48	52	54	60
F.oxysporum	0	0	65.8	64	63	70	60	60.6	58	70	84.8	58	62	59	60.2	57.8	60.4

Figure 1. Percentages of inhibitory of Actinomycetes isolated from cajuput rhizosphere against tested fungi

against fungi. It can be noticed that there were varied produced secondary metabolites.

The antagonistic activity of actinomycetes against 4 fungi tested based on percentage of inhibitory of growth fungi tested were summarized in Figure 1. There were 14 isolates which exhibited potent as antifungal against all the fungi tested, while 2 isolates were showed activity unicellular fungi but not multicellular fungi. High potent broad spectrum antagonists of both unicellular and multicellular fungi were

Tabel 3. Average of inhibition zone of chloroform extract of Actinomycetes isolated from cajuput rhizosphere against tested fungi.

No.	Kode Isolat	Weight of chloroform extract (g)/ paper disk	Diameter of inhibition zone (mm)			
			S. cere-visiae	*C. albi-cans*	*A. flavus*	*F. oxy-sporum*
1	GMR-4	0.0133	-	-	9.90	9.89
2	GMR-5	0.0167	11.54	9.65	-	11.02
3	GMR-6	0.0124	11.33	8.54	10.11	-
4	GMR-9	0.0198	-	10.12	-	11.12
5	GMR-14	0.0091	12.77	-	8.99	13.22
6	GMR-16	0.0079	11.89	-	9.12	12.67
7	GMR-17	0.0049	10.45	9.12	9.23	12.44
8	GMR-18	0.0078	9.33	10.55	-	9.45
9	GMR-19	0.0102	12.76	-	-	9.99
10	GMR-20	0.0106	10.89	9.90	12.01	12.70
11	**GMR-22**	**0.0089**	**19.83**	**19.15**	**14.5**	**24.22**
12	GMR-27	0.0098	9.09	7.99	-	10.90
13	GMR-31	0.0109	10.55	10.23	-	11.10
14	GMR-36	0.0148	11.65	-	-	13.25
15	GMR-37	0.0109	-	11.02	20.43	12.67
16	GMR-41	0.0111	12.23	-	9.21	10.34
17	GMR-44	0.0012	14.01	-	9.34	12.33

[-] : *negative activity*

found in 5 of the 17 samples examined. The ability to inhibit the growth of fungal was observed out of all strain subjected for antagonistic screening process, a single strain was shown strongest antifungal activity

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Antifungal activity of strain

Table 2, 3 and 4 shows the activity resulting from extract of ethylacetate, chloroform and methanol, respectively. Thirteen of isolates showed the antifungi activity against fungi tested. In this case the maximun activity was detected of GMR-37. It is remarkable that GRM-22, although not shows the activity, is able to inhibit the growth of four from the fungi tested in chloroform solvent. However, the GMR-22 strain was selecetd for the ability to produce extracellular metabolite active against several fungi.

We expected, greater inhibition zone was shown, not only in qualitatif but also in degree of activity against pathogen fungi and newly of active compound.

With the exception of GMR-22 strain, all isolates has moderat to strong activity toward the test fungus and the yeast, with somewhat no inhibition zone showing activity against fungi (Table 2). In contrast with chloroform extract, the GMR-22 significantly strongest inhibited the growth of all fungi tested both unicelullar and multicellular fungi (Table 3). The result indicated that the chloroform extract possessed a higher antifungal potential than the ethylacetate and methanol solvent. These finding imply that the antifungal compound was related to the type of solvent was used for extraction. However, most by the antifungal antibiotics are extracted using ethyl acetate (Franco and Countinho, 1991). It was indicated that, the GMR-22 is one of the potensial strain to be explorated of antifungal secondary metabolite. Hence the strain GMR-22 was selected for taxonomical investigation and bioactive compound isolation. For this reason our interest was focused producing antifungal on this strain.

Tabel 4. Average of inhibition zone of methanol biomass extract of Actinomycetes isolated from cajuput rhizosphere against tested fungi.

No.	Strain	Weight of methanol biomass extract (g)/ paper disk	Diameter of inhibition zone (mm)			
			S. cere-visiae	*C. albi-cans*	*A. flavus*	*F. oxy- sporum*
1	GMR-4	0.0289	12.87	7.88	-	-
2	GMR-5	0.0199	9.29	12.78	-	9.44
3	GMR-6	0.0367	8.99	13.78	-	9.42
4	GMR-9	0.0142	12.09	8.90	-	8.77
5	GMR-14	0.0109	9.54	9.45	-	9.56
6	GMR-16	0.0096	8.90	9.56	-	9.67
7	GMR-17	0.0198	13.90	9.90	8.28	-
8	GMR-18	0.0621	15.33	-	9.45	-
9	GMR-19	0.0998	16.23	-	14.33	-
10	GMR-20	0.0399	15.76	12.33	10.34	15.88
11	**GMR-22**	**0.0150**	**20.3**	**12.1**	**13.3**	**20.21**
12	GMR-27	0.0311	9.16	12.22	12.34	-
13	GMR-31	0.0129	10.13	11.45	-	-
14	GMR-36	0.0098	14.21	15.67	10.21	14.33
15	GMR-37	0.0211	15.21	14.34	-	10.22
16	GMR-41	0.0124	14.33	11.21	-	11.21
17	GMR-44	0.0166	8.21	8.90	7.13	14.99

[-] : *negative activity*

Higher total number of antifungal strains were obtained from methanol than ethyl acetate and chloroform extract. Futhermore, the highest antifungal activity was observed from GMR-22 strain of chloroform than ethyl acetate and methanol extract. In contrast were examined from ethyl acetate and methanol extract in all of the antifungal against fungi tested. However, the antifungal activity of chloroform extract was lower than of other strain. This suggested that the antifungal compound of the GMR-22 there are two compounds with different polarity properties, which are soluble in solvents both high polarity and low polarity.

A larger number of actinomycetes produced antifungi substances and greater activity was observed with antagonistic. While, the *in vitro* studies futher indicated that all of the strain isolated were shown antifungal against one or more of the fungal tested, indicate that cajuput to be a interest sources of bioactive antifungal Actinomycetes.

Phylogenetic of GMR-22

The alignment of the nucleotide sequences (900 bp) of GMR-22 strain was done through matching with the 16S rRNA gene sequence of type species of genus. The results exhibited similarity level 83.04% with *Streptomyces* strain type of genus of Actinomycetes from known genus.

For data analysis, the phylogenetic tree in Figure 2. was derived from the distances matrices using neighbour-joining method. It has been shown that representative strain of the related species indicated that strain GMR-22 should be in genus *Streptomyces* and form a separate lineage together with its two closest neighbour *Kitasatospora* and *Streptacidiphilus.*

In conclusion, the molecular data shown that strain GMR-22 has closest relation to Streptomyces genus.

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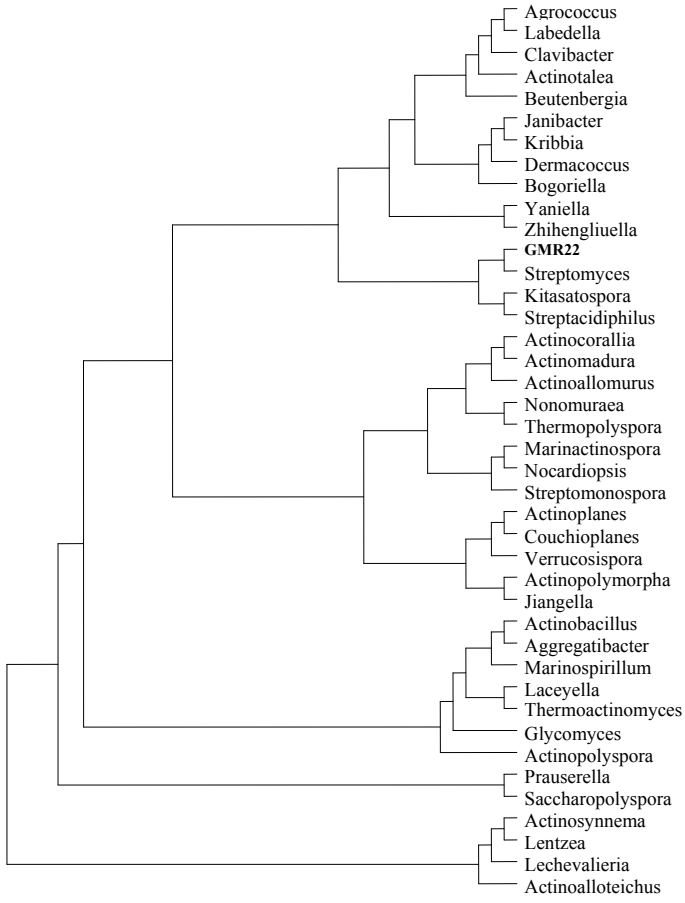

Figure 2. Neighbour-joining tree (Saitou & Nei 1987), based on 16S rRNA gene sequences, showing the phylogenetic relationship of strain GMR-22 with recognized member of the genus Actinomycetes.

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