Isolation and Molecular Characterization of Antifungal Production from Rice Fields Rhizosphere soil, Thailand

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Abstract

One hundred actinomycete strains were isolated from rice field soil Nong Bua, Nakonsawan, Thailand and twenty actinomycete strains were isolated from Happy Rice Organic Farm at Nong sua, Pathumthani, Thailand. A total of one hundred and twenty strains were tested for antifungal activities against rice blast disease (Pyricularia sp. CRI60007). The selected actinomycete strains were chosen from in vitro radial growth inhibition test against Pyricularia sp. CRI60007 that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50%. The results showed eleven strains gave %RGI more than 50% and strain NK05201 gave the highest %RGI (100 ± 0.00%). Analysis of 16S rRNA gene sequence and blast analyses confirmed that the selected actinomycete, ten strains belong to the genus Streptomyces and one strain belong to the genus Amycolatopsis with similarity to Streptomyces albidoflavus (NK01101 (99.56%), NK05201 (99.78%), NK05203 (99.79%), NK05205 (99.79%), NK07201 (99.79%), and NK08202(99.79%)), Streptomyces hydrogenans (NK08203 (100%)), Streptomyces jeddahensis (NK08205 (99.17%)), Streptomyces corchorusii (NK21201 (99.86%)), Streptomyces shenzhenensis (HPF3303 (99.86%)), and Amycolatopsis rhizosphaerae (HPF2102 (99.72%)).

Keywords: Actinomycete, Antifungal, Rice blast disease, 16s rRNA gene

Introduction

Rice and wheat are two main cereal crops that supply the majority of the global daily carbohydrate intake as energy source of human. Any pathogen targeting these two crops supposed to have serious connotations for the economy (John and Jacqueline, 2003). Thailand is one of rice producers to export rice product about 45 percent of rice yield. The main effect that reducing rice yield caused of the outbreaks of rice blast disease that can damaged to rice plant in all stages of growth (Saruda et al.,2017). Rice blast disease caused by the plant pathogenic fungi Magnaporthe grisea (Hebert) Barr. (anamorph, Pyricularia grisea Sacc.) is one of the most economically harmful crop diseases (Seochang and Katherine, 2004). The disease is widespread and distributed about 85 countries around the world where rice is grown. The disease appearance and asperity depending on year, place, environmental conditions and crop management practices. Yield loss appraisals from other areas of the world have ranged from 1% to 50% (Jeffrey, 2016).

Potential use of microbes-based biocontrol-agents as supplement or displacement for agrochemicals has been reported in several reports (Shimizu et al., 2000). Actinomycetes well-known as the source of several drugs such as antibiotics, antiviral, antitumor, antiparasitic agents, and immunosuppressants, microorganisms are responsible for the production of about 23,000 bioactive secondary metabolites (Feitosa et al., 2014; Subbanna et al., 2018). Some strains of actinomycete from rice were found to have antagonism against Magnaporthe grisea, Rhizoctonia solani, Xanthomonas oryzae pv. oryzae. and Fusarium moniliforme (Tian et al., 2004).

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The purpose of this present study to isolate actinomycetes from rhizosphere rice that can produce antifungal activity and searching for potential strains to control Pyricularia sp. CRI60007 that causes rice blast on rice leaves. Characterisation of the selected strains were done based on morphological, biochemical and physiological properties and studied of hydrolytic enzyme production against fungal cell wall. Identification analysis of 16S rRNA gene sequencing were performed. The results from this study can be used for pot experiment test in further study.

Material and methods

Collection of soil sample

Soil samples were collected from Happy Rice Organic Farm at Nong sua, Pathumthani, Thailand. Samples were randomised collecting at rhizosphere soil. These samples were kept in sterile polyethylene bags without sealed and transported immediately to the laboratory (Ruttanasutja and Pathomaree, 2015).

Isolation of Actinomycetes

The soil samples were pre-treated by air-drying method for 3-5 days at room temperature. Ten grams of soil sample was accurately weighed and mashed then transferred to 90 milliliters of 0.1% Tween80, mixed well (10-1). One milliliter of resultant solution was serially diluted with 9 milliliters of 0.85% Sodium Chloride (NaCl) up to 10-5. 200 µl of each intermediate dilution was spread on Modified Zhang's Soil Starch Extract (ZSSE) agar medium that supplemented with nystatin (50 micrograms /milliliter). The plates were incubated for the growth of actinomycetes colonies at 30 ± 2 °C and observed growth of actinomycete intermittently during incubation. After 7 days incubation, colony was picked by micro-needle then cross streak on International Streptomyces Project 2 (ISP2) medium. The pure colonies of actinomycete isolates were selected and maintained on ISP2 medium at 30± 2 °C for 7–14 days. Stocks were preserved in 20% glycerol (w/v), and stored at -80 °C for long time preservation. (Waksman et al., 1961).

In vitro radial growth inhibition test

The antifungal activity was analysed using dual culture method. Potato Dextrose Agar (PDA) medium plates were used, inoculated with the actinomycete isolates by single streak far form edge of petri dish two centimeters and incubated at 30 ± 2 °C for 7 days then using cock borer size 5 millimeters plug the fungi (Pyricularia sp. CRI60007) and placed on opposite site of actinomycete far form edge of petri dish two centimeters and incubated at 28 ± 2 °C. The inhibition zone was measured after the fungal mycelia in the control plates reached the edges of the plates. The antagonism against fungi was recorded by radial growth inhibition percentage was obtained using the formula: %RGI = 100 x (R1-R2)/R1 where R1 was the furthest radial growth distance of the fungus (control) and R2 was the radial growth distance of the fungus in dual culture with the actinomycetes (Mahadtanapuk et al., 2007).

Morphological biochemical and physiological characterization of the selected isolate

The morphological characteristics were observed using light microscopy with long working distance lens (40X) by using 14 days cultures grown at 30 ± 2 °C on ISP2 medium. The colour of aerial mycelium, substrate mycelium and soluble pigment were examined by using ISCC–NBS colour system (Kelly, 1964). Growth at different temperature was test at 4, 10, 20, 28, 30, 37, 40, 50 °C on ISP2 agar plates and visible growth was determined. Growth at different pH range 4 to 12 in ISP2 broth medium was recorded after 14 days, and growth in presence of NaCl (%) concentration 0 to 10 were studied on ISP2 agar plates. All phenotypic properties were recorded after incubation for 14 days at 30 ± 2 °C. Biochemical properties were tested using the several standard methods. Carbon source utilisation was performed by using ISP 9 medium containing different sugar types (Arai, 1975; Shirling and Gottlieb, 1966; Williams and Cross, 1971). Production of melanin pigment was determined by using ISP 6 (Pridham and Lyons, 1969). Hydrolysis of tyrosine, xanthine, hypoxanthine, adenine, and cellulose was recorded positive result by clear zone formation around the colony. Starch hydrolysis was observed as described by Cowan (1974). Urease test was determined by the indicator change phenol red changing pH yellow to pink (Gordon et al., 1974).

DNA isolation, PCR amplification and 16s rRNA gene sequencing

DNA amplification, sequencing and phylogenetic analysis of selected actinomycete strains were identified by using 16S rRNA gene. The selected strains were grown on ISP2 agar medium for 4 days at 30 °C. The colony was picked up by a sterilized toothpick and resuspended in 40 µl of TE buffer pH 8.0 as DNA template. The 16S rRNA gene was amplified and sequenced by using primers 9F (5′ GAGTTTGATCITGCTCAG3′) and 1541R (5′AAGGAGGTGATCCAGCC3′). The temperature for PCR amplification and sequencing reaction followed the method of Yukphan et al., (2005). Each PCR reaction of 50 microliters in total included 25 microliters AccuPower® Taq PCR Master Mix (Bioneer), 18 microliters dH2O, 2.5 microliters the final concentration 10 picomole of each primer and 2 microliters DNA template. The cycling conditions for the amplification of the 16s rRNA gene region were as follow: 3 minutes at 94 °C, 25 cycles at 94 °C for 1 minute, at 50 °C for 1 minute and 2 minutes at 72 °C, then followed by a final extension step for 3 minutes at 72 °C. The sequences of 16s rRNA gene was aligned with the program BioEdit Sequence Alignment Editor (version 7.0.0. Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The Maximum Likelihood method based on the Kimura 2-parameter model of Kumar et al., (2016) was used to construct a phylogenetic tree with the program MEGA7.

In vitro screening of the selected actinomycete isolates for hydrolytic enzyme production against fungal cell wall

Detection of Protease

Protease detection was perform using skim milk agar plate(peptonisation), skim milk broth (coagulation) was determined by following the methods described by Atlas (1997) and bouillon gelatin broth (gelatinisation).

Detection of Lipase

Lipase detection was observed by the production of clear zone in agar plates using Tween80 by following the methods described by Schoofs et al., (1997) and using phenol red by following the methods described by Singh et al., (2006).

Detection of Chitinase

Chitinase detection was detected by following the methods of Nawani et al., (2002). The positive result was determined by formation of clear zone around colony on chitin agar plates.

In vitro screening of the selected actinomycete isolates for plant growth-promoting (PGP) activities

Siderophore production

Siderophore production test of the selected actinomycete strains were screened on Chrom azurol S (CAS) agar medium. The selected actinomycete isolates were spot inoculated on the medium and incubated at 30 ± 2 °C for 7-14 days. A positive CAS reaction was observed by changing to be yellow color around the spot growth. (Alexander and Zuberer, 1991).

Phosphate solubilisation

Solubilization of Phosphate test was used Pikovskaya's agar plates. Plates were divided in four equal sectors and the selected actinomycete isolates were spot on the agar plates and incubated at 30 ± 2 °C for 3-14 days. The present of clear zone around the spot growth indicated phosphate solubilisation activity (Gaur, 1990).

Hydrogen cyanide (HCN) production

Production of hydrogen cyanide, the selected isolates were screened by adapting the method of Lock (1948). Using ISP2 agar medium supplemented with 0.44% glycine. Actinomycetes were streaked on these modified agar plate. Whatman filter paper No.1 dipped in 2% sodium carbonate in 0.5% Picric acid solution placed in the top of the plate. Plates were sealed with parafilm and incubated at 30 ± 2 °C for 3-14 days. The orange to red color of filter paper was indicated a positive test for HCN production.

Ammonia production

The selected actinomycete isolates were tested for ammonia production in peptone water medium. The selected actinomycete isolates were inoculated into 10 milliliters of peptone water and incubated at 30 ± 2 °C for 7-14 days. After that Nessler 's reagent (0.5 milliliter) was applied to each tube. The results showed brown to yellow color was determined to be a positive test for ammonia production (Cappucino and Sherman, 1992).

Results and discussion

Isolation of Actinomycetes

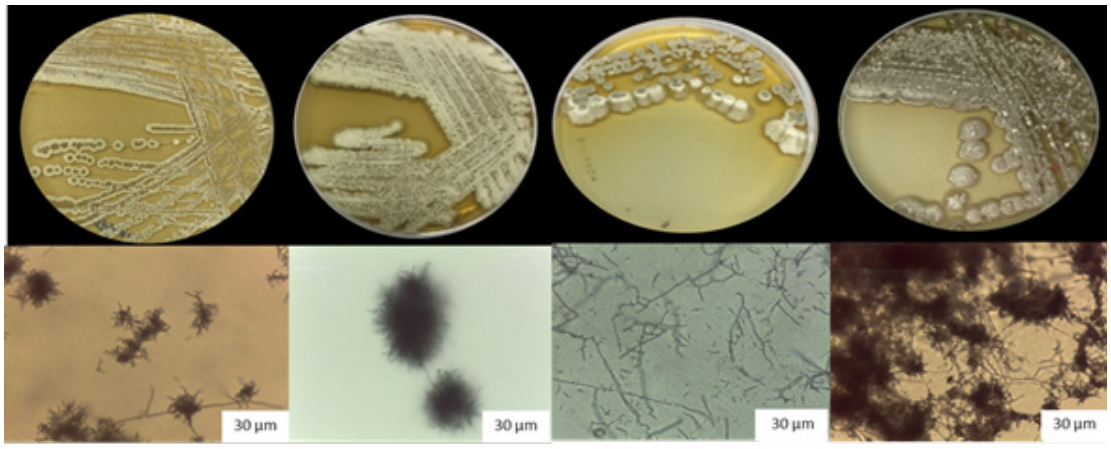
A total of 120 actinomycete isolates were isolated from rice field soil Nong Bua, Nakonsawan, Thailand (Thitiya and Khanungkan., 2019) 100 isolates and 20 isolates from Happy Rice Organic Farm at Nong sua, Pathumthani, Thailand.

In vitro radial growth inhibition test

Screening for antifungal activity of 120 isolates were tested against Pyricularia sp. CRI60007. The results of antifungal activity showed that 11 actinomycete strains were able to inhibit growth of Pyricularia sp. CRI60007 more than 50% including of NK01101 (66.44 ± 0.59%), NK05201(100 ± 0.00%), NK05203 (85.88 ± 0.61%), NK05205 (71.30 ± 0.68%), NK07201 (78.24 ± 0.73%), NK08202 (61.11 ± 0.41%), NK08203 (90.51 ± 0.50%), NK08205 (77.78 ± 0.05%), NK21201 (84.95 ± 0.14%), HPF2102 (99.53 ± 0.03%), and HPF3303 (51.39 ± 0.31%) as shown in Figure 2.

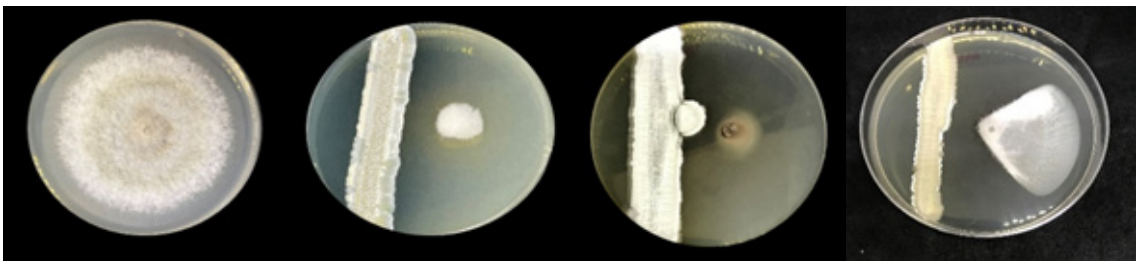
Morphological biochemical and physiological characterization of the selected actinomycetes strains

The selected actinomycete strains were grouped by using ISCC–NBS colour system (Kelly, 1964) of aerial mycelium into beige yellow (NK01101, NK05201, NK05203, NK05205, NK07201, NK08202, and NK08203), light gray (NK21201 and HPF3303), white (HPF2102) and NK08205 strain was not produced aerial mycelium on ISP 2 agar medium. All strains were able to utilise galactose, glucose, mannose and arabinose while strain NK08205 and HPF3303 were able to utilise sucrose. Strain NK01101, NK05201, and NK05203 were unable to utilise raffinose and xylose while NK05205, and NK07201 were unable to utilise raffinose, and strain NK21201, and HPF2102 were unable to utilise mannitol as carbon source. The ability to use differential types of carbon source might be indicate that actinomycetes were able to survive in the different environment (Riddhi et al., 2017). Most of selected actinomyces were able to grow at temperature 20 to 40 °C (Table 1). All of them could not grow at temperature 4 °C. The optimum temperature and pH were found to be 30 °C and pH7, respectively.



(a) (b) (c) (d)

Figure 1. Morphological appearance colonies on ISP2 agar medium and spore arrangement of some selected actinomycete strains NK05201 (a), NK08203 (b), NK21201 (c), and HPF3303 (d).



(a) (b) (c) (d)

Figure 2. Antifungal activity evaluation of some actinomycete strains (a) control, (b) NK07201, (c) (NK21201), and (d) HPF 3303 against Pyricularia sp. CRI60007.

Table 1 Morphological biochemical and physiological characterization of the selected actinomycetes strains.

Characteristics	Actinomycete strains										
	1	2	3	4	5	6	7	8	9	10	11
Colour of											
Aerial mycelium	Beige yellow	Beige yellow	Beige yellow	Beige yellow	Beige yellow	Beige yellow	Beige yellow	-	Light gray	White	Light gray
Substrate mycelium	Yellow-ish-brown	Yellow-ish-brown	Yellow-ish-brown	Yellow-ish-brown	Yellow-ish-brown	Yellow-ish-brown	Yellow-ish-brown	Olive brown	Brilliant orange	Moderate olive-green	Brilliant orange
Melanoid pigment	-	-	-	-	-	-	-	Black	-	-	-
Temperature (°C)	20-40	20-40	20-40	20-40	20-40	20-40	20-40	28-50	20-50	28-37	28-37
pH	5-12	5-12	5-12	5-11	5-11	5-11	5-11	5-11	5-11	5-11	5-11
NaCl (%)	0-8	0-9	0-8	0-8	0-8	0-8	0-9	0-2	0-2	0	0
Carbon sources											
Galactose	+	+	+	+	+	+	+	+	w	w	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	w	w	+
Sucrose	-	-	-	-	-	-	-	+	-	-	+
Mannose	+	+	+	+	+	+	+	+	+	w	w
Raffinose	-	-	-	-	-	w	w	+	w	w	w
Xylose	-	-	-	w	w	w	w	+	+	w	+
Mannitol	+	+	+	+	+	+	+	+	-	-	+

Table 1 (Continue)

Characteristics	Actinomycete strains										
	1	2	3	4	5	6	7	8	9	10	11
Hydrolysis of											
Tyrosine	-	-	-	-	-	-	-	-	-	-	-
Xanthine	-	-	-	-	-	-	-	-	-	-	-
Hypoxanthine	+	+	+	+	+	+	+	+	+	+	+
Adenine	+	+	+	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	+	+	+	+	+	+	+	-
Urea	-	+	-	+	-	-	w	-	-	-	-

Strains.; 1. NK01101, 2. NK05201, 3. NK05203, 4. NK05205, 5. NK07201, 6. NK08202, 7. NK08203, 8. NK08205, 9. NK21201, 10. HPF2102, and 11. HPF3303.

The symbol, + means the positive reaction or appearance of growth while symbol – means the negative or absence of growth.

Molecular identification

The 16S rRNA gene sequences and Blast analyses confirmed that selected ten strains of actinomycete belong to the genus Streptomyces spp. with similarity of 99.56-100 % including of S. albidoflavus (NK01101, NK05201, NK05203, NK05205, NK07201, and NK08202), S. hydrogenans (NK08203), S. jeddahensis (NK08205), S. corchorusii (NK21201), S. shenzhenensis and (HPF3303) with similarity percentage of 99.56, 99.78 99.79, 99.79, 99.79, 99.79, 100, 99.17, 99.86 and 99.86, respectively. One strain belongs to the genus Amycolatopsis spp. As A. rhizosphaerae (HPF2102) with similarity 99.86 %. These sequences from selected actinomyces strains have been deposited in the GenBank with accession number as showed in Table 2 and the maximum likelihood phylogenetic tree construction showed in Figure 2.

Several strains of the selected actinomycetes have been reported their antifungal activities such as strain NK01101, NK05201, NK05203, NK05205, NK07201, and NK08202 tend to be Streptomyces albidoflavus. Ahmed et al., (2018) reported that Streptomyces albidoflavus AS25 produced antifungal against. Strain NK 08203 tend to be Streptomyces hydrogenans. Rajesh and Talwinder, (2016) emphasized Streptomyces hydrogenans DH16 and its culture metabolites can be developed as biofungicides. Strain NK21201 tend to be Streptomyces corchorusii. Tamreihao et al., 2016 reported that Streptomyces corchorusii strain UCR3-16 showed antifungal activities against rice fungal pathogens.

Table 2 Molecular identification of the selected actinomycetes strains determined by 16s rRNA gene sequencing.

Strains	Source of isolation	Identification as	Similarity (%)	Accession
NK01101	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.56	LC516418
NK05201	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.78	LC516413
NK05203	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.79	LC516409
NK05205	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.79	LC516410
NK07201	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.79	LC516411
NK08202	Rice field soil, Nakhonsawan, Thailand.	Streptomyces albidoflavus	99.79	LC488882
NK08203	Rice field soil, Nakhonsawan, Thailand.	Streptomyces hydrogenans	100	LC516412
NK08205	Rice field soil, Nakhonsawan, Thailand.	Streptomyces jeddahensis	99.17	LC477341
NK21201	Rice field soil, Nakhonsawan, Thailand.	Streptomyces corchorusii	99.86	LC516414
HPF2102	Rice rhizosphere soil, Pathumthani, Thialand.	Amycolatopsis rhizosphaerae	99.86	LC516416
HPF3303	Rice rhizosphere soil, Pathumthani, Thialand.	Streptomyces shenzhenensis	99.72	LC516417

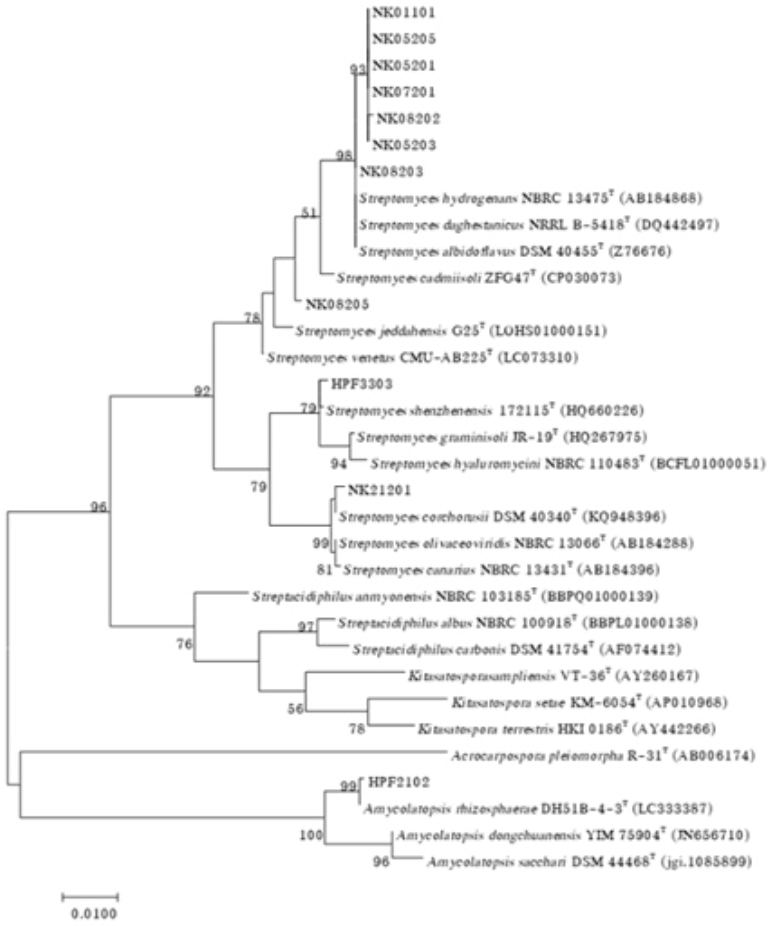


Figure 2. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of selected actinomycetes 11 strains and their closely related type strains.

In vitro screening of the selected actinomycete strains for hydrolytic enzyme production against fungal cell wall

The possible mechanisms of antifungal activity from actinomycetes to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes including of protease, lipase, chitinase, and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls (Prapagdee et al., 2008). The result of hydrolytic enzyme production against fungal cell wall showed all most of the selected actinomycete strains could be produced enzyme protease and lipase (Table 3).

In vitro screening of the selected actinomycete strains for plant growth promoting (PGP) activities

Despite their preliminary track record as BCAs and plant growth promoting (PGP) activities actinomycetes. have been scarcely reported in the literature. Some reports exist for their ability to solubilize phosphate, and production of indole acetic acid (IAA), siderophores, 1-amino cyclopropane-1-carboxylic acid (ACC) deaminase (Sadeghi et al., 2012; Passari et al., 2015; Qin et al.,2015). The result of plant growth promoting (PGP) activities showed strain NK05205, NK07201, and NK08205 could be produced siderophore while strain NK05201, NK05203, NK05205, NK07201, NK08202, and NK08203 were determined to be a positive test for ammonia production. Strain HPF3303 showed positive result of hydrogen cyanide production. Strain NK21201 and HPF2102 showed positive result of phosphate solubilisation (Table 4).

Table 3 In vitro screening of the selected actinomycete strains for hydrolytic enzyme production against fungal cell wall.

Characteristics	Actinomycete strains										
	1	2	3	4	5	6	7	8	9	10	11
Protease Production											
Peptonization	+	+	+	+	+	+	+	-	w	-	-
Coagulation	+	-	-	+	+	+	+	-	w	-	-
Gelatinization	-	+	-	-	+	-	w	-	-	-	-
Lipase production											
Tween80	+	+	+	+	+	+	+	-	+	+	+
Phenol red	-	-	-	-	-	+	-	-	+	-	+
Chitinase	-	-	-	-	-	-	-	-	-	-	-

Strains.; 1. NK01101, 2. NK05201, 3. NK05203, 4. NK05205, 5. NK07201, 6. NK08202, 7. NK08203, 8. NK08205, 9. NK21201, 10. HPF2102, and 11. HPF3303. The symbol, + means the positive reaction while symbol, – means the negative reaction and w, weak.

Table 4 In vitro screening of the selected actinomycete strains for plant growth-promoting (PGP) activities

Characteristics	Actinomycete strains										
	1	2	3	4	5	6	7	8	9	10	11
Production of											
Siderophore	-	-	-	+	+	-	-	+	-	-	-
Ammonia	-	+	+	+	+	+	+	-	-	-	-
HCN	-	-	-	-	-	-	-	-	-	-	+
Phosphate solubilisation	-	-	-	-	-	-	-	-	+	+	-

Strains.; 1. NK01101, 2. NK05201, 3. NK05203, 4. NK05205, 5. NK07201, 6. NK08202, 7. NK08203, 8. NK08205, 9. NK21201, 10. HPF2102, and 11. HPF3303.

The symbol, + means the positive reaction while symbol, – means the negative reaction and w, weak.

Conclusion

In this study the selected actinomycetes strains were chosen from in vitro radial growth inhibition test against Pyricularia sp. CRI60007 that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50%. The results showed eleven strains gave %RGI more than 50%. Strain NK05201 gave highest %RGI (100 ± 0.00%) and followed by strain HPF2102 (99.53 ± 0.03%), NK08203 (90.51 ± 0.50%), NK05203 (85.88 ± 0.61%), NK21201 (84.95 ± 0.14%), NK07201 (78.24 ± 0.73%), NK08205 (77.78 ± 0.05%), NK05205 (71.30 ± 0.68%), NK01101 (66.44 ± 0.59%), NK08202 (61.11 ± 0.41%), and HPF3303 (51.39 ± 0.31%) respectively. Molecular identification of the selected actinomycetes strains belong to the genus Streptomyces spp. with similarity of 99.56-100 % and one strain belong to the genus Amycolatopsis spp. with similarity 99.86%. The results from this study can be used for further study.

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