ISOLATION OF ACTINOMYCETES FOR PRODUCT FORM DEVELOPMENT TO CONTROL RICE BLAST DISEASE AND THE IDENTIFICATION OF A POTENTIAL STRAIN

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DEPARTMENT OF BIOLOGY SCHOOL OF SCIENCE
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Title	Isolation of Actinomycetes for Product Form Development to Control Rice Blast Disease and the Identification of A Potential Strain
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Abstract

One hundred strains of actinomycetes were isolated from rice paddy field soils at Nong Bua, Nakonsawan, and "Happy Rice Organic Farm" at Nong sua, Pathumthani. All isolates were preliminary screened for antimicrobial properties and antifungal activities against *Pyricularia* sp. CRI60007 that caused rice blast disease. Sixteen isolates that showed the inhibitory against the tested microorganisms and rice blast disease were secondary screened by agar disc diffusion method. Sixteen isolates with antimicrobial and antifungal ability on rice blast disease were analysed for 16S rRNA genes sequences. Strain NK08203 was selected to be a potential strain. This strain showed 90.51% against *Pyricularia* sp. CRI60007, presented ability to grow in sodium chloride 0-8%, temperature at 28-40 °C, pH 5-11, tolerance to agriculture chemical products, and produced 4.44 $x10^{13}$ spore/mL in submerged culture at 96 hrs. Identification of 16S rRNA gene tend to be *Streptomyces hydrojenans* with 100% similarity. Whole genome sequencing was used to analysed secondary metabolite gene cluster of strain NK08203 that showed revealed antifungal gene expression. Development biocontrol agent of strain NK08203 was performed by microencapsulation techniques. The results of yield production presented 20.43 % and cell survival as 4.73 x 10^9 CFU/mL.

Keywords: Actinomycete, antimicrobial activity, biocontrol, rice blast diesease

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(Miss. Thitiya Rattanakavil)

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Chapter 1
Introduction

1.1 Research Motivation

Rice (*Oryza sativa*) is a majority cereal crop, and rice is the main food of people around the world, specifically in Asia. Nevertheless, rice is infeceted by pathogenic fungi that causing of crop yield drecrease or losses (Chaiharn *et al.,* 2009 ; Asibi *et al*., 2019). The rice blast disease causing *Pyricularia* spp. is one of the most damaging airborne pathogens (Kato, 2001). Rice plants can be infected by this fungal at any growth stage (Asibi *et al*., 2019). The yield of rice could be loosed 10 to 30% in various rice-producing countries and can raise by up to 50% during blast disease pandemic (Asibi *et al*., 2019 ; Wilson and Talbot, 2009 ; Ashkani *et al*., 2015). Many synthetic chemicals were used against blast disease for instance carbendazim, carpropamid, tricyclazole, and pyroquilon (Kato, 2001 ; Pooja and Katoch, 2014). However, the synthetic chemicals that used in agriculture to control pests and disease were produced pollution in environmental, reduced soil quality, damaged ecosystems, and developmented of pesticide resistance (Pooja and Katoch, 2014 ; Law *et al*., 2017). To control rice blast disease by using fungicide could be effected to the environment and soil microorganism (Yoon *et al*., 2013) and human risk to pesticides that could be harmful to human health.

Biological control can be one of alternative way that is an environment friendly approach. A definition of biological control is biological agent that used for managed pests or disease in environment permanently or over the long term (Kenis *et al*., 2017). Biological agent applied can be a parasitoid, a pathogen or a predator of enemy organism that is causing yield of production losses (Hajek *et al*., 2007).

Many strains of actinomycetes are considered to be biocontrol agents because of their abilities in antifungal and antimicrobial production as well as plant growth-promoting (PGP). It also efficiently colonises the rhizosphere of different plant species including rice (Patel *et al*., 2018). Many *Streptomyces* strains had been studied in several laboratories to control bacterial and fungal diseases of rice such as rice blast disease fungus caused by *Pyricularia* sp. (Hastuti *et al*., 2012 ; Law *et al*., 2017 ; Chaiharn *et al*., 2020), but there is poorly found to improve the actinomycetes as

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biocontrol products for agicultures (Suárez-Moreno *et al*., 2020). Shelf-stable of biocontrol development is one of the major bottle necks for commercial biocontrol products (Droby *et al*., 2009 ; Navatar *et al*., 2011). Biological control product should be contain enough colony forming units (CFU) to inhibit phytopathogens, low cost to produce, and provide a long shelf-life (Melin *et al*. 2007 ; Teixido´*et al*., 2011). Eventually, biological products should be showed the ability to control phytopathogenic plant like fresh cells. Biological formulations could be accomplished by various appoarchs including liquid or dry preparations (Gotor *et al*., 2017). Microencapsulation technique could be protected sensitive substances or microorganism from the external environment (Ghosh, 2006) by the addition of certain carriers to the formulation medium for providng a dry particle and also to maintain and against cell damage during the drying process, this technique will be used in this research (Abadias *et al*., 2001 ; Sabuquillo *et al*., 2010 ; Melin *et al*., 2011).

1.2 Objectives of the study

1) Isolation and screening of antimicrobial activity and searching for potential strains to control rice blast disease of actinomycete from rice rhizospheric soil and rice field soil.

2) Identification of potential strains based on phenotypic and genotypic characterisation.

3) To develop spore formation of the potential strain in submerged cultivation.

4) To study of biocontrol against properties of the potential strain include plant growth promoting and hydrolytic enzyme to fungal cell wall.

5) Immobilize the potential strain with encapsulated formulation by using spray-drying technique.

1.3 Scopes of the study

1) Isolation actinomycetes from rice rhizosphere soil that can produce antimicrobial and potential strains to control rice blast disease on rice leaves.

2) Preliminary screening of antimicrobial activity to test microorganisms by “T-streak” method and antifungal activity by dual culture method.

3) Screening for secondary metabolite by crude extracts preparation by agar disc diffusion to test microorganisms.

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4) In *vitro* screening hydrolytic enzyme production of the potential actinomycete strains that can produce strong antifungal activity to *Pyricularia* sp. CRI60007.

5) Characterisation of the potential strains based on morphological, biochemical, physiological properties and identification analysis of 16S rRNA gene sequencing.

6) Improving spore formation of the potential strain in submerged cultivation conducted by comparing the effect of two differences medium on spore formation including of International *Streptomyces* Project No.2 (ISP2) and modified ISP2.

7) To develop spore formation of the potential strain in submerged cultivation.

8) Immobilize the potential strain with encapsulation technique by using spray-drying technique.

1.4 Benefit of this study

1) Actinomycetes can produce antimicrobial activity to microorganism test and showed good inhibition to rice blast disease fungus caused by *Pyricularia* sp.

2) The potential strain will be identified based on phenotypic and genotypic characterisation.

3) The potential strain can be produced spore in submerged cultivation.

4) The potential strain had a great potential to be used as biocontrol against.

5) Encapsulated formulation of the potential strain is performed by using spray-drying technique.

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Chapter 2

Theory and literature reviews

2.1 Actinomycetes

2.1.1 The general classification of actinomycetes

Actinomycetes are gram-positive filamentous bacteria with high G+C content approximately 57-75% (Lo *et al*., 2002). Actinobacteria displays the differential morphology from gram-positive bacteria by performed aerial mycelium, substrate mycelium, absent of nucleus and mitochondria. Actinomycetes are classified into class *Actinobacteria*, subclass *Actinobacteridae*, and order *Actinomycetales*. The cell wall of actinomycetes compose of mucorpeptide (*N*-acetyl glucosamine links with *N*-acetyl muramicacid), 2,6-diaminopimeric acid, glutamic acid, glycine, and alanine (Witt and Stackebrandt, 1990). *Actinobacteria* represent one of the largest taxonomic units in Domain Bacteria among the 18 major lineages currently recognised (Ventura *et al*., 2007). Actinomycete shows differential morphology among gram-positive bacteria. However, actinobacteria displays typical prokaryote and produce mycelia like fungi but totally different with fungi. The differential of morphology and function, mycelium of actinomycete could be divided into aerial and substrate mycelium. Some actinomycete could be performed complex structures for instance type of spore, spore chain (short or long chains), sporangia, or sporangiospore. The position, number, and surfaces structure of spore, the shape of sporangia, and present or absent flagella on sporangiospore. All above data are important to classify morphological characteristics of actinomycetes. *Actinobacteria* are widely distributed environments such as water, sea water, hot spring, rhizosphere, endophytic in plants, desert, and mainly in soil. Actinomycetes produced a variety of secondary metabolites or bioactive compounds for instance antibiotics, degraded enzyme, anti-parasitic, plant growth promoting, herbicides, and pesticides. Especially, *Streptomyces* is a majority genus for antibiotics production in the microbial world. More than 80 % of all antibiotics produce by *Streptomyces* genus (Goodfellow and Williams 1983 ; McCarthy and Williams 1992 ; Stach and Bull, 2005 ; Girish and Lakshmi, 2012). Actinomycetes could performed various volatile matters such as geosmin of the characteristic “wet earth odor” (Wilkins, 1996).

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2.1.2 Isolation of actinomycetes from soil

Isolation of actinomycetes from soil depending on the abundant of an environment around that soil sample, the composition of isolating medium, and preparing soil sample (Poter *et al*., 1960). Major composition of a medium is nitrogen sources and carbon sources. Actinomycetes could be grown in complex carbon source such as casein, chitin, dextran, D-glucose, D-mannose, and humic acid.

Preparing soil sample to isolate actinomycetes is one of a methodology to reduce the number of other bacteria and fungi. It could be induced spore in a dormant spore state. The preparing soil sample methodologies for instance using high temperature, using chemicals, or mixing antibiotic to the medium as shown in table 2.1.

Table 2.1 Preparing soil sample to isolate actinomycetes.

Preparing methodology	Reference
Mixing 50 mg/L of nystatin into the isolation medium.	Porter *et al*. (1960)
Centrifugation at 4500 rpm/1 min.	Porter *et al*. (1960)
Heating soil sample at 100 °C for 15 mins.	Athalye *et al*. (1960)
Drying soil sample at room temperature until the soil completely dry.	Xu *et al*. (1996)
Heating soil sample at 70 °C for 15 mins.	Seong *et al*. (2001)
Heating soil sample at 55 °C for 15 mins.	Sirivibool *et al*. (1960)
Dilution soil sample with 0.9% sodium chloride (NaCl).	Thakur *et al*. (1960)

2.2 Development of actinomycete classification

Actinomycetes are prokaryotic cells that characterized by using a simple cell structure. In the past, prokaryotic classification was clearly demonstrated that newly discovered techniques (Schleifer, 2009). The classification of prokaryotic was developed different stages.

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2.2.1 Phenotypic characteristics

Normally, the classical or traditional classification method were determined by using microbial morphological attributes, growth requisitions, physiological characterisation and biochemical characterisation. Morphological characteristics were determined by using colour of substrate mycelium, aerial mycelium, and types of spores. The colour of substrate mycelium, aerial mycelium and soluble pigments diffusion are compared by using the ISCC-NBS colour charts (Centore, 2016). Spores need to observe by using long working distance lens and scanning electron microscope (SEM).

2.2.1.1 Morphological characteristics of actinomycetes

2.2.1.1.1 Actinomycetes mycelium

- Substrate mycelium

The substrate or vegetative mycelium could be grown on the surface of the agar medium cultivation or into the agar culture medium (Figure 2.1 (2)). Some substrate mycelium could be produced solubilised water pigment or solubilised fat pigment (non-water-soluble). Water soluble pigments could be diffused into the medium cultivation and provide important information to determine a novel species (Buchanan, 1955).

- Aerial mycelium

Aerial mycelium is the hyphae that developing form the vegetative mycelium (Figure 2.1 (3)). Sometimes, actinomycetes are hard to distinguish by observing aerial and substrate mycelium. The easy approach to distinguish by using slide culture technique with a light microscope. The hyphae of the aerial mycelium are characterised by a fibrous sheath, excepted from *Pseudonocardia* genus and *Amycolata* genus (Warwick *et al*., 1994). Ultramicroscopic was used for observing a characteristic pattern of formed fibrillar components and short rodlets. The fibrous sheath was appeared in aerial hyphae sporulation, causing the various type of the spore surface (Wildermuth *et al*., 1994 ; Kalakoutski and Agre, 1976). The aerial hyphae of all actinobacteria were depended upon nutritional conditions (carbon source or nitrogen source), environmental factor (pH or temperature) or species characterisation.

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Some genus, aerial mycelium developed a certain stage in the top form spore chain, which is a reproductive hypha spore formation.

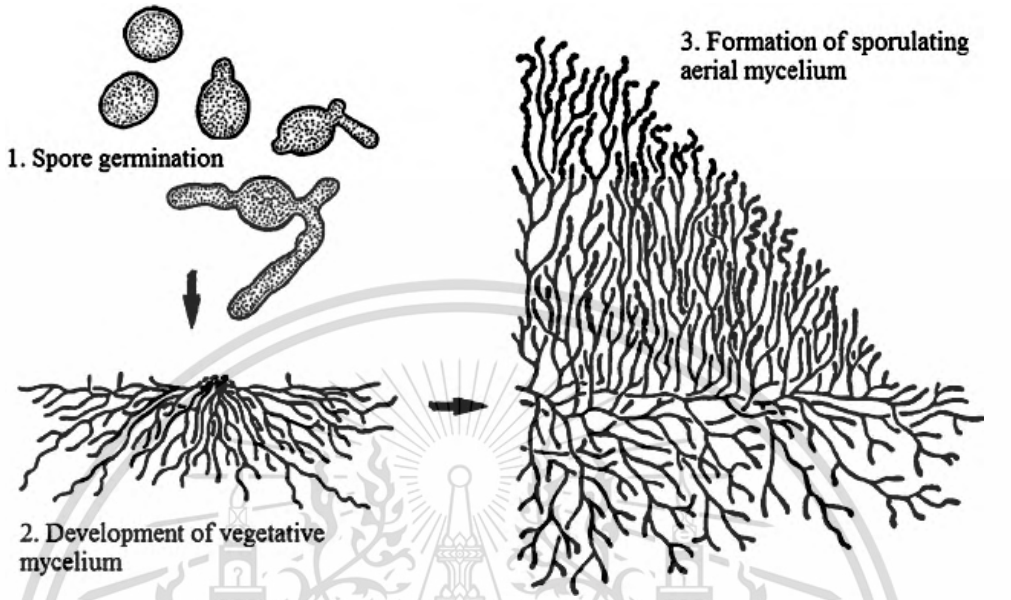

Figure 2.1 Actinomycetes mycelium ; (1) spore germination, (2) development of vegetative mycelium, (3) formation of sporulating aerial mycelium (Vobis, 1997).

2.2.1.1.2 Actinomycetes spore

At a certain stage of actinomycete growth, differentiated of aerial hyphae could be reproduced spore-bearing mycelium. Truly, spore-bearing mycelium was appeared in most genus of actinobacteria. According to the observation by Lechevalier and Lechevalier (1989), spore chains could be determined their length (spore chain) and amount of spore for instance two spores can be called di- or bisporous, a few spores call oligosporous, and poly-sporous with many spores as shown in Figure 2.2. The basic classification of actinomycete spore chain including of length, appearance, position, and colour.

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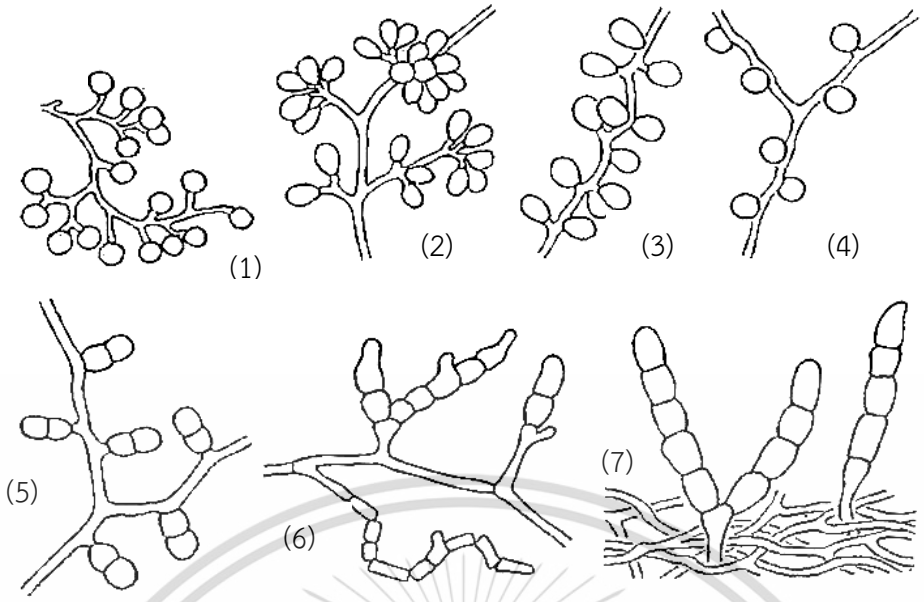

Figure 2.2 Single spore formation and short chain of spores in differences genus: (1) *Micromonospora*, (2) *Thermomonospora*, (3) *Saccharomonospora*, (4) *Thermoactinomyces*. Disporous: (5) *Microbispora* Oligosporous: (6) *Nocardia brevicatena*, (7) *Catellatospora* (Qinyuan *et al*., 2016).

Monosporous or single spore formulation presents in genera *Micromonospora*, *Thermomonospora*, *Saccharomonospora*, and *Thermoactinomyces*. Almost of them were developed single spore form at the end of a hyphal branch as shown in Figure 2.2 (1-4).

Disporous chain contains a longitudinal pair of spores. The species of *Microbispora* genera were represented this type of spore formulation as shown in Figure 2.2 (5).

Oligosporous chain forms short chain of spores. Normally, oligosporous present 7 to 20 spores/chain and the lowest is three spores. The chains can be presented open loop, hooked, straight, or arranged in irregular spirals one. The spore chains in *Nocardia brevicatena* genera were performed branched or short chains of 2 to 7 spores as shown in Figure 2.2 (6).

Polysporous also found in *Streptomyces* genus which produce more than 50 spores in long chains. Cross (1970) described the arthrosporous is spores of

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Streptomyces genus and other polysporous in actinomycetes genera. Spore chains of *Streptomyces* can be described the differentiated of spore chains into the following main types (Figure 2.3):

(1) Rectiflexibiles or flexuous type, straight (rectus) or flexuous (flexibilis) spore chains, partly in clusters as shown in Figure 2.3 (1).

(2) Retinaculiaperti or retinaculum type, spore chains with hooks, open loops or short, irregular spirals having 1 to 4 turns as shown in Figure 2.3 (2).

(3) Spira or spiral type, spiral spore chains divided into two subtypes: Closed spiral may be compact or open, extended, and simple spiral (stretched spirals) as shown in Figure 2.3 (3).

(4) Verticillati or verticillate type, main axis of true verticillate presented wider diameter than branching hypha and verticillate could be divided into four subtypes: monoverticillate (no spirals), monoverticillate (elements of verticils spiraled), biverticillate (no spirals), biverticillate (secondary verticils spiraled) as Figure 2.3 (4). Moreover, *Nocardiopsis* genera could be performed spore in long chains with zigzag shape, has well-developed aerial hyphae, which may either be straight-flexuous or zigzag shaped, straight – flexuous chains or fragmented spores in several lengths (Meyer, 1989) as shown in Figure 2.3 (5).

An important basis for classification of actinomycetes is length, type, or shape of spore and colour.

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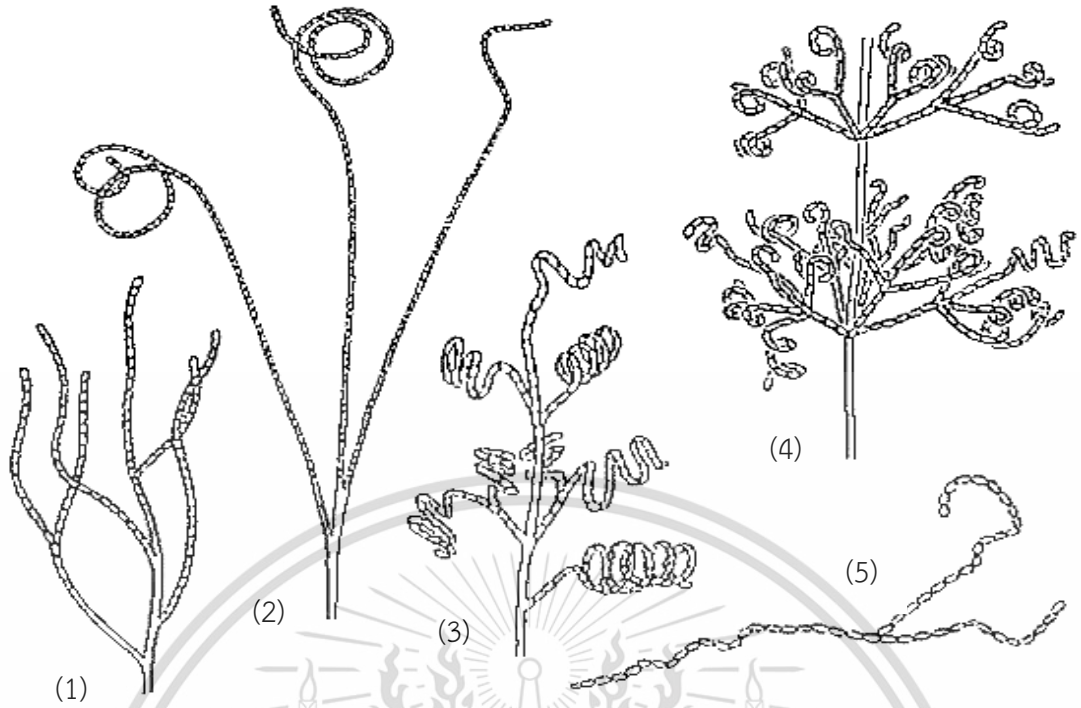

Figure 2.3 Long chain sporulation of actinomycetes. *Streptomyces* ; (1) Rectiflexibiles type, (2) Retinaculum type, (3) Spiral type, and (4) or Verticillate type. *Nocardiopsis*: (5) fragmenting branched aerial hyphae (Qinyuan *et al*., 2016).

2.2.1.2 Cultural characteristics of actinobacteria

The cultural characterisatics are refer to the growth properties (such as source of isolation or actinomycete habits) and morphological characterisation in differential culture media according to the method that used in the International *Streptomyces* Project (ISP) (Shirling and Gottlied, 1966).

2.2.1.2.1 Actinomycetes isolation culture medium

The selective isolation medium should be promoting the growth of actinomycetes and inhibit another microorganism. The culture should be selected based on actinomycete target genus, habitat of actinomycetes, medium composition, and inhibitors (Li *et al*., 2016). For instance, to isolate alkalophilic the pH of the media should be adjusted pH 10 to pH 12 or to isolate halophilic actinomycetes, the isolation culture medium should be contained salt

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concentration 15% to 25%. Typically, isolation culture mediums were found to be soil extracts medium, starch-casein medium, and humic vitamin (HV) medium supplemented with chemicals or antibiotic for selective specific genus as presented in Table 2.2 (Hayakawa and Nonomura, 1987 ; Connon and Giovannoni, 2002).

Table 2.2 Chemicals or antibiotics used for the selective isolation of different genus actinomycetes (Jiang *et al*., 2016).

Chemicals	Target genera
Breuneomycin	*Actinomodura*
Benzoate	*Micromonospora*
Fradiomycin	*Actinokineospora*
Gentamycin	*Actinomodura* and *Streptosporangium*
Kanamycin	*Actinokineospora*, *Thermomonospora,* and *Microtetraspora*
Strptomycin	*Actinomodura* and *Glycomyces*
Neomycin sulfate	*Amycolatopsis*
Polymyxin	*Streptomyces*
Nalidixic acid	*Actinokineospora*

2.2.1.2.2 International *Streptomyces* Project (ISP) medium

The standard culture medium of ISP media for morphological characterisation will determine on ISP2 (yeast extract-malt extract medium), ISP3 (oatmeal agar medium), ISP4 (inorganic salts-starch agar medium), and ISP5 (glycerol-asparagine agar medium). Physiological characterization by using ISP6 (peptone iron agar) for observe melanoid pigment and ISP7 (tyrosine agar) for observe other organic medium (peptones). Carbon source utilisation used ISP9 (Pridham and Gottlieb carbon source utilisation medium) as basal medium.

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2.2.1.2.3 Spore formulation of actinomycetes in submerged cultivation

Most of actinomycetes do not sporulate in submerged cultivation but industrial secondary metabolite production is mostly produced in flasks and bioreactors (submerged culture). In solid medium, development starts from spore germination and performed substrate mycelium into the medium (early MI). Programmed cell death (PCD) appears (red cellular segments in Figure 2.4) after hyphae develop into the culture which induces the variation of the multinucleated (MII) antibiotic-producing hyphae (late substrate mycelium, early MII). Then, the aerial mycelium begins to grow (late MII) and spore formation at the end of the process.

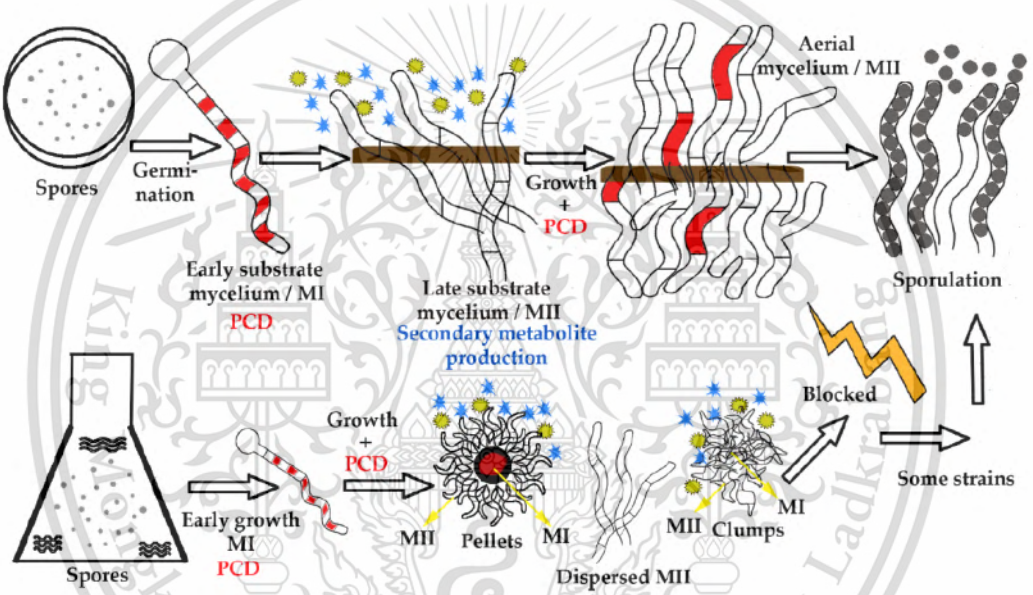

Figure 2.4 Spore formulation of actinomycetes in submerged cultivation (Manteca and Yagüe, 2018) : mycelium stage (MI), programmed cell death (PCD), multinucleated (MII).

In submerged cultivation, development starts from mycelium stage or MI with programmed cell death (PCD). After that, the mycelium transformed to be pellets and clumps (Manteca *et al.,* 2008) . Formation of pellets and clumps with secondary metabolite production are incomprehensible. Some authors have reported that pellets and clumps are basal on antibiotic production such as nikkomycins in *Streptomyces tendae* (Vecht *et al*., 1992) and hybrid antibiotics in *Streptomyces lividans* (Sarra *et al*., 1997). While some authors have reported that formation of pellets and clumps are

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reduced secondary metabolite production such as nystatin in *Streptomyces noursei* (Jonsbu *et al*., 2002) and tylosin in *Streptomyces fradiae* (Park *et al*., 1997). However, some strain of actinomycetes could be performed spore in submerged cultivation with nutrient induction. Daza and collaboration (1989) affirmed that sporulation of several species of *Streptomyces* in liquid culture by using complex R2YE medium with high concentration of Ca^{2+} (20 mM) and using sonication to release spores from mycelium.

2.2.1.3 Physiological and biochemical characteristics

2.2.1.3.1 Carbon source utilisation

Carbon source utilisation is one of the basic classification of bacteria nutrient tests. Variation carbon source utilisation of actinomycetes cloud be classified in different species of actinomycetes (Geraldine and Klaus, 1979).

2.2.1.3.2 Nitrogen source utilisation

Nitrogen source utilisation is one of the basic classification of bacteria nutrient tests. Variation nitrogen source utilisation of actinomycetes cloud be classified different species of actinomycetes (Pelezar *et al*., 1993).

2.2.1.3.3 Determination of temperature on actinomycetes growth (Waksman and Henrici, 1943)

Microorganism could be classified by the optimal growth temperature. The optimal growth temperature for cloud be divided five classifications into hyperthermophile (60 ℃ and more than 60 ℃), thermophile (55 and 110 ℃), mesophile (25 and 40 ℃), psychrotrophs (mesophilic temperature is essential to grow but could be survive at 0 ℃), and psychrophiles (growing between lower -15 and 10 ℃)

2.2.1.3.4 Determination of sodium chloride concentration on actinomycetes growth (Bernard *et al*., 1994)

Halophile characterisation could divide into three groups based on their grow on culture medium that contained sodium chloride. Slight halophiles (optimally growth at 0.34 to 0.85 M or between 2 and 5% NaCl), moderate halophiles (optimally growth at 0.85 to 3.4 M or between 5 to 20% NaCl), and extreme halophiles (optimally growth at 3.4 to 5.1 M or 20 to 30% NaCl).

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2.2.1.3.5 Determination of pH on actinomycetes growth

Microorganism or actinomycetes could be grown in differential pH values. Actinomycetes that could be grown at a pH less than 5 called acidophile. The neutrophile was determined by optimising actinomycetes that could be grown at pH values 5.4-8.0 and alkaliphiles could be optimally grown at pH value more than 8.0 (Krap, 2010).

2.3 Genotypic characteristic of actinobacteria

The genotypic characterisation based on genetic relatedness of microorganism. The genetic of bacteria including of DNA and RNA. RNA could be separated in to 3 types of constituent mRNA, tRNA and rRNA. In the present, rRNA plays an importance role to identify microorganism base on like or unlike of sequencing. The sequencing analysis of DNA could solve the differential by comprising of base adenine (A), tyrosine (T), cysteine (C), and guanine (G) (Takamustsu *et al*., 1999). Sequencing analysis of DNA was used for identification and phylogenetic tree relatedness, and it could be indicated microorganism in genus, family, and order level. The other supported information is DNA-DNA hybridization (DDH) by comprising genome sequencing analysis of homologous base (Stackebrandt and Goebel, 1994 ; Goris *et al*., 2007). At present, the actinobacteria classification was comprised more and more genotypic approaches such as average nucleotide identity (ANI) (Rosselló, 2003 ; Kim *et al*., 2014), multilocus sequence analyses (MLSA) (Serrano *et al*., 2009), and whole genome analysis (Kim *et al*., 2014 ; Meier *et al*., 2014). Polyphasic approach describe by Vandamme and colleagues (1996) is the most widely accepted system. The approach combines various differential results such as phenotypic, chemotypic, and genotypic data.

2.3.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is used in genome sequencing based on DNA polymerization reaction and including the Human genome project. A piece of genome could be amplified by using the random primers, dNTPs, DNA template, and DNA polymerase (*Taq* polymerase) (Lawyer, 1989). The main component of polymerase chain reaction including of template, primers, DNA polymerase, dNTPs, and magnesium chloride concentration.

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2.3.1.1 Template

DNA or RNA template can be extracted in several available methods. A template strand was referred to the strand used by DNA polymerase during DNA replication or RNA polymerase during RNA transcription to attach complementary bases. Either molecule moves along 3' to 5' direction. Each subsequent base was complementary bind to the current DNA base in the 5' to 3' direction.

2.3.1.2 Primers

Typically, length of oligonucleotide primers is 15–25 nucleotides with 40 to 60% of GC and a Tm that is similar for each member of the pair. During sequencing, denatured DNA template was annealed with primers and formed initiation site for elongation of the new DNA molecule. Universal primers are complementary to nucleotide sequences. This is specific to conservative region to DNA molecules and cloning vectors. Primers can either be specific to a particular DNA nucleotide sequence "specific primer" or they can be "Universal" (Green and Sambrook, 2019).

2.3.1.3 DNA Polymerase

DNA polymerase enzymes were selected depended on experimentation. Some DNA polymerase enzymes were required hot start and inactive at low temperature to reduce nonspecific, low error rate, and specific fragments for amplification (Gelfand and White, 1990 ; Lubin *et al*., 1991).

2.3.1.4 dNTPs

dNTPs are including of dATP, dCTP, dGTP, and dTTP. However, the dNTPs are essential for long fragments amplification or highly abundant targets (Green and Sambrook, 2019).

2.3.1.5 Magnesium chloride concentration

Free magnesium ions play role as a co-factor for DNA polymerase activity. Varying concentration of magnesium chloride (1-8 mM) is essential for optimising in each experiment. Typically, magnesium chloride concentration is between 1.5- and 5.5-mM for each experiment.

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2.3.1.6 Polymerase Chain Reaction (PCR) Cycling Process (Wetmur, 1991)

A typical polymerase chain reaction (PCR) consists of five stages as following:

Initial denaturation stage: Complex or double-stranded DNA (dsDNA) molecules are degraded into single strands. Degrading temperature is required an incubation at 95 °C for 2-5 mins to hydrolyse double stand DNA.

Denaturation stage: The temperature is increased to 95 °C to hydrolyse all double stand DNA into single-stranded DNA (ssDNA) by disrupting hydrogen bonds between complementary bases.

Annealing stage: In this stage, primers were bind to DNA template and the temperature was set up lower (approximately 5 °C) than primers melting temperature (often 45–60 °C).

Extension stage: Hybidisation of primer to DNA template is required DNA polymerase enzyme activity to be extended. The temperature in this stage is increased to 72 °C.

Repeat: Seps 1 to 3 were repeated in a cyclical manner, resulting in exponential amplification of the amplicon.

2.3.1.7 Electrophoresis

After using polymerase chain reaction amplification, the PCR product was determined quality by using gel electrophoresis observation. Electrophoresis is one of laboratory technique that used for separating molecule of DNA, RNA, or protein by determining size and electrical charge. Molecules could be moved through a gel pore. Larger molecules could be moved slower than small molecules. Dyes such as ethidium bromide or SYBR Green II was added to the gel and used to visualise band under fluorescent (Scull, 2014).

2.3.1.8 16S rRNA gene

16S rRNA gene sequencing is highly useful about bacterial classification. Nowadays, a systematic classification divided into eight ranks from general to specific including of domain, kingdom, phylum, class, order, family, genus, and species. 16S rRNA gene contained the small ribosomal subunit of RNA molecules that used to

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convert genetic message to functional cell and translated mRNA to proteins. All self-replicating system of ribosomal RNA are included conservative region that is readily isolated, its sequence does not variation to changes or slowly change and permitting detection of relatedness taxonomy among the distant species (Woese and Fox, 1977).

2.3.1.9 Phylogenetic tree

Studying of phylogenetics is study of individual relatedness genetic between the same or different species. The phylogenetic tree was presented to be rooted (known ancestral root) or unrooted (unknown ancestral root) and individual studied of a phylogenetic tree's root is the origin of evolution. Branches between leaves represent the evolutionary relationships between sequences, individuals, or species, and branch length display evolutionary time. Several approach can be used for constructing a phylogenetic tree (Xiong, 2018).

2.3.2 Bioinformatic

2.3.2.1 Whole Genome Sequencing (WGS)

Revolution of DNA sequencing of the biological sciences could be discovered gene functions. In 1980s, the Sanger sequencing method was used for DNA sequencing (Sanger *et al*., 1977 ; Slatko *et al*., 2001). In 1990s, DNA sequencing techniques made it possible for automated sequencing which allowed the sequencing of whole genomes (Heather and Chain, 2016). Whole genome sequencing (WGS) is a laboratory process that determined an organism's genome sequence at once. It involves uncovering the order of bases in a complete genome of an organism, which is backed by automatic DNA sequencing methods and *de novo* techniques to assemble the enormous biological sequence data (Saraswathy and Ramalingam, 2011). In 1979, whole genome shotgun sequencing was developed for small genomes ranging from 4000 to 7000 base pairs (Staden, 1979). Nevertheless, early techniques for whole genome sequencing were slow, labor-intensive, and expensive. Specially, in the era of enormous genomic data growth (Kwong *et al*., 2015).

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2.3.2.2 Next-Generation Sequencing

In 2005, next-generation sequencing (NGS) technologies were started. The emanation and widely used next generation sequencing (NGS) techniques for whole genome analysis and significantly enhanced the capacity to perform efficient and low-cost whole genome sequencing. Generally, next generation sequencing processes contain parallel sequencing, where millions of DNA fragments from a single sample are sequenced (Moorthie *et al*., 2011). Among the new sequencing technologies that have been released over the last 10 years, the most used are the 454 GS FLX (Roche), HiSeq (Illumina), SOLiD (Sequencing by Oligonucleotide Ligation and Detection; Thermo Fisher Scientific), Ion Torrent (Thermo Fisher Scientific), and PacBio (Pacific Biosciences) platforms (Pareek *et al*., 2011 ; Liu *et al*., 2012 ; Rhoads and Au, 2015).

2.3.2.3 G+C content

Taxonomy description of G+C content in genome is used to indicate species and genera. In the past, G+C content was determined by using conventional methods. Nowadays, G+C content could be calculated by using genome sequencer.

2.3.2.4 antiSMASH database

The antiSMASH database is one of a *de novo* computing database that available at http://antismash-db.secondarymetabolites.org/. The antiSMASH used to determine secondary metabolite or bioactive compounds in genome sequencing. The identification of biosynthetic gene clusters (BGCs) of *in silico* was reported gene cluster and similarity of known cluster. The antiSMASH is limited for registered user who submitted genomes and could be only analysed individual genomes (Kai *et al*., 2016).

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2.4 Substances producing actinomycete

Actinomycetes have ability to produce a variety secondary metabolite substance such as hydrolytic enzyme to against plant pathogen or plant growth-promoting (PGP) substances to protect plants.

2.4.1 Hydrolytic enzyme production

2.4.1.1 Starch hydrolysis

Some actinomycetes could be produced extracellular enzymes and released outside of cell. In this case, medium contained starch, the starch molecule is too large to be transported through membrane cell. Starch molecule can be hydrolysed into smaller molecules and adsorbed into cell membrane via diffusion. Growth of actinomycetes on International *Streptomyces* Project No.4 (ISP4) were added iodine solution to indicate starch hydrolysis by a transparent clear zone around the colonies (Tille, 2014).

2.4.1.2 Gelatin liquefaction

Gelatin liquefaction was used for determination the ability of actinomycetes to perform proteolytic enzyme or gelatinase that liquefy gelatin. Gelatin liquefied process divided into two stages of reaction. The first reaction, gelatinases hydrolyse gelatin to polypeptides. After that, polypeptides form was changed into amino acids. Metabolic processes of actinomycetes could be used these amino acids.

2.4.1.3 Protease

Protein matrix plays embedded role of chitin and β-glucan in fungal cell wall. Proteases could be hydrolyse the protein matrix into peptide chains or amino acids (Elad and Kapat, 1999). Protease test could be determined on skim milk medium. If the actinomyces can be digested casein to soluble nitrogenous form, a clear zone around the colonies was produced (Wehr and Frank, 2004).

2.4.1.4 Lipase

Lipases are ubiquitous enzymes performed by plants, animals and microorganisms belonging to sub class 1 of hydrolytic enzymes class 3 (Pahojar *et al.*, 2001). Lipase test could be determined by using rhodamine B agar medium (shows

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pink colour around colonies under UV 254 nm.), phenol red agar medium contained olive oil or tributyrin, and tween 80 or tween 20 agar medium.

2.4.1.5 Chitinase

Chitinase is an enzyme that plays role to hydrolyse fungal cell wall composition (chitin polymer). Chitin hydrolysed related to digest chitin polymer into monomer, random breakage at internal micro-fibril, of chitin or more releasing diacetylchitobiose without discharging monosaccharide or oligosaccharides (Harmen *et al*., 1993 ; Sahai and Manocha, 1993).

2.4.1.6 Glucanase

β-1,3-Glucans are one of a composition of yeast and fungal cell wall and higher plants (Simmon, 1994). β-1,3-Glucanases is an enzyme that displays functional to hydrolyse yeasts and fungal cell wall. β-1,3-Glucanases could be divided into exo or endo-β-1,3-glucanases. Glucanase causes, hydrolysation of fungal cell wall and adsorbed into the host mycelium (Fridlender *et al*., 1993). Two possible mechanism that β-1,3-Glucanases could be hydrolysed the substrate are cleaving glucose residues at the end of non-reducing and cleaving random sites of cross linkages (Noronha and Ulhoa, 1996).

2.4.1.7 Cellulase

The function of cellulase enzyme is hydrolyse the 1,4- β -D-glucosidic linkages in cellulose. Cellulose chains form many inter and intramolecular of hydrogen bonds, which formation of crystalline micro-fibrils and insoluble compound. Complete hydrolysation of cellulose needs a complex interaction between different cellulolytic enzymes such as cellulose and exo or endo-glucanases, exo-cellobiohydrolase and β-glucosidases transform cellulose into β-glucose (Lee *et al*., 2002).

2.4.2 Plant growth-promoting (PGP) activities

2.4.2.1 Hydrogen cyanide (HCN) production

Hydrogen cyanide production play an importance role to control fungal disease by producing cyanide. Cyanide presents metabolic inhibitor to escape predation or competition. HCN production of microorganism was suggested as a control root fungal pathogen (Banchio *et al*., 2008 ; Hata *et al*., 2015).

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2.4.2.2 Phosphate solubilisation

Phosphorus (P) is one of phospholipids, adenosine triphosphate (ATP), and nucleic acid components that are required nutrient for plants (Khan *et al*., 2009 ; Richardson and Simpson, 2011). Plants can be absorbed phosphorus in two soluble forms, the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}). Microorganism can be solubilised an inorganic soil phosphate, such as calcium phosphate ($Ca_3(PO_4)_2$), iron(III) phosphate ($FePO_4$), and aluminum phosphate ($AlPO_4$), through the organic acids production. However, plants can utillise phosphorus from soil only in the free available form (Rodríguez *et al*., 2006, Sharma *et al*., 2013).

2.4.2.3 Ammonia production

Biomolecule synthesis of all microorganisms required nitrogen (N) to grow, and nitrogen fixation process is essential for transform nitrogen (N_2) to ammonia (NH_3) (Newton, 2000 ; Franche *et al*., 2009) .

2.4.2.4 Siderophore production

Plants and microorganism are required micronutrient for growth. One of an essential micronutrient for plant is iron (Fe) that plants could not be directly adsorbed. Their need chelator agents or siderophore to bind Fe^{3+} and transported within bacterial (Schwyn and Neilands, 1987 ; Krewulak and Vogel, 2008). The direct effect of siderophores from rhizospheric bacteria could be improved essential nutrition for plant growth. An indirect effect is presented the ability of inhibition to phytopathogens through the sequestration of iron from the environment.

2.4.2.5 Indole acetic acid (IAA) production

Indolic compounds (ICs) could be synthesis in several rhizospheric bacteria that produced auxin phytohormone or indole-3-acetic acid (IAA). The ability of indolic compounds production was distributed several plant-associated bacteria. Approximately 80% of bacteria that isolated from rice rhizospheric could be produced indolic compounds. L-tryptophan was determined to be a main precursor for indolic compound biosynthesis of rhizospheric bacteria. Intermediate compounds were characterised based on different pathway that utilise L-tryptophane (Spaepen *et al*., 2007 ; Souza *et al*., 2013).

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2.5 Growth stages and disease of rice

Rice is the world's staple food source, with more than 958 million hectares of rice cultivation area spread across the world. Especially, in the Asian continent. Rice is one of majority of cereal crops that supply the main of the global daily carbohydrate intake as energy source. Rice is a primary carbohydrate source for 2.5 to 3.5 billion people that largely located in rapidly growing low-income countries (LICs) (Maclean *et al*., 2013) . In 2008, approximately 685 million tons of rice were cultivated around 160 million ha. Nowadays, the number population is increasing and the requirement of rice production in 2020 is increasing too. (Chauhan *et al*., 2017). In Thailand, rice has been cultivated for more than 5,000 years, so rice has become a part of the tradition, culture, and way of life of Thai people. In addition, the domestic consumption of rice is approximately 21 million tons per year. Rice exports are considered to be the main export of Thailand, with ranked as one in five of the world. Although, Thailand exports rice product about 45 percent of rice yield (Saruda *et al*., 2017). Fungal diseases to rice are reduced 14 % globally of annual rice production (Agrios, 2005). The climate change of air temperature increased and raised carbon dioxide concentration in atmosphere was promoted rice disease to new areas (Anderson *et al*., 2004).

2.5.1 Growth phases and stages of rice plant

The growth phases and stages of rice plants take around 3 to 6 months and it could be divided in to 4 growth phases including of germination phase, vegetative phase, reproductive phase, and ripening phase as presented in Figure 2.5.

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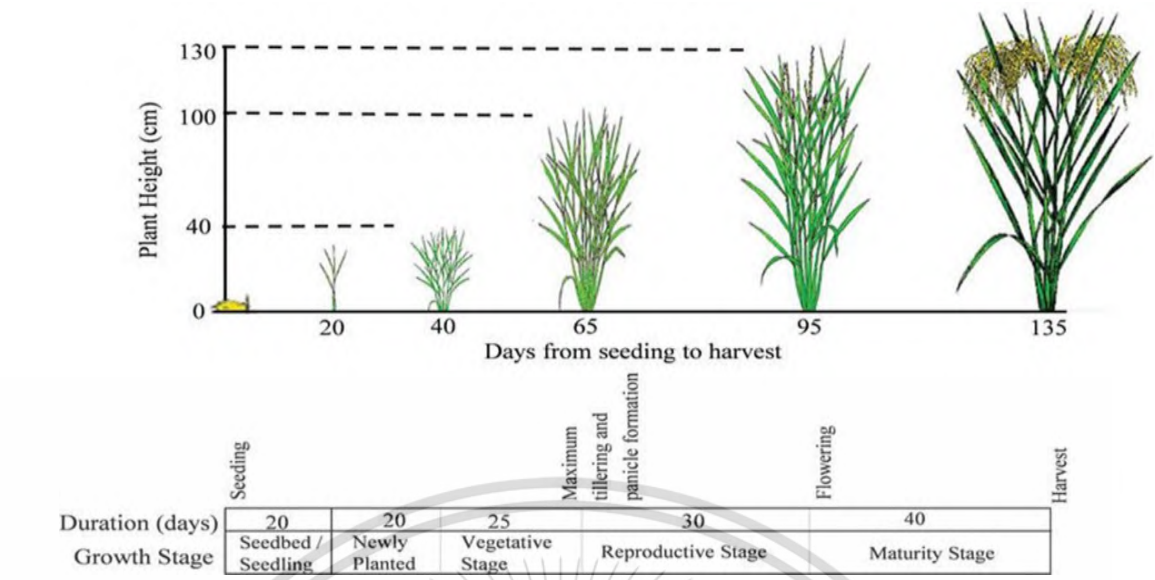

Figure 2.5 Growth phases and stages of rice plant (International Rice Research Institute (IRRI), 2007).

Germination phase: In this phase, root will develop depending on soil humidity. When planted into high humidity soil, the roots were emerged after the first shoot raised to the air. If the seeds are planted into low humidity soil, the roots were emerged before the first shoot.

Vegetative phase: characterisation of this phase based on more leaves production, tillers development, and a gradual rise in plant height. Typically, this phase takes between 55 and 85 days. In this phase could be divided into 3 stages including of early vegetative, newly planted, and late vegetative stage. The early vegetative stage starts from germinates into a seeding stage begins after the shoot and root emerge up to five leaves develop. Newly planted stage, leaves continue to develop every 3 to 4 days during the initiated stage. Late vegetative stage begins while tillering and panicle formation. This stage extends from the presented of the first tiller to the maximum number of tillers. Generally, this stage happens 40 days after sowing. The final of vegetative phase stem starts, and height of growing is stopped.

Reproductive phase: The reproductive phase takes approximately 45 days started from panicle initiation. While the panicle changes to be a visible naked eye, the booting stage is already started. This phase takes approximately 15-20 days and farmer also called “rice pregnant” in this phase. The booting stage was followed after the heading stage that takes about 10 days . 90% of the panicle from the covering flag

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leaf sheath development in late heading stage. Flowering stage starts from the appearance of the first anthers form the uppermost spikelets on each panicle and continues for approximately 15 days.

Ripening phase: the ripening phase lasts approximately 25-35 days. This phase occurs when fertilisation and continue, through grain filling and ripening stages. First is the milk stage, the first endosperm produces a milky liquid. Second stage is the dough stage, the milky liquid performs solidify into a sticky. Third is the maturity stage started when the grains were ripened more than 90%. Mature grains could be indicated by transforming to a golden brown colour under wet climeate conditions.

2.5.2 Disease of rice

Disease consideration was examined based on the host plant, pathogen (pest or phytopathogen) and environment occurs together. These three components also called disease triangle as presented in Figure 2.6.

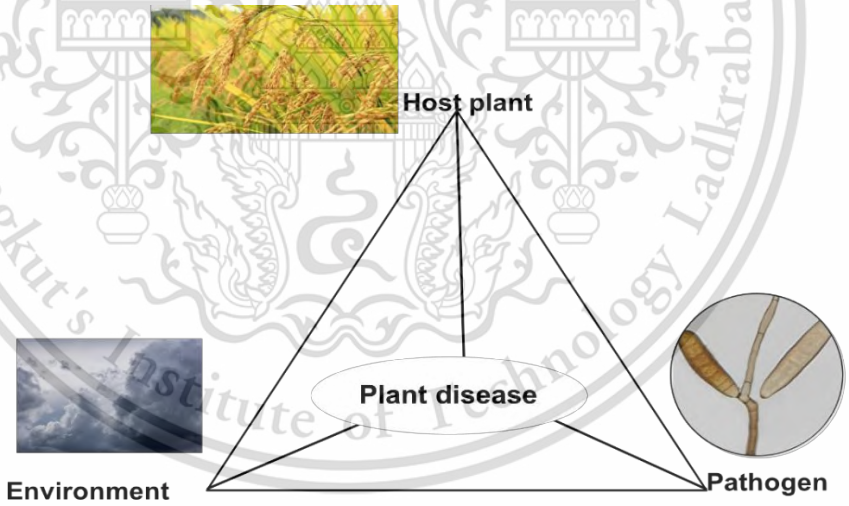

Figure 2.6 Disease triangle of rice plant.

Rice blast disease is one of the most serious cereal crops disease that causing *Pyricularia oryzae* (*Magnaporthe oryzae*) negative effects to globally rice production including lowland and upland around the world (Kato, 2001 ; Talbot, 2003 ; Dean *et al.*, 2005 ; Wilson and Talbot, 2009 ; Mathurot *et al.*, 2019). Yield loss causing rice blast

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disease about 10% to 30% annually. During the disease outbreak, yield loss could be up to 50% (Wilson and Talbot, 2009 ; Ashkani *et* al., 2015 ; Asibi *et al*., 2019). *Pyricularia* sp. (Figure 2.7) could be infected at any growth phases and stages of rice plants (Figure 2.9). It could be infected and displayed the symptoms every part of rice plants even leaves (leaf blast), nodes (node blast), stems (node and collar blast) and panicles (panicle blast) (Kato, 2001 ; Talbot, 2003 ; Dean *et al.,* 2005 ; Wilson and Talbot, 2009).

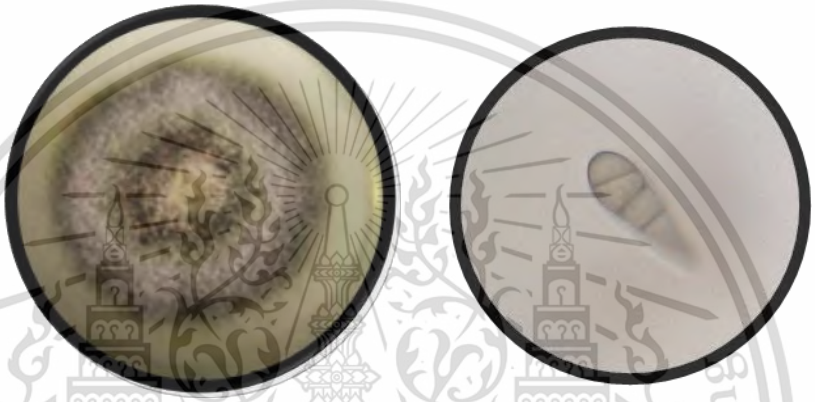

Figure 2.7 *Pyricularia* sp. on agar plate and conidia under light microscopic.

2.5.3 The symptom of rice blast disease in differentiate growth stages (Rice department, 2020)

2.5.3.1 Seeding stage

The seedling stage has brown spots like eyes. It shows a gray colour in the middle of the wound. The width of the incision is 2 to 5 mm. And the length is 10 to 15 mm. The lesion can expand and spread over the leaf area. If the disease is severe, the rice seedlings will dry up and die (Figure 2.8 (1)).

2.5.3.2 Tilling stage

Tilling stage, symptoms are found in the leaves, leaf joints and stem joints. The wound size is larger than that found in the seedling stage. The wound spreads to be contagious at the joint area. The leaves display bruised, brownish black (Figure 2.8 (2)).

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2.5.3.3 Maturity stage

The maturity stage if the rice has just begun to grind when destroyed by fungi. The seeds will be withered. The disease occurs when the ear grows near harvest. The symptoms have brown bruises on the chest area and all most of Every single ear has died (Figure 2.8 (3)).

Figure 2.8 Rice blast disease in differentiate growth stages of rice plants: (1) seeding stage, (2) tilling stage, and (3) maturity stage.

2.5.4 Prevention form rice blast disease (Rice department, 2020)

2.5.4.1 Using disease-resistant rice

Use of different disease-resistant rice or different strain of Thai rice can be divided according to the region of Thailand as following:

Central of Thailand: using disease-resistant rice including of Suphanburi 1, Suphanburi 2, Suphanburi 60, Suphanburi 90, Chainat 1, Prachinburi 1, Plai Ngam, Khlong Luang 1, Phitsanulok 1.

North and Northeast of Thailand: using disease-resistant rice including of Surin 1, Nyeu Ubon 2, Nyeu Phrae, San Patong 1, Hang Yee 71.

Southern of Thailand: using Dok Phayom for disease-resistant rice.

Caution: Suphanburi 1, Suphanburi 60 and Chainat 1 rice that cultivated in the lower northern regions were found to show severe symptoms in some areas and some years, especially when rain or fog, frost, cold weather and excessive fertiliser.

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2.5.4.2 Prevention seed with fungicide

Mix the seeds with a fungicide such as casugamycin, tricyclazol, carbendasimprochloraz, at the rate specified. In sites that have previously been infected with blast and common blast lesions, 5% of the leaf area should be sprayed with anti-fungal agents such as casugamycin, edifenphostricclasol, isopotrolane and carbendazim according to the specified rates. However, agricultural chemical pesticides are main source of soil, air, and water pollution. Pesticides also presented significant risk factors on health because and environment (Selcen and Murat, 2017).

2.6 Biological control agent (BCA)

Health problems can be divided into acute and chronic toxicity. Acute toxicity, the patient will have symptoms immediately after exposure to chemicals such as nausea, vomiting, headache, muscle pain, diarrhea, shortness of breath, blurred vision, etc. Chronic toxicity, which is caused by the accumulated toxins causing other diseases or problems such as cancer, diabetes, paralysis, skin diseases, infertility, neonatal disability, or sexual dysfunction, etc. Due to the limitations of the information system, statistics of the patients who have been poisoned by pesticides. (Not counting self-harm) In 2010, there were people in 2015, while the reports from the insurance system database were more than four times. However, these statistics report the incidence of patients from acute poisoning, mostly due to chemical use. But this excludes patients who may not be admitted to a medical facility or who are suffering from chronic cumulative poisoning. Which is expected that the actual number of patients may reach 200,000-400,000 people per year.

2.6.1 Pesticide residues; a crisis in the Thai food system

Problems and dangers from pesticides do not only affect farmers but also consumers, due to the consumption of vegetables and products that contain chemical residues. As reports of blood testing in farmers and consumers in Chiang Mai It was found that farmers and housewives had a total of 75% of unsafe and risky chemical residues 89.22% of the civilian university workforce (Chaing Mai provincial public health

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office, 2008) The main reason for this difference may be that farmers have safer food options than conventional consumers.

2.6.2 Pesticides increase the risk of serious diseases

Since 2004, cancer has been the country's number one cause of death, overtaking AIDS, heart disease and road accidents. The number of deaths is estimated at 55,000 per year (Umpol, 2008). Residues of pesticides in food are one of the leading risk factors for cancer and many other health problems leading to the disease. Canadian research in 2004 found that insecticides of the carbamate group and the herbicides in the phenoxy group (carbamate and phynoxy herbicide) such as carbofuran methomylcabaril can cause lung cancer. Organophosphate chemicals such as chlorophyll, phosphos, diacinon, dichloros can cause brain cancer and blood cancer.

Agrochemicals, considered less acutely toxic, also cause many serious diseases, such as chemical pyrethrines can cause psychoactive disorders. Anomalous chromosome can lead to a slow growth in the fetus, although the US Department of Agriculture has indicated that these chemicals may be the safest for humans, such as pesticides. Phasinet (glufasinate) can cause birth defects or miscarriages and chemicals in the phenoxyl group (phenoxyl) like 2,4-D can cause lymphoma. In addition, there have been research reports that chemical pesticides may be involved in the development of type 2 diabetes and Parkinson's disease as well (Margaret *et al*., 2004).

Tackling the pesticide problem should be a national agenda. They must jointly drive-up policies, laws and measures that will make the countries agricultural and food systems consider health and the environment also it should have access to sufficient and safe food.

At the present, all over the world are used pesticide more than two billion tons per year. Biological control was recognised to replace of pesticide. Nevertheless, biological control products are an alternative product which cheaper than pesticide and less harmful to human and environment (Noemi *et al*., 2019).

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2.6.3 Biological control agent (BCA) from microorganism

Biological control is one of alternative way to reduce chemical using in agriculture. Some of chemical residue toxic compounds which are harmful to animal, agriculturist, and consumption. Biological control agent involves the use of potential antagonistic, their ability to produce such as metabolites or antibiotics that could be reduced the negative effects of plant pathogens and induced plant growth.

2.6.4 Developing microorganism for commercial biological control agent

Developing microorganism for commercial biological control agent product through the standard pesticide application. In the ASEAN region, microbial control agent or MCA not only performed by bacteria and fungi but also included of protozoa, insect viruses and entomopathogenic nematodes. In Thailand, application also developed for practical use, for instance by government research programmes in Thailand (Ratanasatien *et al*., 2005).

2.6.4.1 Bacteria

Using bacteria for commercial biological control agent products are almost form genus *Bacillus*. *Bacillus* spp. is the most important biological control agent for control plant disease and some insect. The *Bacillus* species that considerable to be commercial product for plant disease include *Bacillus subtillis* and *Bacillus pumilus*. The *Bacillus* species that considerable to be commercial product for insecticidal bacterium is *Bacillus thuringiensis*. *Bacillus thuringiensis* was divided into three sub-groups with different functions specific to coleoptera (*Bacillus thuringiensis* var tenebrionis), lepidoptera (*Bacillus thuringiensis* [sero] var thuringiensis, *Bacillus thuringiensis* var morrisoni, *Bacillus thuringiensis* var kustaki, *Bacillus thuringiensis* var aizawai), and diptera (*Bacillus thuringiensis* var israelensis). Nowadays, commercial biocontrol performed by *Bacillus* in Thailand presented in Table 2.2.

2.6.4.2 Fungi

The properties of fungi could be control pest disease (*Metarhizium* and *Beauvaria*) and plants disease (*Trichoderma* spp.). But all the fungal strain must not be assumed that it would be safe. For example, some may be risky to man health. At present, commercial biocontrol performed by fungi in Thailand presented in Table 2.3.

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2.6.4.3 Protozoa

A few protozoa could be developed to be protozoan parasites for rodent because of it specific to each local to use. In ASEAN, only *Sarcocystis singaporensis* is used for control rats (genera *Bandicota* and *Rattus*) and a boid snake. *Sarcocystis singaporensis* product is now available in Thailand, Lao, Vietnam, and Indonesia.

2.6.4.4 Insect viruses and entomopathogenic nematodes

Insect viruses and nematodes are worldwide use but in ASEAN only nuclear polyhedrosis virus (NPV) was registered product in Vietnam according to the ASEAN Biocontrol for Sustainable Agrifood Systems database. However, department of agriculture in Thailand has been developed baculoviruses and nematodes that use for biocontrol against lepidopteran vegetable pests in Thailand.

2.6.4.5 Actinomycete

Actinobacteria was shown ability to be biocontrol agent for instance, the research of Jodi *et al*., (2017) reported biocontrol agents from *Streptomyces globisporus* JK-1 could be reduced rice blast (*M. oryzae*) up to 88.3% in pot experiment test. Other research also reported the potential of actinobacteria could control the rice blast disease (Ningthoujam *et al*., 2009; Awla *et al*., 2017 ; Law *et al*., 2017). Actinobacteria showed good activities to be biocontrol agents, but it was not found to be commercial biocontrol in Thailand. In Vietnam, one strain of *Streptomyces lydicus* has been registered to control mold disease.

Table 2.3 List of commercial biocontrol performed by microorganism in Thailand.

Biocontrol agent	Product	Manufacturer
Bacillus subtilis	ROTER®	AP-Biotech Thailand, Co., Ltd.
Bacillus subtilis	BIO-SENSOR ™	Thai Green Agro, Co., Ltd
Bacillus subtilis AP-01	LAMINAR®	AP-Biotech Thailand, Co., Ltd.

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Table 2.3 List of commercial biocontrol performed by microorganism in Thailand (continued).

Biocontrol agent	Product	Manufacturer
Bacillus subtilis NSRS 89-24 and MK-007	BIOBASE®	AP-Biotech Thailand, Co., Ltd.
Bacillus thuringiensis (aizawi)	BTTECH	SV Group
Bacillus thuringiensis var.*kurstaki*	BIOTACT ™	Thai Green Agro, Co., Ltd.
Beauveria bassiana BCC 2660 and *Metarhizium anisopliae* BCC 4849	MIXTECH	SV Group
Beauveria bassiane	CUTOFF ™	Thai Green Agro, Co., Ltd
Metarhizium anisopliae	FORTRAN ™	Thai Green Agro, Co., Ltd
Metarhizium anisopliae BCC 4849	METATECH	SV Group
Trichoderma asperellum CB-Pin-01	TRICHOTECH POWDER	SV Group
Trichoderma harazium	TRISAN®	AP-Biotech Thailand, Co., Ltd.
Trichoderma harazium	INDUCER ™	Thai Green Agro, Co., Ltd

2.6.5 The actions of biological agent mechanisms

Biocontrol mechanisms have several actions to control plant disease (Jamalizadeh *et al*., 2011). The actions have five possible actions include competition, antibiosis, parasitism, and induced resistant in the host tissue.

2.6.5.1 Competition

Competition occurs when two or more microbial populations are in the same location at the same time (Droby and Chalutz., 1994). The effects of competition have several possibilities for biocontrol agent such as nutrient competition by reducing inoculum potential of pathogens, decreasing several pathogens, and raising saprotrophic competition for initial resources in substrate colonisation. If antagonistic

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grows in the same place with pathogen, the pathogen will eliminate as shown in Figure 2.9.

Figure 2.9 Competition mechanism of biological agent.

2.6.5.2 Antibiosis

A definition of antibiosis is a biological interaction of two or more microorganism that one of them could be produced metabolic substances to inhibit another microorganism as presented in Figure 2.10. Actinomycete plays an importance role in a source of antibiotic and agroactive compounds such as kasugamycin from *Streptomyces kasugaensis* to control rice blast disease and disease causing *Psuedomonas* or valindamycin to against rice sheath blight disease.

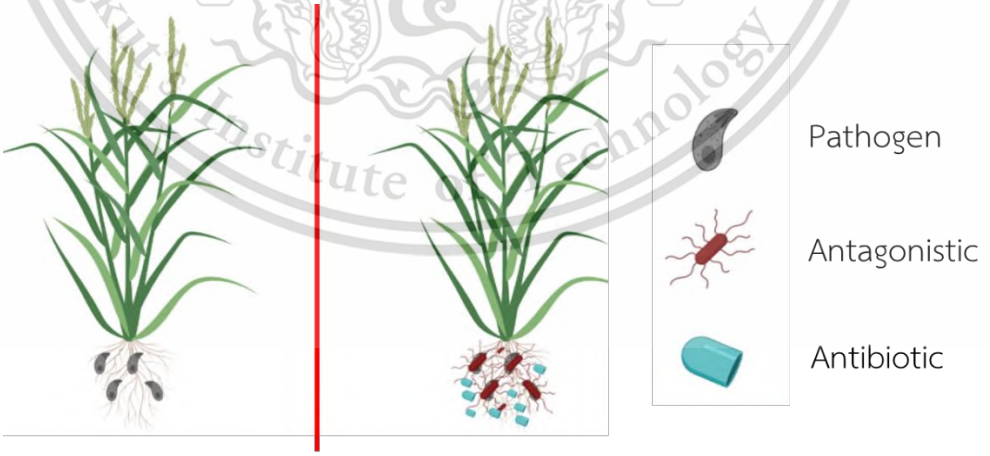

Figure 2.10 Antibiosis mechanism of biological agent.

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2.6.5.3 Parasitism

Parasitism or mycoparasitism occurs when antagonistic microorganism could be produced enzyme for utilised fungal cell wall such as protease, glucanase, and chitinase to eliminate the composition of fungal cell wall as shown in Figure 2.11 (Elad *et al*., 1983).

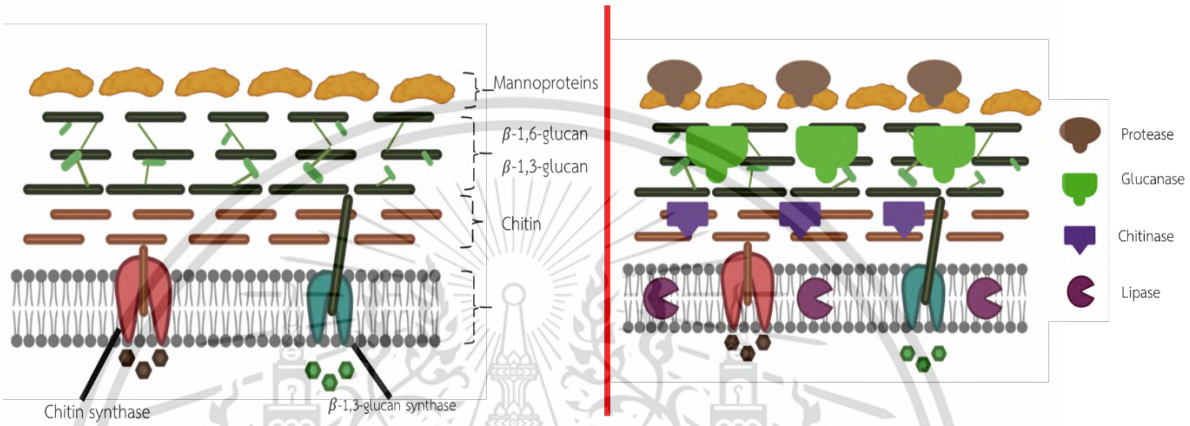

Figure 2.11 Mycoparasitism mechanism of biological agent (Modified from Fesel and Zuccaro, 2016).

2.6.5.4 Induced resistance in the host tissue

Induced resistance of plant caused by antagonistic can be protected plant from plant pathogens (Castoria *et al*., 2001) such as plant growth promoting rhizobacteria (PGPR). When PGPR treated into the soil, they could be showed abilities of nitrogen fixation, phosphate solubilisation, phytohormone synthesis, IAA production, or siderophore production (Glick, 1995) to induced resistance in the plant tissue as shown in Figure 2.12.

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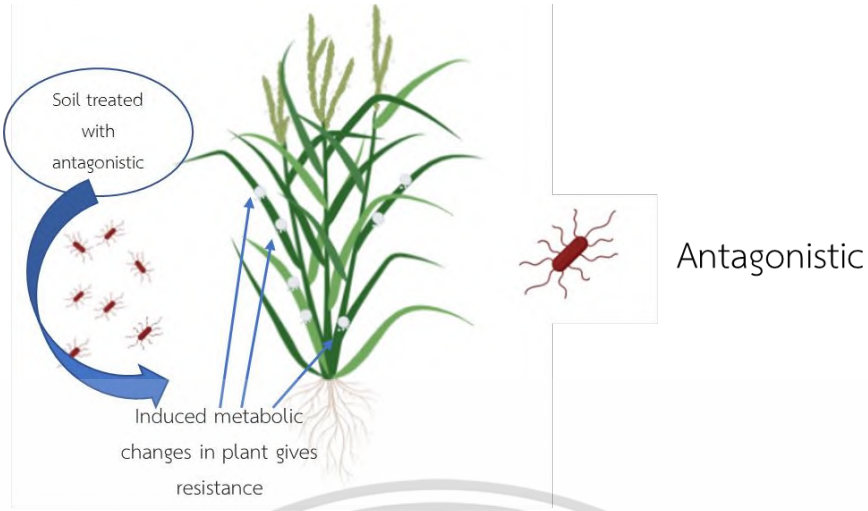

Figure 2.12 Induced resistance in the host tissue mechanism of biological agent.

2.6.6 General required information for biocontrol agent registration

The necessary information about microbial product registration is based on various parameter. The parameter include colony forming unit (CFU) count, strain specifications, moisture content, pathogenic contamination, type of formulation, shelf life and storage.

2.6.6.1 Colony forming unit (CFU) count

Limitation of cfu count is depending on antagonistic fungi or antagonistic bacteria. CFU count of antagonistic fungi should not be less than $2x10^6$ spore/ mL or g on selective medium (SM) and a minimum of antagonistic bacteria is $1x10^8$ spore/ mL or g. These principles according to the Central Insecticide Board and Registration guideline.

2.6.6.2 Strain specifications

Strain specifications required the information about identification (genus and species), growth parameters (pH and temperature), potential of biological control, and growth promotion. Moreover, study on field and lab condition are required.

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2.6.6.3 Moisture content

Moisture would be affected physical or chemical properties to the final product. Maximum moisture content of dry fungal or bacterial formulation is not exceeded more than 8% and 12%, respectively.

2.6.6.4 Pathogenic contamination

Other microbial contaminants must not over $1x10^4$ spore/ mL or g and should not be present *Shigella*, *Vibrio* and *Salmonella* in formulation product.

2.6.6.5 Type of formulation

Biological agent products could be formulated in several approach such as dry formulations (dusts, granules, and microgranules), dry powder formulations for dilution in water (dispersible granules and wettable powder), seed dressing formulations (powder for seed dressing), liquid formulation for dilution in water (emulsions, capsule suspensions, oil dispersion, and suspension concentrates), and ultralow volume formulations. However, biological agent product in the market is found in granular, liquid and powder formulations.

2.6.6.6 Shelf life and storage

Commercial of biological product in the market should be storage in long term or long shelf life and could be kept at room temperature. The Central Insecticide Board and Registration Committee required for claiming 1-year shelf life of the product. Product storage at room temperature is a necessary condition for farmers who cannot afford the refrigerator to store at any temperature.

2.7 Formulation of biological agent

Formulation of biocontrol agents is one of an importance steps to develop a commercial product. Biological formulations should be presented the abilities to against plant pathogens as fresh cell. The formulation must be provided the information about colony forming unit (CFU) count, strain specifications, moisture content, pathogenic contamination, type of formulation, shelf life and storage (Kinay and Yildiz, 2008; Teixido´ *et al*., 2011).

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2.7.1 Formulation methods

Biological control agent formulation approach could be performed in liquid, powder, or cell immobilised forms (Keswani *et al*., 2016).

2.7.1.1 Liquid formulations

Liquid formulations contain 10 to 40 percent of antagonistic cells or suspension and adding additive to preservation a viability of cells. Basically, the additive might be oil, mineral, polymer, or surfactant that could be improve viability of the antagonistic (Brar *et al*., 2006).

2.7.1.2 Powder and granular formulation

Powder and granular formulations were performed by adding carrier (soil, inorganic, or organic) and mixing with antagonistic. In addition, antagonistic could be prepared in different ways such as fresh cell solution, cells lyophilisation, or spray-drying. Fresh cell solution is usually made powder formulation in granules by spraying, mixing, or making a powder carrier with a fresh cell solution and using air-drying to dry the powder. Wettable powder form is produced as granules but generally contain carrier (50–80%), diluent (15–45%), dispersant (1–10%), wetting agent (3–5%). The final concentration of the antagonistic in residue is approximately 5 up to 20%, while in powders is less than 10% (Brar *et al*., 2006). Cell lyophilized preparation is considered a relatively dehydration approach in which cells are initially incorporated into a matrix that maintain them from damage during dehydration. Spray-drying cell preparation was conducted by transforming liquid solution or mixture into dried particles and mixing with carrier to perform powder formulation.

2.7.1.3 Cell immobilisation

Cell immobilisation or encapsulation was produced in different technique, including spray drying, temperature and ionic gelation, ionic polymer coating, co-acervation and solvent extraction, sodium alginate gel immobilisation, interfacial polymerisation, and layer-by-layer coating used for determining size, shape, and the texture of the capsules (Vemmer and Patel, 2013).

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2.8 Microencapsulation

Microencapsulation is one of cell immobilised formulation methods that used in the present study. Microencapsulation is a preservation technique of sensitive substances or microorganism for instance, the reported Locatelli *et al*., (2018) using microencapsulation technique with *Trichoderma* sp. to encapsulate in alginate. Microencapsulation is an approach to produce materials with new potential properties and it is the process to encircle micron-particles size of droplets of liquids, solids, or gasses in an inert shell. This technique could be protected sensitive substances or microorganism from the external environment (Ghosh, 2006). The products of microencapsulation technique that displayed the diameter of particles between 3–800 μm by this process are called microcapsules, microparticles, or microspheres which differential morphological of internal structure as shown in Figure 2.13. Particles less than 1 μm are known as nanocapsules, nanoparticles, or nanospheres (Remun and Alonso, 1997). The macroparticles are displayed particle size larger than 1000 μm (Thies, 1996).

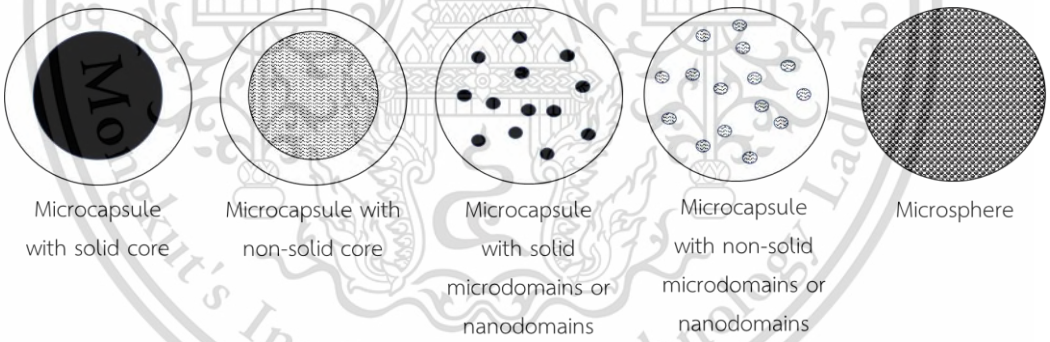

Figure 2.13 Differential structures by using microencapsulation technique (Singh *et al*., 2010).

At present, many biological controls from microorganism that contained spores or live cells were used microencapsulation technique. Causing the commercial formulations based on fresh cells or fresh spores required storing at temperatures 4 to 28 ℃ or keeping in refrigerator which a short shelf life. (Paula *et al*., 2011 ; Vemmer and Patel, 2013). Briefly, it is necessary to improve approaches that could be increasing bioavailability and shelf life of microorganism biological control in the field. (Lewis and

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Papavizas, 1985 ; Fraceto *et al*., 2018). Powder or dried products contained less viable cell causing of high temperature (spray-drying formulation) or thermal and dehydrated during drying process (Abadias *et al*., 2001 ; Melin *et al*., 2007). Even so, cell viability could be increased by adding carrier or certain stabilising substances for instance polymers, sugars (maltodextrin), albumin, milk (skim milk), salts, honey, polyols, or amino acids) to the formulation component solution. Components of the formulation solution have two main benefits in preserving the cell viability of formulated: to increase a dry residue in the rehydration process and to avoid cells and biochemical damage through drying process (Abadias *et al*., 2001 ; Sabuquillo *et al*., 2010 ; Melin *et al*., 2011). In this study, microencapsulation was conducted by using spray-drying technique causing the commercial formulation of actinomycetes poorly found to improve as biocontrol products for agriculture (Zulma *et al*., 2020). Commonly, microencapsulation can be performed by using spray dryer equipment with spray-drying technique in industrial process as described in title 2.8.1.

2.8.1 Spray dryer

Spray dryer is an equipment that used for drying liquid solution to be particles by using spray drying technique. The scale of spray dryer including of a laboratory scale and industrial scale. Büchi (Switzerland) is one of developing dependable and versatile spray dryers that used for several intents such as Mini Spray Dryer and Nano Spray Dryer (Lee *et al*., 2011 ; Beck *et al*., 2012 ; Sosnik and Seremeta, 2015). The major components of spray dryer B-290 including of nozzle, peristatic pump, drying chamber, cyclone separator, collection bottle, and controller switch as shown in Figure 2.14.

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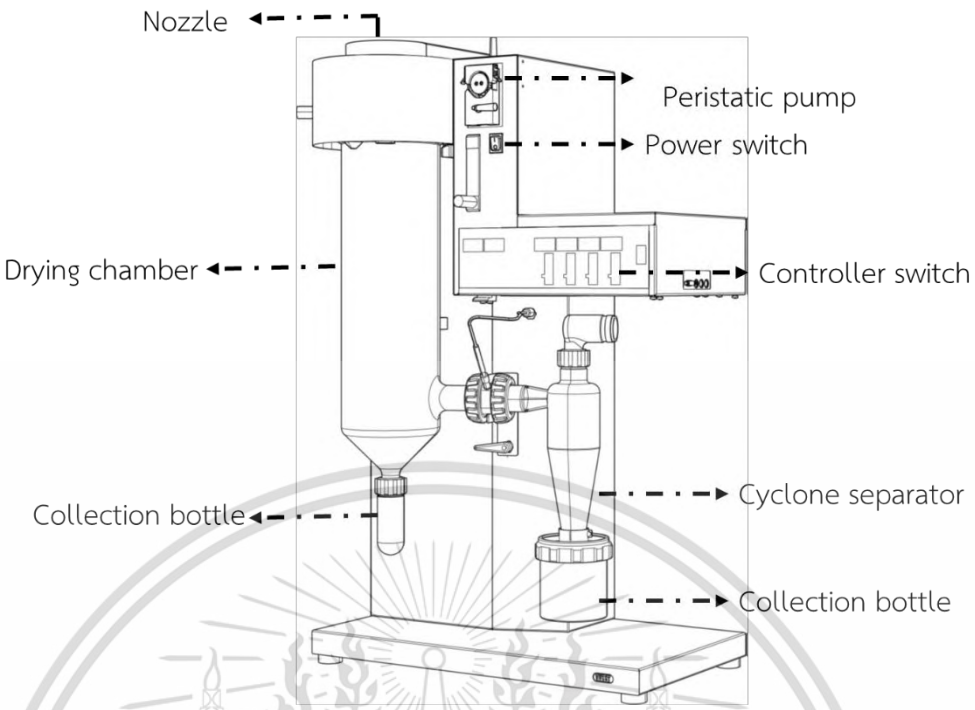

Figure 2.14 Spray dryer B-290 components (B-290 Mini Spray Dryer Operation Manual, 2020).

Spray-drying technique is one of the encapsulation techniques which consists of transformation liquid solution or mixture into dried particles and elimination the moisture of sample. The process could be divided into three major stages as shown in Figure 2.15 (Patel *et al*., 2009).

Atomisation: liquid solution was pumped to an atomiser, at the end liquid feed into a spray of fine droplets.

Droplet-to-particle: the droplets are discharged to drying chamber, the moisture was vaporization and dry particles were formation.

Particle collection: dry particles were passed through cyclone separator to collecting bottle, dried particles are separated from the drying solution, being then collected in a collecting chamber.

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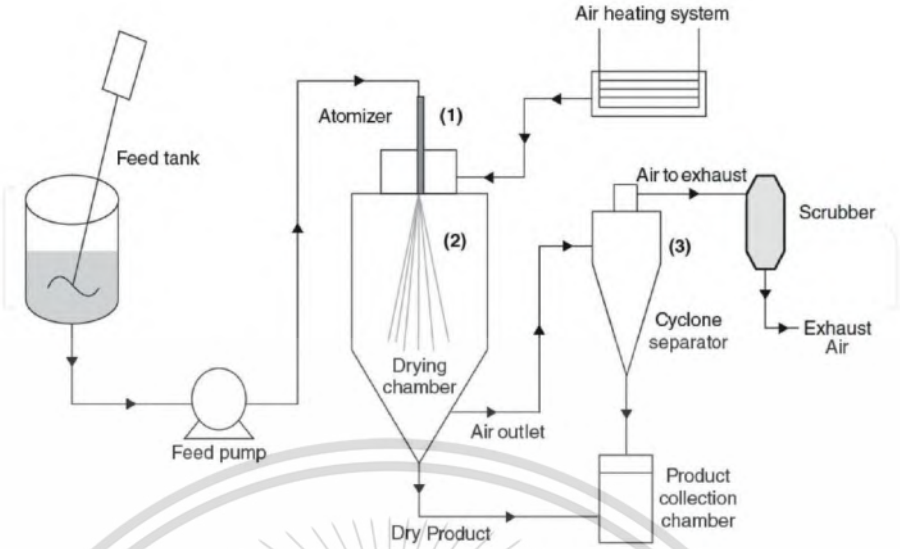

Figure 2.15 Spray drying process; atomization (1), droplet-to-particle (2), and particle collection (3) (Anandharamakrishnan, 2017).

2.9 Literature Reviews

Actinomycetes, especially *Streptomyces* are well known as huge sources of secondary metabolites especially antibiotics (Berdy, 2005). Commercially important antibiotics are majorly produced by *Streptomyces*, *Actinoplanes*, *Amycolatopsis, Saccharopolyspora*, and *Micromonospora*. Briefly, around 80% of commercial antibiotics have been conducted by *Streptomyces* (Khanna *et al*., 2011).

Rice is one of the main cereal crops that supply most of the global daily carbohydrate intake as energy source of human. Any pathogen targeting this crop supposed to have serious connotations for the economy. 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 oryzae*. (Anamorph stage, *Pyricularia oryzae*) is main economically harmful crop diseases. 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 properties, and crop management. 1% to 50% of yield loss appraisals from other areas of the world (Jeffrey, 2016).

Many strains of actinomycetes are biocontrol agents because of their abilities not only in antimicrobial production as well as plant growth-promoting (PGP) but also

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actinomycetes could be produced agroactive compound to control rice disease as presented in Table 2.4. Actinomycetes also have efficiently colonises the rhizosphere of different plant species including rice. Many *Streptomyces* strains had been studied in several laboratories to control bacterial and fungal diseases of rice such as rice blast disease fungus caused by *Pyricularia* sp. (Ratih *et al*., 2012 ; Jodi *et al*., 2017 ; Chaiharn *et al*., 2020), but there is poorly found to improve the *Streptomyces* as biocontrol products for agriculture (Zulma *et al*., 2020).

Table 2.4 Agroactive metabolite (antibiotic) from actinomycetes.

Agroactive metabolite/ antibiotic	Actinomycete(s)	Disease control	Reference
Kasugamycin	*Streptomyces kasugaensis*	Rice blast disease	Umezawa *et al*. (1965)
Polyoxin B and D	*Streptomyces cacaoi* var. *asoensis*	Rice sheath blight	Isono *et al*. (1965)
Mildiomycin	*Streptoverticillium rimofaciens*	Powdery mildew	Iwasa *et al*. (1978)
Validamycin	*Streptomyces* spp.	Rice sheath blight	Shirvastava and Kumar (2018)
Cyclo (L-leucyl-L-prolyl)	*Streptomyces* sp. KH-614	Rice blast disease	Shirvastava and Kumar (2018)
AC-1	*Streptomyces* sp. AB-88M	Rice blast disease	Matsuyama. (1991)
Gopalamicin	*Streptomyces hygroscopicus*	Rice blast disease	Nair *et al*. (1994)
2,3- dihydroxybenzoic acid, phenylacetic acid, cervinomycin A1 & A2	*Micromonospora* sp. M39	Rice blast disease	Ismet *et al*. (2004)

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Table 2.4 Agroactive metabolite (antibiotic) from actinomycetes (continued).

Agroactive metabolite/ antibiotic	Actinomycete(s)	Disease control	Reference
Fungichromin	*Streptomyces padanus* Strain PMS-702	Rice sheath blight	Shih *et al*. (2003)
Nigericin and Guanidylfungin A	*Streptomyces violaceusniger*	Rice sheath blight	Trejo-Estrada *et al.* (1998)
Geldanamycin	*Streptomyces hygroscopicus* var. *geldanus* and *Streptomyces griseus*	Rice sheath blight	Rothrock and Gottlieb. (1984)
Antifungal mycin 702	*Streptomyces padanus* JAU4234	Rice blast disease	Xiong *et al*. (2013)
Resistomycin and tetracenomycin D	*Streptomyces canus* BYB02	Rice blast disease	Zhang *et al*. (2013)
Bafilomycins B1 and C1	*Streptomyces cavourensis* NA4	Rice blast disease	Pan *et al*. (2015)

A considerable amount of literature has been reported many studies focused on the use of genera *Bacillus* as potential biocontrol formulation by spray-drying against rice disease. *Streptomyces* has been published powder formulation as biocontrol agents rice disease but their produced it by using lyophilized cell or fresh cell mixed with powder (Tamreihao *et al*., 2016). Limitation of lyophilized are cost, production time, and complication in industrial process. Compared the cost of spray-drying to freeze-drying method, the cost of spray-drying is cheaper than freeze-drying 30-50 times (Desorbry *et al*., 1997). Spray-drying commonly used for transforming liquids into solid powders. This technology is the most general and cheapest technique to produce encapsulation. However, biocontrol formulation by spray-drying against rice disease from *Streptomyces* has not been published and encapsulation technique was used to

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immobilize in this study because it provides higher cell viability and lower cost of industrial production.

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Chapter 3

Research methodology

3.1 Isolation and preservation of actinomycetes

3.1.1 Collection of soil sample

Twenty-one soil samples were collected from rice field soil at Nong Bua, Nakonsawan, Thailand (Code strain started with NK) and four soil samples were collected from "Happy Rice Organic Farm" at Nong sua, Pathumthani, Thailand (Code strain started with HPF). Samples were randomised by collected the sediments approximately 10 to 20 cm. depth. The area of soil sampling was 5 x 5 m^2. These samples were kept in sterile polyethylene bags sealed and transported immediately to the laboratory. The soil samples were determined some properties of soil as following approach :

3.1.1.1 Measuring soil temperature

At each of the location, soil was measured temperature by using a temperature probe. Recording the data on the soil temperature.

3.1.1.2 Measuring soil pH

Weight the soil sample and added deionised water in ratio 1:1. Cover and stand for an hour. Rinse electrode with deionised water and place probes in soil sample, measure pH, and record measurement.

3.1.1.3 Measuring soil moisture

Soil samples were examined soil moisture by weight 10 g of the soil sample and drying oven at 105 ℃ for 24 hrs. After drying, the soil moisture was calculated as equation 1.

$$Soil\ moisture\ (\%) = \frac{Weight\ of\ soil\ moisture\ in\ gram - Weight\ of\ dry\ soil}{Weight\ of\ dry\ soil} \times 100 \quad (1)$$

3.1.2 Isolation of actinomycetes

These soil samples were pre-treated by air-dried samples for 3 to 5 days at room temperature. Ten grams of soil sample was accurately weighed and transferred to 90 mL of 0.1% Tween80 (10^{-1}), mixed well and then 1 mL of resultant solution was serially

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diluted with 0.85% Sodium Chloride (NaCl) 9 mL up to 10^{-5}. 200 µl of each intermediate dilution was spread on Modified Zhang's Soil Starch Extract (ZSSE) agar (appendix A) medium that contained 50 µg/mL nystatin. The plates were incubated at 30 ± 2 °C and observed every day during incubation. During incubation time, colony was picked by micro-needle then cross streak on International *Streptomyces* Project 2 (ISP2) medium (Shirling and Gottlieb, 1966 ; appendix A).

3.1.3 Preservation of actinomycetes

The selected colonies of actinomycetes isolates were maintained on ISP2 medium at 30 ± 2 °C for 7 to 14 days and preserved in 20% glycerol (w/v) stocks stored at -20 and -80 °C for long time preservation (Waksman *et al*., 1961).

3.2 Identification of actinomycetes

Identification of actinomycetes in this study are determined phenotypic characteristics and genotypic characteristics. All actinomycete strains were studied phenotypic characteristics based on morphological characteristic. The morphological identification was examined colour of aerial mycelium on ISP2 medium comparing with ISCC–NBS colour system and type of spore chains was observed by light microscopy. The potential strains that showed antimicrobial activity were determined genotypic characteristics by using 16S rRNA gene analysis. Moreover, the actinomycete strains that showed antifungal activity (%RGI > 50%) were determined biochemical, and physiological characteristics.

3.2.1 Phenotypic characteristics

Phenotypic characteristics including of morphological characteristic, cultural characteristic, biochemical and physiological characteristics were performed.

3.2.1.1 Morphological characteristics

All actinomycete strains were observed spore chains under light microscope (Leica DM750, Leica, Germany) with long working distance lens (40X) to interpret type of spore chains.

3.2.1.2 Cultural characteristic

All actinomycete strains were performed by using 7 to 14 days cultures grown on ISP2 agar medium at 30 ± 2 °C for 14 days. The colour of aerial mycelium was indicated with ISCC–NBS colour system (Paul, 2016).

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3.2.1.3 Physiological and biochemical characteristics

3.2.1.3.1 Sodium chloride tolerance

The selected actinomycete strains were grown in differential concentration of NaCl (1 to 12%) with ISP2 medium and incubated at 30 ± 2 °C for 14 days.

3.2.1.3.2 pH tolerance

The selected actinomycete strains were grown in differential pH (pH 4 to pH 12) with ISP2 medium and incubated at 30 ± 2 °C for 14 days.

3.2.1.3.3 Temperature tolerance

The selected actinomycete strains were grown in differential temperature at 4, 10, 20, 25, 30, 37, 40, 45, 50, and 55 °C with ISP2 medium and incubated at 30 ± 2 °C for 14 days. Temperature tolerance at 80 °C for 15 mins was tested for microencapsulated formation.

3.2.1.3.4 Carbon source utilisation

The selected actinomycete strains were observed carbon source utilisation by using basal agar medium (ISP9) and added 1% of carbon source then incubated at 30 ± 2 °C for 14 days. The carbon source including of galactose, glucose, arabinose, sucrose, mannose, raffinose, xylose, and mannitol. The positive control was glucose and negative control was ISP9.

1) Positive utilisation (+), when the actinomycete could grow equal or more than positive control.

2) Weakly positive utilisation (w), when the actinomycete could grow more than negative control and less than positive control.

3) Negative utilisation (-), when the actinomycete could grow equal or less than negative control.

3.2.1.3.5 Insoluble compound

The selected actinomycete strains were grown in differential insoluble compound including of hypoxanthine, xanthine, adenine, cellulose, and tyrosine with ISP2 medium and incubated at 30 ± 2 °C for 14 days. A clear zone around the colonies were considered as positive result.

3.2.1.3.6 Amylase production

The selected actinomycete strains were streaked on International *Streptomyces* Project no.4 (ISP4) and incubated at 30 ± 2 °C for 7 days. After incubation

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time, 3 mL of 1% iodine (appendix B) was added to each plate. The development of clear zone around the colonies were considered amylase production (Hankin and Anagnostakis., 1975).

3.2.1.3.7 Gelatin liquefaction

The selected actinomycete strains were grown in bouillon gelatin broth and incubated at 30 ± 2 °C for 21 days. The result was determined by keeping in 4 °C for 30 mins. After that, the positive result will show liquefaction when lay the tube down.

3.2.1.3.8 Nitrate reduction

The selected actinomycete strains were grown in peptone KNO_3 broth incubated at 30 ± 2 °C for 14 days. After incubation time, sulfanilic acid 2 drops and 3 drops of *N, N*-dimethyl-1-naphthylamine (appendix B) were dropped into the medium. If nitrate (NO_3^-) change to nitrite (NO_2^-), the medium will be turned to pink or red.

3.2.1.3.9 Melanin production

The selected actinomycete strains were observed melanoid pigments on International *Streptomyces* Project no.6 and incubated at 30 ± 2 °C. The positive result will show brown to black diffusible pigment.

3.2.2 Genotypic characteristics

Identification of the selected actinomycete strains by 16S rRNA gene analysis. The selected actinomycete strains (The strains that showed antibacterial activity and showed %RGI more than 50%) were classified and identified based on 16S rRNA gene analysis. The potential strain was identified based on whole genome sequencing.

3.2.2.1 DNA extraction

The selected actinomycetes were grown on ISP2 broth at 30 ± 2 °C for 24 to 120 hrs (not over 5 days) at 180 rpm. After growth, the culture broth was separated by centrifuged (13,000 rpm/10 mins) then discarded the supernatant and washed cell with 1mM SEDTA pH 8.0 for three times. Added 20 µL of lysozyme (concentration 100 mg/mL) mixed well and incubated at 37 °C for 30 mins. After that, added 40 µL of 10% SDS mixed well then incubated at 56 °C for 1 to 3 hrs or overnight (If cells were not lysed as seen by cleared solution with increased viscosity). Added 100 µL of 5M sodium chloride (NaCl) and 100 µL of 10% CTAB (10%CTAB/0.7M NaCl) then mixed well and incubated at 65 °C 10 mins. Added 400 µL of chloroform mixed

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well and centrifuged (13,000 rpm/10 mins). Transferred aqueous phase to 1.5 mL clean microcentrifuge tube. Added 400 µL of phenol: chloroform to the aqueous phase mixed well by hand and centrifuged (13,000 rpm/10 mins). Pipet the top layer into new 1.5 mL microcentrifuge tube. Add 4 µL of RNase A (concentration 100mg/mL) incubated at 37 °C for 1.30 hrs. After that, added 400 µL of phenol: chloroform, mixed well by hand and centrifuged (13,000 rpm/10 mins). Transferred aqueous phase to new 1.5 mL microcentrifuge tube and extracted DNA by adding cold absolute ethanol (keep absolute ethanol in -20 °C), mixed well by hand and discarded ethanol then dried DNA by reversed the tube on sterile tissue. The DNA was used to be DNA template for polymerase chain reaction by adding 50 to 100 µL of TE buffer (appendix B).

3.2.2.2 Polymerase Chain Reaction, PCR

DNA amplification, sequencing and phylogenetic analysis of active isolates were identified by using 16S rRNA gene. The active isolates were grown on ISP2 agar medium for 4 days at 30 ± 2 °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′GAGTTTGATCITIGCTCAG3′) 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 µL in total included 25 µL AccuPower® *Taq* PCR Master Mix (Bioneer), 18 µL dH_2O, 2.5 µL the final concentration 10 pMol of each primer and 2 µL DNA template. The cycling conditions for the amplification of the 16S rRNA gene region were as follow: 3 mins at 94 °C, 25 cycles at 94 °C for 1 min, at 50 °C for 1 min and 2 mins at 72 °C, then followed by a final extension step for 3 mins at 72 °C. 3 µL of PCR product was determined by using 0.8% agarose gel buffer (appendix B) supplemented with SYBR® Safe DNA gel strain (Invitrogen, USA). Gel was developed in 1X TAE buffer with 100 volts for 30 mins then observed DNA band by using UltraSlim® LED illuminator.

3.2.2.3 Purification of PCR product

PCR product was purified by using Invitrogen™ Purelink™ PCR Purification kit, USA. DNA purity was determined by using NanoDrop™ Lite Spectrophotometer (Thermo Scientific, USA). The absorbent of pure DNA A_{260}/A_{280} approximately 1.85-1.88

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3.2.2.4 16S rRNA gene sequencing

Purified PCR product was analysed at First BASE Laboratories Sdn Bhd (Malaysia) by using BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The analysis primers were using 518F (5'-CCAGCAGCCGCGGT AATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3').

3.2.2.5 Analysis of 16S rRNA gene and phylogenetic tree construction

The sequences of 16S rRNA gene was cap contig assembly with the BioEdit Sequence Alignment Editor (version 7.0.0) software. Comparing the actinomycete strains with type strains in EzTaxon-server (https://www.ezbiocloud.net; Kim *et al*., 2012). Alignment nucleotides of the potential strains and nucleotide of type strains from EzTaxon by using clustal W in BioEdit software. Gap and unidentified nucleotides were eliminated. Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The maximum likelihood method of Kumar *et al*., (2016) was used to construct a phylogenetic tree with the program MEGA7. Accession number for a nucleotide sequence of the potential strains were submitted in DDBJ-server for (https://www.ddbj.nig.ac.jp/ddbj/submission-e.html).

3.2.2.6 Analysis of whole genome sequencing

Whole genome sequencing was analysed by Omics Sciences and Bioinformatics Center (OMICs), Chulalongkorn university, Thailand. The annotation fasta file of whole genome sequencing was analysed secondary metabolite in antiSMASH database (http://antismash-db.secondarymetabolites.org/) (Kai *et al*., 2016) and accession number for a whole genome sequence was submitted in NCBI database (https://submit.ncbi.nlm.nih.gov/). Analysed digital of DNA-DNA hybidisation was performed in Type (Strain) Genome Server (https://tygs.dsmz.de). Comparisation of whole genome sequencing of actinomycete strain with type strain for Average Nucleotide Identity (ANI) by using Average Nucleotide Identity (ANI) calculator (http://jspecies.ribohost.com/jspeciesws/ ; Richter *et al.,* 2015).

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3.3 Antimicrobial activities

3.3.1 Preliminary screening for antibacterial activities

The preliminary screening of the actinomycetes isolates against test microorganisms that including of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, and *Xanthomonas oryzae X3* was performed by T' streak method (Figure 3.1) and against test fungi was performed by dual culture method (Figure 3.2). The T' streak method, ISP2 agar plates were used inoculated with the actinomycete isolate by single streak at the middle of petri dish plate and incubated at 30 ± 2 °C for 7 days. The test microorganisms were streaked perpendicularly to the actinomycetes and incubated (bacteria were incubated overnight at 30 ± 2 °C). The antagonism against each test microorganism was measured in millimeter unit (Naif *et al*., 2016).

Figure 3.1 Preliminary screening for antimicrobial activities.

3.3.2 Crude extracts preparation and screening for secondary metabolite

The potential strains were cultivated with ISP2 broth medium in incubator shaker at 150 rpm, 30 ± 2 °C for 14 days. The broth culture was filtered through Whatman No. 1 filter paper to separate supernatant and cells. Using ethyl acetate as an organic solvent for extracting the bioactive compounds by adding an equal volume (v/v) of the solvent to supernatant filtrate in separation funnel for 3 times. The mixture filtrate with ethyl acetate solvent was appearing in two layers, the organic solvent layer contained the secondary metabolites (upper layer). The upper layer was collected and evaporated solvent using rotary evaporator. The crude extract was obtained and stored in a desiccator. Harvested cells were fermented in equal volume of methanol for another 3 days in incubator shaker at 150 rpm, 30 ± 2 °C and then also evaporated. The completely dried residues were dissolved in methanol to be used for agar disc

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diffusion studies. Antibacterial activity of partially purified extracellular (ethyl acetate crude extract) and intracellular (methanolic crude extract) cell extracts; were determined by agar disc diffusion method. Cell concentration of all test microorganisms were adjusted at 0.5 McFarland turbidity standards and swab on Mueller Hinton Agar (MHA) for bacterial cells by using sterilized cotton swabs. Sterile disc containing 20 µL of each crude extract (concentration 1 and 50 mg/mL) were placed on the agar plates. Plates were incubated at 37 ± 2 °C for 24 hrs (Selvameenal *et al*., 2009).

3.3.3 Antifungal activities

In dual culture method, potato dextrose agar (PDA) plates were used, inoculated with the actinomycete strains by single streak method. The steak line far from the edge of petri dish 2 cm. and incubated at 30 ± 2 °C for 7 days then using cock borer size 5 mm. plug the fungi and placed on opposite site of actinomycete far from edge of petri dish 2 cm. 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 inhibition percentage.

The inhibition percentage of the growth of the fungi was measured as equation (2):

$$\%inhibition = \frac{(R1 - R2)}{R1} \times 100 \quad \textbf{(2)}$$

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)

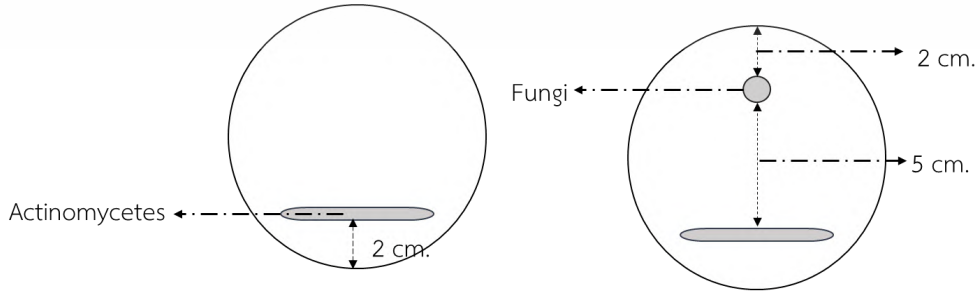

Figure 3.2 Dual culture method.

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3.4 In *vitro* screening of degrading enzyme production to fungal cell wall

Actinomycete strains that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50% were studied of hydrolytic enzyme production.

3.4.1 Screening of protease production

3.4.1.1 Skim milk agar plate

The selected actinomycete strains were spot inoculated on skim milk agar plates and incubated at 30 ± 2 ℃ for 7 days. A clear zone of hydrolysis appeared after 3-14 days incubation indicative of protease synthesis (Atlas, 1997).

3.4.1.2 Coagulation test medium

The selected actinomycete strains were inoculated in 10% skim milk and incubated at 30 ± 2 ℃ for 7 to 14 days. A positive result was determined by producing coagulation.

3.4.2 Screening of lipase production

3.4.2.1 Tween80

Tween80 agar plates (appendix A) were divided into four parts then the selected actinomycete strains were spot inoculated the agar cultivation test and incubated at 30 ± 2 ℃ for 7 days. A clear zone of hydrolysis appeared after 3 to 14 days incubation indicative of lipase synthesis (Schoofs *et al*., 1997).

3.4.2.2 Phenol Red

Phenol red agar plate (appendix A) was prepared by using olive oil as basal of lipid. The plate was divided into four wells in each plate (Singh *et al*., 2006). The selected actinomycete strains were spot inoculated into each well and incubated at 30 ± 2 ℃ for 7 days. The positive result was determined by producing yellow zone around well.

3.4.3 Screening of chitinase production

Chitin agar plate (appendix A) was prepared by following method of Nawani and colleague (2002). The selected actinomycete strains were spot inoculated on the agar cultivation test and incubated at 30 ± 2 ℃ for 7 days. A clear zone of hydrolysis appeared after 3 to 14 days incubation indicative of chitinase synthesis.

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3.5 In *vitro* screening of plant growth-promoting (PGP) activities

Actinomycete strains that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50% were studied of siderophore production, phosphate solubilisation, hydrogen cyanide (HCN) production, and indole acetic acid (IAA).

3.5.1 Siderophore production

Before preparing the Chrom azurol S (CAS) agar medium. All of glass wears that used prepare the medium, there were rinsed with 6M Hydrochloric acid to get rid of any trace elements, then rinse with distilled water.

Siderophore production test of the selected actinomycete isolates were screened on CAS agar medium. The selected actinomycete strains were spot inoculated on the medium and incubated at 30 ± 2 ℃ for 7-14 days. A positive CAS reaction was observed by changing to be yellow colour around the growth spot (Schwyn and Neilands, 1987 ; Krewulak and Vogel, 2008).

3.5.2 Indole acetic acid (IAA) production

Indole acetic acid (IAA) production test of selected actinomycete isolates from Yeast extract - Malt extract (YM) agar was cultivated in YM broth with 0.2% L-tryptophan and incubated in an incubator shaker at 180 rpm, 30 ± 2 ℃ for 7 days. The cultivation was centrifuged at 10,000 rpm for 10 minutes to separate supernatant and cells. Supernatant was collected and determined IAA production by adding Salkowski reagent (appendix B) in a ratio of 2:1 and incubated for 25 mins at room temperature. Colour transformed to a pink colour indicated the IAA production, and quality determination of IAA was measured by using a spectrophotometer at 530 nm. The IAA production from each strain was compared with the IAA standard and a standard curve was prepared using different concentrations of pure IAA (0, 25, 50, 75, 100, and 125 µg of IAA/mL). The supernatant from uninoculated test tubes in which no visible colour was observed and used as a control (Kaur *et al*., 2013).

3.5.3 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

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agar plates and incubated at 30 ± 2 ℃ for 3 to 14 days. The present of clear zone around the spot growth indicated phosphate solubilisation activity (Gaur, 1990).

3.5.4 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. The selected actinomycete strains were streaked on these modified agar plate. Whatman filter paper No.1 was dipped in 2% sodium carbonate in 0.5% picric acid solution (appendix B) then placed on the top of the plate. Plates were sealed with parafilm and incubated at 30 ± 2 ℃ for 3 to 14 days. The orange to red colour of filter paper was indicated a positive test for HCN production (Anwar *et al*., 2016).

3.5.5 Ammonia production

The selected actinomycete strains were tested for ammonia production in peptone water medium. The selected actinomycete isolates were inoculated into 10 mL of peptone water and incubated at 30 ± 2 ℃ for 7 to 14 days. After that 0.5 mL Nessler ‘s reagent (appendix B) was applied to each tube. The results showed brown to yellow colour was determined to be a positive test for ammonia production (Cappucino and Sherman, 1992).

3.6 Study of potential antifungal strain *Streptomyces* sp. NK08203

3.6.1 Determined of tolerance to commercial chemical and biological product using for agriculture

The potential strain was determined of tolerance to commercial chemical and biological product as shown in Table 3.1. The potential strain was spread on ISP2 medium then 200 µL of final concentration were applied 80 grams paper (1 x 7 cm.) and placed on the middle of petri dish plate. The positive result was determined by no showing a clear zone around the paper.

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Table 3.1 Commercial chemical and biological product using for agriculture.

Commercial name	Final concentration	Commercial name	Final concentration
Amigo	0.25%	Provado	0.0075%
Thaibact	3.00%	*Trichoderma* spp.	0.4%
Greendive	0.05%	Beauverin	0.4%
Carbendazim	0.05%	Metarhzan	1%
Beam 75	0.075%	TAB-1	0.25%
Omycin	0.20%	Quinclorac	0.25%
Paraquat	1.00%	Siamese neem	1.5%

3.6.2 Study of spore formation in submerged cultivation

Standard inoculum of each strain was prepared by using conical flasks (250 mL volume) containing 100 mL of ISP2 medium then inoculated with *Streptomyces*. There were incubated in an incubator shaker at 30 ± 2 °C for 3 days and shaken at 150 rpm. The appropriated cultivation condition was conducted by comparing the effect of two differences medium on spore formation including of ISP2 and modified ISP2. The medium was prepared in conical flasks (2,000 mL volume) and containing 1,000 mL of the medium. 100:1000 of the standard inoculum (approximately 5 to 7 mg dried biomass) was applied to each medium then incubated in an incubator shaker at 30 ± 2 °C and shaken at 150 rpm for 4 days. Six milliliters of sample were taken every 24 hrs to determined pH, dry biomass, total glucose, and colony forming unit (CFU).

3.6.2.1 pH

The sample was detected pH by using pH meter (Horriba, USA)

3.6.2.2 Dry biomass

One mL of each strain was filtrated pass-through glass microfiber filter (GF/C). The pellets were twice washed with distilled water and determine dry cell weight (two replicates).

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3.6.2.3 Total glucose

300 µl of taken sample was determined total sugar in culture by using Biochemistry Analyser (YSI 2900D, USA).

3.6.2.4 Spore preparation

Four days incubation time, one mL of harvested spores was sonicated at 50% amplitude at 30 °C for 2 mins (Antonio *et al*., 1989). Comparing with ISP2 medium that used as control sample. The physical morphology of spore formation was also observed by using light microscope.

3.6.2.5 Colony forming unit (CFU)

The sample was diluted with ten-fold dilution and spread on ISP2 agar plate then incubated for 3 days and the colonies were counted.

3.6.3 Minimum inhibitory concentration (MIC): Minimum fungicidal concentration (MFC)

Minimum inhibitory concentration (MIC) determination was used broth dilution method. The spore suspension of actinomycete was prepared (as 3.6.2.4) and enumerated the CFU/mL by spread plate technique. The actinomycete suspension was serially diluted with water up to 10^{-12}. The spore suspension of *Pyricularia* was determined by using hemacytometer (10^6 CFU/mL).

The test bottle contained potato dextrose broth media (8 mL). One mL of actinomycete spore suspension and one mL of *Pyricularia* spore suspension was added to the test bottle. One mL of water and one mL actinomycete spore suspension was added to PDB medium as actinomycete control. One mL of water and one mL *Pyricularia* spore suspension was added to PDB medium as *Pyricularia* control. The bottles were incubated in shaker at 150 rpm, 30 ± 2 °C for 4 days. After incubation, each bottle was spread on PDA medium for minimum fungicidal concentration (MFC) determination. MIC determination was examined by comparing mixture solution in different dilution with actinomycete control and *Pyricularia* control. MFC determination was examined by observing fungal colony on PDA medium.

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3.6.4 Powder formulation

3.6.4.1 Encapsulation formulation by spray-drying technique

Microparticles were conducted by spray-drying technique (Mini Spray-Dryer BÜCHI B-290, BÜCHI, Switzerland). The carriers to immobilize potential strain as biocontrol formulation were comparing as shown in Table 3.2

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Table 3.2. The composition of encapsulation formation.

Formulation no.	1% Wall material (A)	2% Preserved spore (B)	Wall material (C)			Wall material (D)			20% Spore
			5%	10%	15%	0.05%	0.1%	0.3%	
1	✓	✓	✓	-	-	✓	-	-	-
2	✓	✓	✓	-	-	-	✓	-	-
3	✓	✓	✓	-	-	-	-	✓	-
4	✓	✓	✓	-	-	✓	-	-	✓
5	✓	✓	✓	-	-	-	✓	-	✓
6	✓	✓	✓	-	-	-	-	✓	✓
7	✓	✓	-	✓	-	✓	-	-	-
8	✓	✓	-	✓	-	-	✓	-	-
9	✓	✓	-	✓	-	-	-	✓	-
10	✓	✓	-	✓	-	✓	-	-	✓

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Table 3.2. The composition of encapsulation formation (continued).

Formulation no.	1% Wall material (A)	2% Preserved spore (B)	Wall material (C)			Wall material (D)			20% Spore
			5%	10%	15%	0.05%	0.1%	0.3%	
11	✓	✓	-	✓	-	-	✓	-	✓
12	✓	✓	-	✓	-	-	-	✓	✓
13	✓	✓	-	-	✓	✓	-	-	-
14	✓	✓	-	-	✓	-	✓	-	-
15	✓	✓	-	-	✓	-	-	✓	-
16	✓	✓	-	-	✓	✓	-	-	✓
17	✓	✓	-	-	✓	-	✓	-	✓
18	✓	✓	-	-	✓	-	-	✓	✓

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3.6.4.2 Determination of spray-dried powder yield

Spray-drying powder was measured as equation ratio of the weight of the resultant powder after spray drying in collecting chamber and the weight of all solids (including wall and core materials) in the emulsion, expressed as percentage. This yield will be only approximate, any particle sticking to the cyclone wall or drying chamber were not determined.

3.6.4.3 Determination of moisture content

The moisture content (%M) of the spray-dried powders was determined using moisture analyzers (RADWAG, Poland).

3.6.4.4 Determination of viable cell after spray-drying

One gram of spray-dried powders was dissolved in 9 mL of sterile distilled water. Colony forming unit (CFU) was measured by spread plate technique using ISP2 medium and the plates were incubated at 30 ± 2 °C for 4 days.

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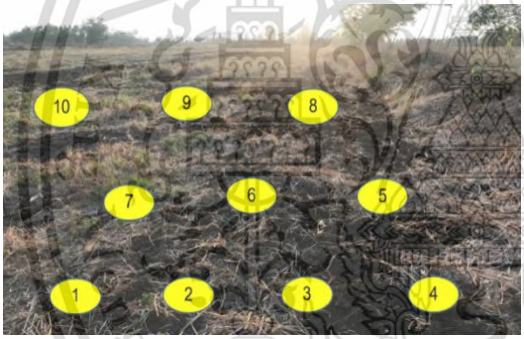
Chapter 4

Main results and discussion

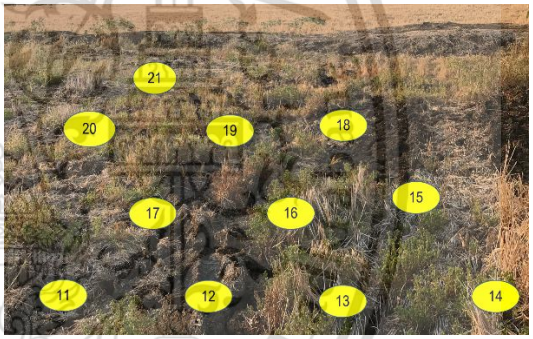
4.1 Isolation and preservation of actinomycetes

4.1.1 Collection of soil sample

Twenty-one soil samples were collected from rice field soil at Nong Bua, Nakonsawan, Thailand (Figure 4.1 (1)) on January 12, 2019 (area 1 to 10) and February 28, 2019 (area 11 to 21) (Figure 4.1 (2)). The code strain of actinomycete isolated from Nakonsawan province started with "NK". Four soil samples were collected from "Happy Rice Organic Farm" at Nong sua, Pathumthani, Thailand (Figure 4.1 (3)) on September 25, 2019 (area 22 to 25). The code strain of actinomycete isolated from Pathumthani province started with "HPF".



(1)



(2)

(3)

Figure 4.1 Rice field soil at Nong Bua, Nakonsawan, Thailand (1), (2) and Happy Rice Organic Farm at Nong sua, Pathumthani, Thailand (3).

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Soil samples were measured some properties of soil including of temperature, pH, and moisture. The results showed that the soil temperature range is between 30 and 39 °C, pH range of soil samples is between 5.7 and 8.5, and the percentage of soil moisture range is between 6 and 41.5 % as shown in Table 4.1.

Table 4.1 Soil properties measurement.

Area	Temperature (°C)	pH	Soil moisture (%)	Source
1	37	8.0	7.42	Nakonsawan, Thailand
2	37	8.2	9.60	Nakonsawan, Thailand
3	39	8.2	6.95	Nakonsawan, Thailand
4	38	7.9	7.90	Nakonsawan, Thailand
5	38	7.6	6.05	Nakonsawan, Thailand
6	37	7.9	11.43	Nakonsawan, Thailand
7	39	7.5	2.93	Nakonsawan, Thailand
8	38	7.6	9.88	Nakonsawan, Thailand
9	37	7.6	13.39	Nakonsawan, Thailand
10	38	7.3	10.37	Nakonsawan, Thailand
11	36	8.2	3.03	Nakonsawan, Thailand
12	37	8.5	11.35	Nakonsawan, Thailand
13	35	8.2	12.93	Nakonsawan, Thailand
14	35	8.3	7.80	Nakonsawan, Thailand
15	37	8.4	14.32	Nakonsawan, Thailand
16	36	8.5	13.18	Nakonsawan, Thailand
17	35	7.9	8.28	Nakonsawan, Thailand
18	35	7.9	5.79	Nakonsawan, Thailand
19	36	7.9	5.48	Nakonsawan, Thailand
20	36	8.0	6.64	Nakonsawan, Thailand
21	37	7.7	15.50	Nakonsawan, Thailand

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Table 4.1 Soil properties measurement (continued).

Area	Temperature (°C)	pH	Soil moisture (%)	Source
22	28	5.7	40.85	Pathumthani, Thailand
23	27	6.2	38.79	Pathumthani, Thailand
24	25	5.8	41.54	Pathumthani, Thailand
25	28	6.1	37.52	Pathumthani, Thailand

4.1.2 Isolation of actinomycetes

Ten grams of soil sample was spread on modified Zhang's Soil Starch Extract (ZSSE) agar plate and the actinomycete colonies were observed every day. Commonly, colonial morphology of actinomycetes were found to be white, purple, brown, pink, orange, or gray with chalky, matt, or velvety colonies appearance as presented in Figure 4.2 (UK Standards for Microbiology Investigations, 2016). All actinomycete isolates were studied on morphological characteristic as presented in Table 4.2. and cultural characterisitic as presented in appendix C.

Figure 4.2 Colonies appearance of actinomycetes on ZSSE agar medium.

4.1.3 Preservation of actinomycetes

The actinomycete colonies were picked and cross streak on ISP2 agar medium and preserved in 20% glycerol (w/v) stocks stored at -20 and -80 °C for long term preservation. In this study, one hundred of actinomycetes were isolated (Waksman *et al*., 1961).

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4.2 Identification of actinomycetes

Identification of actinomycetes was determined phenotypic characteristics and genotypic characteristics. Phenotypic character of all actinomyces strains were studied morphological characteristic on ISP2 medium by indicating the colour of aerial mycelium comparing with ISCC–NBS colour system (Centore, 2016). Type of spore chains was observed under light microscopy with long working distance lens. The potential strains were determined biochemical, physiological characteristics, genotypic characteristics by using 16S rRNA gene analysis and whole genome sequencing.

4.2.1 Phenotypic characteristics

All actinomycetes isolates were identified based on morphological characteristic, cultural characteristic, biochemical and physiological characteristics were performed.

The morphological characteristics of actinomycete spore are one of an important basic for classification. Normally, actinomycete spores could be determined their length, shape, position, and colour. The length of spore divided into four categories include monosporous, disporous, oligosporous, and polysporous such as spore formation in long chains form tend to be the genus *Streptomyces* and *Norcadiopsis*. The main type of *Streptomyces* spore chain can be differentiated from *Norcadiopsis* genus by performing straight or flexuous spore chains (rectiflexibiles type), spore chains with hooks or loops (rectinaculiaperti type), spore chains in spirals with closed or open spirals (spira type), and spore chains in spirals with umbel branch (verticillati type) but spore chains of *Norcadiopsis* genus might be straight or zigzag shaped in various lengths (Meyer, 1989). Spore chain could be classified into four types as shown in Figure 4.3 and Table 4.3 including of rectinaculiaperti type (1), spira type (2), verticillati type (3), and rectiflexibiles type (4) (Qinyuan, 2016).

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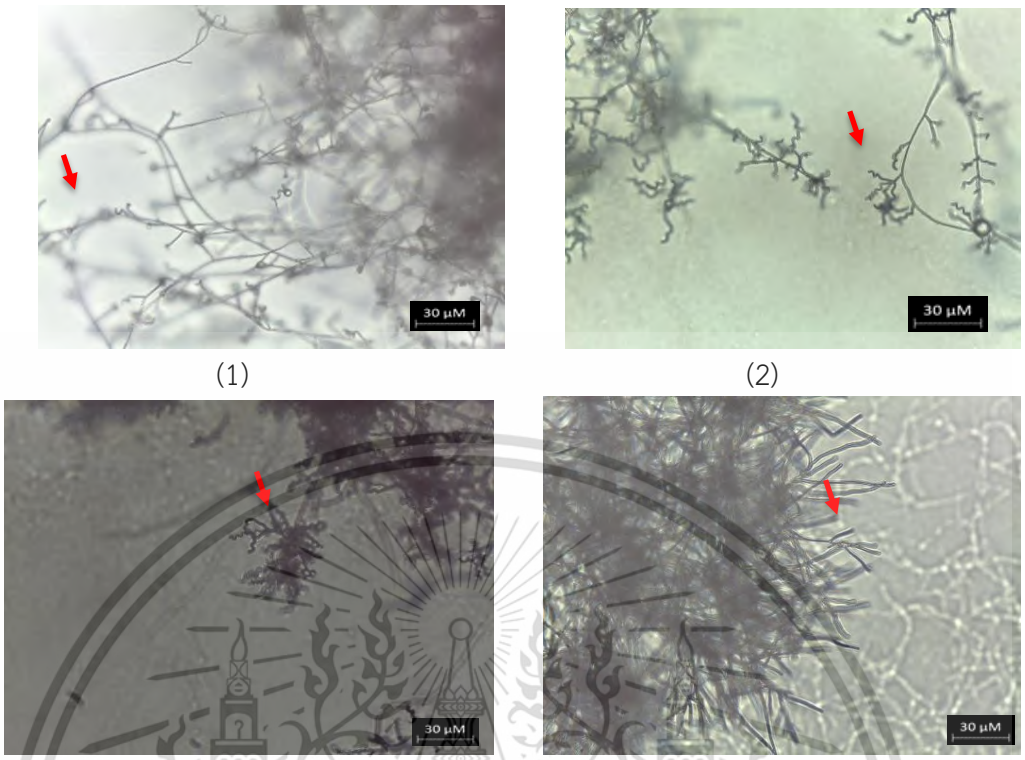

(1) (2)

(3) (4)

Figure 4.3 Classification of actinomycetes spore type: Rectinaculiaperti type of isolate HPF4301 (1), Spira type of isolate NK15104 (2), Verticillati type of isolate NK16301 (3), and Rectiflexibiles type of isolate NK17205 (4).

Table 4.2 Type spore chains of actinomycetes isolate.

Category	Spore type	Isolate no.
1	**Rectinaculiaperti type**	Six actinomycete isolates presented rectinaculiaperti type of spore including of isolate no. : NK16201, NK16302, NK16303, HPF3101, HPF3303, HPF4201, and HPF4301.
2	**Spira type**	Twenty-five actinomycete isolates presented spira type of spore including of isolate no. : NK03101, NK03201, NK05202, NK05206, NK09102, NK09201, NK10101, NK10105, NK11302, NK15103, NK15104, NK15202, NK16101, NK16102, NK16103, NK16202, NK16204, NK16205, NK16206, NK17203, NK17204, NK19301, NK21202, HPF1301, and HPF4203.

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Table 4.2 Type spore chains of actinomycetes isolate (continued).

Category	Spore type	Isolate no.
3	**Verticillati type**	Seven actinomycete isolates presented verticillati type of spore including of isolate no. : NK01104, NK11301, NK15101, NK16104, NK16203, NK16301, and HPF2203.
4	**Rectiflexibiles type**	Forty actinomycete isolates presented rectiflexibiles type of spore including of isolate no. : NK01101, NK01102, NK01103, NK03202, NK04101, NK04103, NK05101, NK05104, NK05105, NK05203, NK05207, NK07201, NK07203, NK08201, NK08202, NK08203, NK08209, NK10102, NK10103, NK10107, NK13101, NK13102, NK13201, NK14101, NK15203, NK16105, NK17101, NK17200, NK17205, NK18101, NK18201, NK21200, NK21201, HPF1201, HPF1202, HPF1203, HPF2102, HPF2201, HPF2209, and HPF4303.
5	**Non-producing spore**	Twenty-one isolates non-producing aerial mycelium on ISP2 agar medium including of isolate no. : NK01105, NK08204, NK08205, NK10106, NK15201, NK15301, NK15302, NK15303, NK17201, NK17202, NK20201, HPF1204, HPF1205, HPF2202, HPF2204, HPF2206, HPF2207, HPF2301, HPF4302, HPF4304, and HPF4308.

All actinomycetes isolates were grown for 7 to 14 days at 30 ± 2 °C on ISP2 agar medium and examined the colour of aerial mycelium, substrate mycelium and soluble pigment by using ISCC–NBS colour system (Centore, 2016). Formally, the ISCC–NBS colour system recognises by distinguishing three levels of precision. Level 1 was described the simplest colour contained 13 names: pink, orange, brown, yellow, olive, yellow green, green, blue, purple, white, gray, and black. Level 2 contained 29 names and showed more specific colour than level 1 such as reddish purple, yellowish pink, olive gray or yellowish green. Level 3 is formed by subdividing a level 2 name, using modifiers such as pale, moderate, grayish, or dark and contained 267 names of colour.

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Level 3 of the ISCC-NBS system were used for assigning the colour of actinomycetes in this study and it could be classified by the colour of aerial mycelium into fourteen categories and one category was not producing aerial mycelium on ISP2 agar medium as presented in Figure 4.4 and Table 4.3

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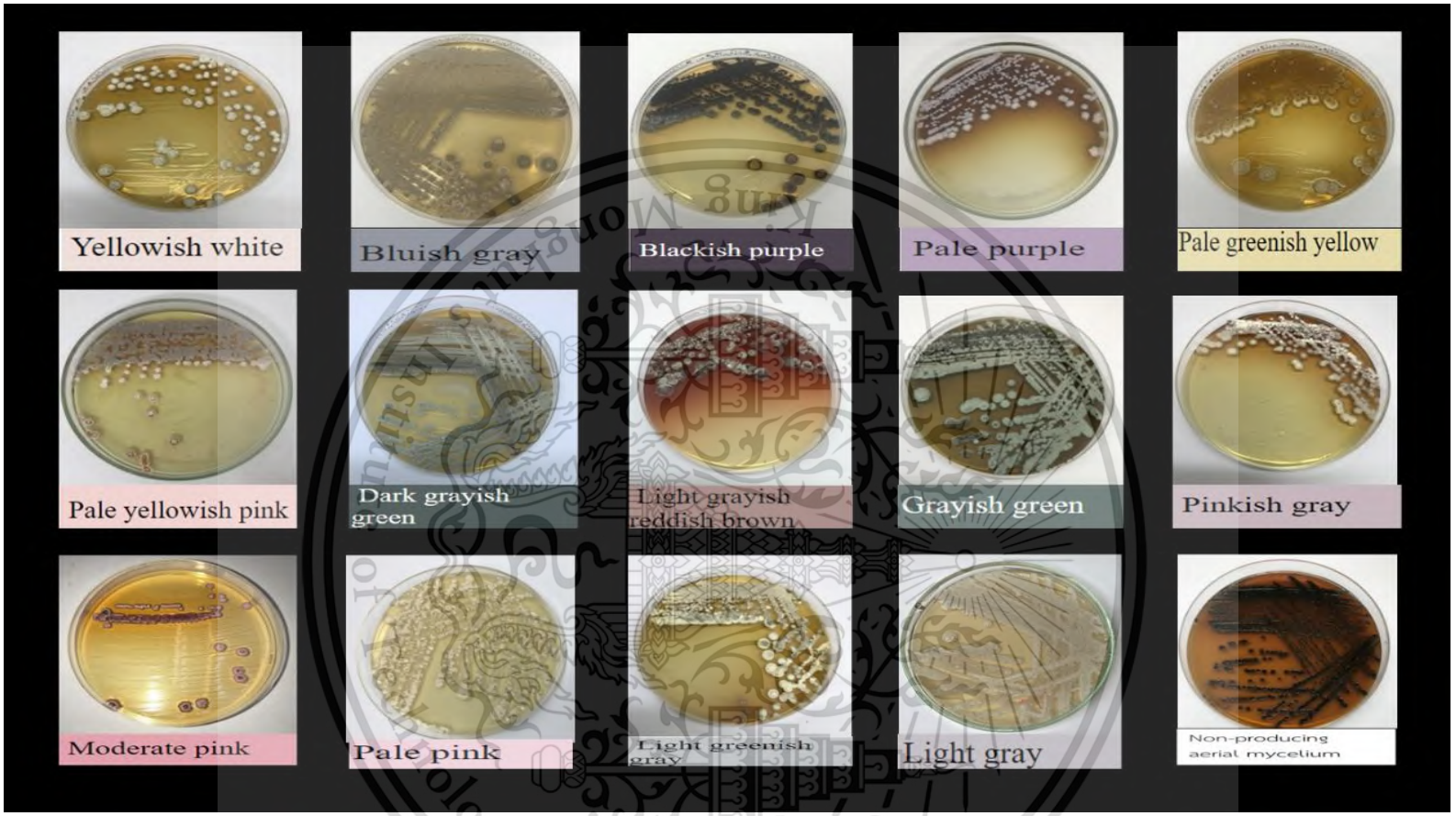

Figure 4.4 Colour classification of actinomycetes aerial mycelium isolated from rice field soil at Nakornsawan and Pathumthani province by using ISCC-NBS colour system.

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Table 4.3 Colour classification of actinomycetes isolate from rice field soil, Nakornsawan and Pathumthani province by using ISCC-NBS colour system.

Category	ISCC-NBS colour system	Isolate no.
1	Yellowish white	Twenty-eight actinomycete isolates presented yellowish white aerial mycelium including of isolate no. : NK01101, NK04101, NK05104, NK05105, NK05202, NK05203, NK05206, NK05207, NK07201, NK08201, NK08202, NK08203, NK08209, NK09102, NK11301, NK13201, NK14101, NK15101, NK15203, NK16105, NK16302, NK21200, HPF1201, HPF1202, HPF2102, HPF2201, HPF3101, and HPF4203
2	Bluish gray	Fifteen actinomycete isolates presented bluish gray aerial mycelium including of isolate no. : NK01102, NK01104, NK04103, NK10101, NK10103, NK16201, NK17204, NK18101, NK21202, HPF1301, HPF2203, HPF3303, HPF4201, HPF4301, and HPF4303
3	Blackish purple	Two actinomycete isolates presented blackish purple aerial mycelium including of isolate no. : NK01103 and NK13102
4	Pale purple	NK03201
5	Pale greenish yellow	Three actinomycete isolates presented pale greenish yellow aerial mycelium including of isolate no. : NK03202, NK05101, and NK10102
6	Pale yellowish pink	Three actinomycete isolates presented pale yellowish pink aerial mycelium including of isolate no. : NK03201, NK17205 and NK09201
7	Dark grayish green	Four actinomycete isolates presented dark grayish green aerial mycelium including of isolate no. : NK07203, NK10105, NK10107, and NK11302
8	Light grayish reddish brown	NK13101

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Table 4.3 Colour classification of actinomycetes isolate from rice field soil, Nakornsawan and Pathumthani province by using ISCC-NBS colour system (continued).

Category	ISCC-NBS colour system	Isolate no.
9	Grayish green	Sixteen actinomycete isolates presented grayish green aerial mycelium including of isolate no.: NK15103, NK15104, NK15202, NK16101, NK16102, NK16103, NK16104, NK16202, NK16203, NK16204, NK16205, NK16206, NK16301, NK16303, NK17203, and NK19301
10	Pinkish gray	NK17101
11	Moderate pink	NK17200
12	Pale pink	NK17205
13	Light greenish gray	NK18201
14	Light gray	Two actinomycete isolates presented light gray aerial mycelium including of isolate no. : NK21201 and HPF1203
15	Non-producing aerial mycelium	Twenty-one isolates non-producing aerial mycelium on ISP2 agar medium including of isolate no. : NK01105, NK08204, NK08205, NK10106, NK15201, NK15301, NK15302, NK15303, NK17201, NK17202, NK20201, HPF1204, HPF1205, HPF2202, HPF2204, HPF2206, HPF2207, HPF2301, HPF4302, HPF4304, and HPF4308

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Actinomycetes that showed antifungal activity (%RGI > 50%) were indicated to be the selected strain. Physiological and biochemical characteristics of the selected strains were determined including sodium chloride tolerance, pH tolerance, temperature tolerance, carbon source utilisation, insoluble compound utilisation, amylase production, gelatin liquefaction, nitrate reduction, and melanin production.

Sodium chloride tolerance test: Actinomycetes were grown in differential concentration of NaCl (1 to 12%) with ISP2 medium. The result showed that isolate NK05201 and NK08203 could be tolerance up to 9% of sodium chloride. NK01101 and NK05203 could be tolerance up to 8% of sodium chloride. NK05205, NK07201, and NK08202 could be tolerance up to 7% of sodium chloride. NK08205 and NK21201 could be tolerance up to 2% of sodium chloride. HPF2102 and HPF3303 cannot grow if present of sodium chloride (Table 4.4).

Table 4.4 Sodium chloride tolerance test of the selected actinomycetes.

NaCl (%)	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
0%	+	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	-	-
2%	+	+	+	+	+	+	+	+	W	-	-
3%	+	+	+	+	+	+	+	-	-	-	-
4%	+	+	+	+	+	+	+	-	-	-	-
5%	+	+	+	+	+	+	+	-	-	-	-
6%	+	+	+	+	+	+	+	-	-	-	-
7%	+	+	+	+	+	+	+	-	-	-	-
8%	+	+	+	-	-	-	+	-	-	-	-
9 %	-	W	-	-	-	-	W	-	-	-	-
10 %	-	-	-	-	-	-	-	-	-	-	-

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

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pH tolerance test: Actinomycetes were grown in differential pH (4 to 12) with ISP2 medium. The result showed that eleven strains could be grown in pH range from 5 to 11 but could not be grown at pH 4 and three strains showed ability to grow at pH 12 (NK01101, NK05201, and NK05203) as presented in Table 4.5.

Table 4.5 pH tolerance test of the selected actinomycetes.

pH	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
4	-	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	-	-	-	-	-	-	-	-

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

Temperature tolerance test: Actinomycetes were grown in differential temperature at 4, 10, 20, 25, 30, 37, 40, 45, 50, and 55 °C with ISP2 medium. Temperature tolerance at 80 °C for 15 mins was tested for microencapsulated formulation using spray-drying process. The results showed that eleven strains could be grown at temperature 28 °C to 30 °C but could not grow at 4 °C to 10 °C, ten strains could be grown at 40°C excepted from isolate HPF3303, nine strains could be tolerance

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to 80 ℃ for 15 mins excepted from isolate NK08205 and NK21201, and isolate NK21201 and HPF2102 could be grown up to 55 ℃ as presented in Table 4.6.

Table 4.6 Temperature tolerance test of the selected actinomycetes.

Temperature (°C)	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
4 ℃	-	-	-	-	-	-	-	-	-	-	-
10 ℃	-	-	-	-	-	-	-	-	-	-	-
28 ℃	+	+	+	+	+	+	+	+	+	+	+
30 ℃	+	+	+	+	+	+	+	+	+	+	+
40 ℃	+	+	+	+	+	+	+	+	+	+	-
50 ℃	-	-	-	-	-	-	-	-	+	+	-
55 ℃	-	-	-	-	-	-	-	-	+	+	-
80 ℃ (15 mins)	+	+	+	+	+	+	+	-	-	+	+

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

Carbon source utilisation tests, all of the selected actinomycete strains could be utilised glucose, galactose, arabinose, and mannose. Almost the selected actinomycete strains could be utilised raffinose (except NK01101, NK05201, NK05203, NK05205, and NK07201), xylose (except NK01101, NK05201, and NK05203), and mannitol (except NK21201 and HPF2102). Only strain NK08205 and HPF3303 could be utilised sucrose (Table 4.7).

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Table 4.7 Carbon source utilisation tests of the selected actinomycetes.

Characteristics	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
Carbon source utilisation											
Galactose	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	+	-	-	+
Mannose	+	+	+	+	+	+	+	+	+	W	W
Raffinose	-	-	-	-	-	W	W	+	W	W	W
Xylose	-	-	-	W	W	W	W	+	+	W	+
Mannitol	+	+	+	+	+	+	+	+	-	-	+

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

The results of insoluble compound utilisation tests showed that the selected strain could be utilised hypoxanthine (NK01101, NK0520, NK05203, and NK08202), and adenine (all the selected strain except NK21202); but could not be utilised tyrosine, xanthine, and cellulose. The results of amylase production test, isolates NK05205, NK07201, NK08202, NK08203, NK08205, and NK21201 have ability to hydrolyse starch soluble. NK01101, NK05201, NK05203, NK05205, NK07201, and NK08205 showed the ability to reduce nitrate. Isolate NK08205 could be produce melanoid pigment as presented in Table 4.8.

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Table 4.8 Biochemical characteristics test of the selected actinomycetes.

Characteristics	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
Insoluble compound utilisation											
Tyrosine	-	-	-	-	-	-	-	-	-	-	-
Xanthine	-	-	-	-	-	-	-	-	-	-	-
Hypoxanthine	+	+	+	-	-	+	-	-	-	-	-
Adenine	+	+	+	+	+	+	+	+	-	+	+
Cellulose	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	+	+	+	+	+	+	-	-
Gelatinisation	+	-	-	+	+	+	+	-	W	-	-
Nitrate reduction	+	W	W	W	W	-	-	+	-	-	-
Melanin production	-	-	-	-	-	-	-	+	-	-	-

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

4.2.2 Genotypic characteristic

Identification of the actinomycetes that presented antibacterial and antifungal activity were classified and identified based on 16S rRNA gene analysis. The sequencing was deposited at DDBJ for accession number. Phylogenetic tree was evaluated based on the Kimura 2-parameter model (Kimura, 1980) by using MEGA7 (Kumar *et al*., 2016) .

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The strains that showed antibacterial activity include of NK01105, NK08204, NK13102, NK14101, and NK17205 was amplified by using universal primer (9F and 1541R) and detected band by using gel electrophoresis as Figure 4.5.

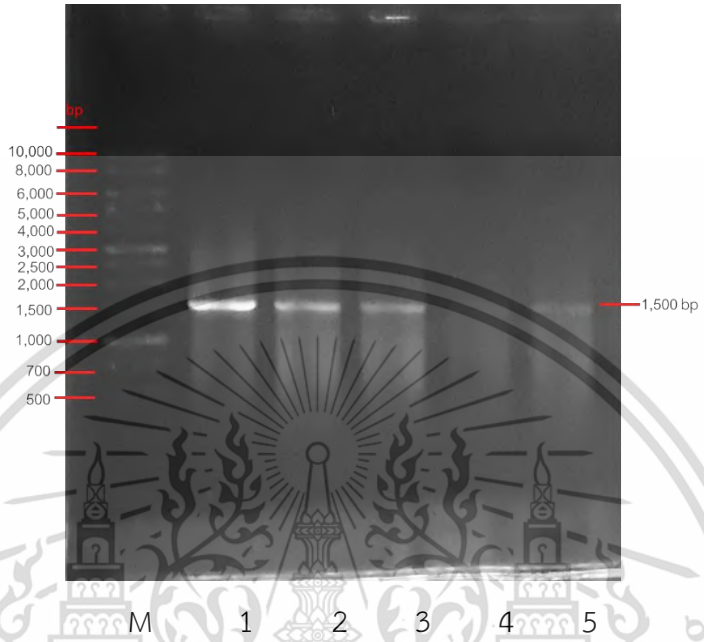

Figure 4.5 DNA amplification and agarose gel electrophoresis analysis of the actinomycete strains showed antibacterial activity : NK01105 (1), NK08204 (2), NK13102 (3), NK14101 (4), and NK17205 (5), comparing with DNA marker size 1 kb (M).

The sequences of 16S rRNA gene was cap contig assembly with the BioEdit Sequence Alignment Editor (version 7.0.0) software. Comparing the actinomycete strains with type strains in EzTaxon-server (https://www.ezbiocloud.net; Kim *et al*., 2014). Alignment nucleotides of the potential strains and nucleotide of type strains from EzTaxon by using clustal W in BioEdit software. Gap and unknown nucleotides were deleted. Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The results identification of the actinomycete strains showed antibacterial activity by 16S rRNA gene were showed in Table 4.9 and phylogenetic tree construction in Figure 4.6.

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Table 4.9 Identification actinomycete strains showed antibacterial activity by 16S rRNA gene.

Isolate no.	Nearest strain	Type strain	% Similarity	Length (bp)	Accession Number
NK01105	*Streptomyces echinatus*	NBRC 12763^{T}	99.42	1,408	LC488880
NK08204	*Streptomyces echinatus*	NBRC 12763^{T}	99.16	1,423	LC477342
NK13102	*Streptomyces avellaneus*	NBRC 13451^{T}	99.51	1,467	LC488884
NK14101	*Streptomyces bungoensis*	DSM 41781^{T}	99.86	1,440	LC488885
NK17205	*Streptomyces laurentii*	ATCC 31255^{T}	99.37	1,422	LC488888

NK01105 and NK08204 tend to be *Streptomyces echinntus* with similarity 99.42 and 99.16%, respectively. Gauvreau and Warin, (1984) reported that *S. echinntus* produces echinomycin (quinomycin A). Echinomycin is powerfully active against tumors and inhibit gram-positive bacteria. Isolate NK13102 tend to be *Streptomyces avellaneus* with similarity 99.51%. *S. avellaneus* produces novel chromomycin antibiotics, 02-3G in addition several known related antibiotics Tsuyoshi *et al*. (1989). Isolate NK14101 tend to be *Streptomyces bungoensis* with similarity 99.86%. *S. bungoensis* showed anti-Methicillin-resistant *Staphylococcus aureus* /Vancomycin-resistant *Enterococcus* activity (Jinhua *et al*., 2013). NK17205 tend to be *Streptomyces laurentii* with similarity 99.37%. *S. laurentii* produces thiostrepton (Mocek *et al*., 1993) that could be against breast cancer cells (Kwok *et al*., 2008 ; Kwok *et al*., 2011).

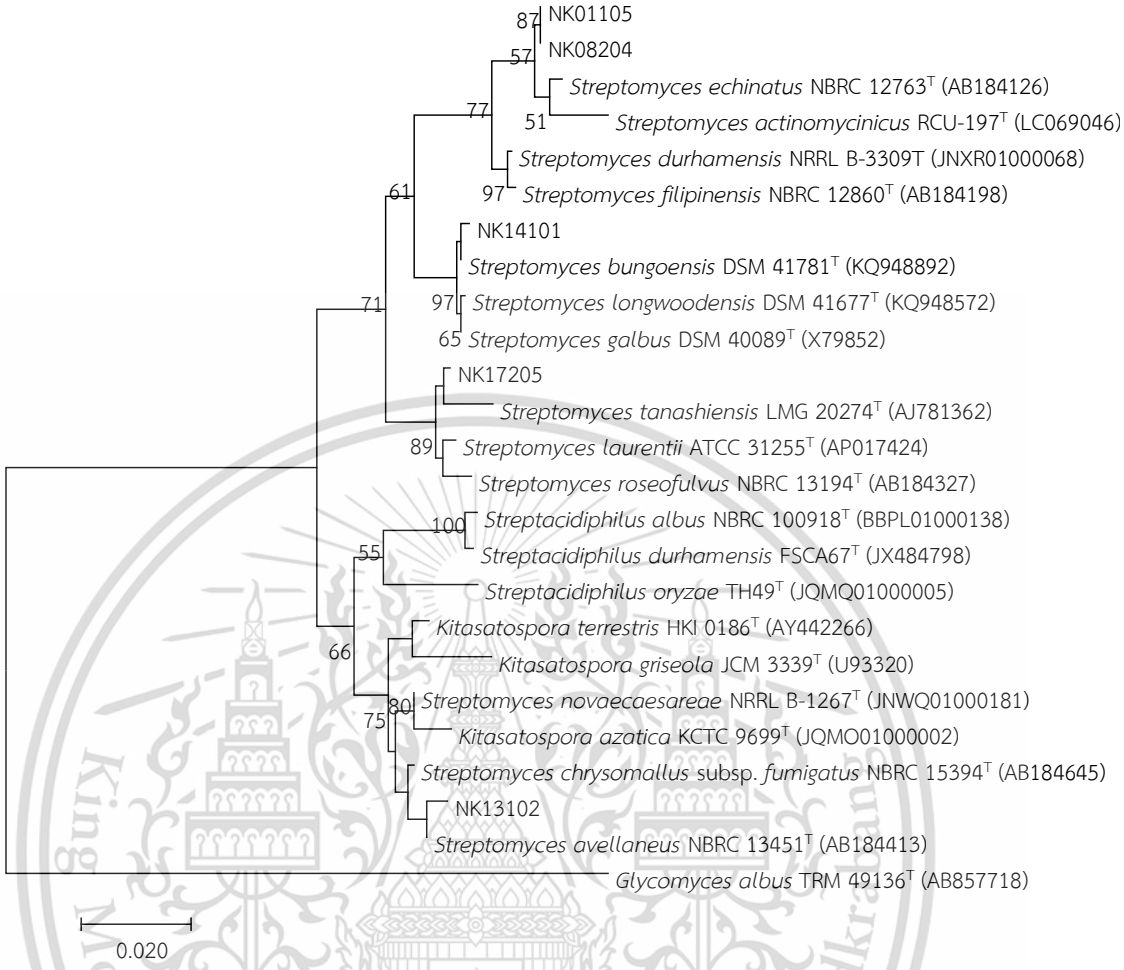

Figure 4.6 Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method for five isolates that showed antibacterial activity and their closely related type strains.

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The strains that showed antifungal activity included NK01101, NK05201, NK05203, NK05205, NK07201, NK08202, NK08203, NK08205, NK21201, HPF2102, and HPF3303 was amplified by using universal primer (9F and 1541R) and detected band by using gel electrophoresis as Figure 4.7.

Figure 4.7 DNA amplification and agarose gel electrophoresis analysis of the actinomycete strains showed antifungal activity: NK01101 (1), NK05201 (2), NK05203 (3), NK05205 (4), NK07201 (5), NK08202 (6), NK08203 (7), NK08205 (8), NK21201 (9), HPF2102 (10), and HPF3303 (11), comparing with DNA marker size 1 kb (M).

The results identification of the actinomycete strains showed antifungal activity by 16S rRNA gene were showed in Table 4.10 and phylogenetic tree construction in Figure 4.8.

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4.10 Identification actinomycete strains showed antifungal activity by 16S rRNA gene.

Isolate no.	Nearest strain	Type strain	% Similarity	Length (bp)	Accession Number
NK01101	*Streptomyces albidoflavus*	DSM 40455^T	99.56	1,406	LC516418
NK05201	*Streptomyces albidoflavus*	DSM 40455^T	99.78	1,405	LC516413
NK05203	*Streptomyces albidoflavus*	DSM 40455^T	99.79	1,435	LC516409
NK05205	*Streptomyces albidoflavus*	DSM 40455^T	99.79	1,453	LC516410
NK07201	*Streptomyces albidoflavus*	DSM 40455^T	99.79	1,413	LC516411
NK08202	*Streptomyces albidoflavus*	DSM 40455^T	99.79	1,424	LC488882
NK08203	*Streptomyces hydrogenans*	JCM 4771^T	100	1,452	LC516412
NK08205	*Streptomyces jeddahensis*	G25^T	99.17	1,339	LC477341
NK21201	*Streptomyces corchorusii*	DSM 40340^T	99.86	1,415	LC516414
HPF2102	*Amycolatopsis rhizosphaerae*	DH510-4-3^T	99.86	1,420	LC516416
HPF3303	*Streptomyces shenzhenensis*	172115^T	99.72	1,512	LC516417

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Six actinomycete strains showed antifungal activity include isolate NK01101, NK05201, NK05203, NK05205, NK07201, and NK08202 tend to be *Streptomyces albidoflavus* with similarity 99.56 - 99.79%. *Streptomyces albidoflavus* showed antifungal activity, produced antimycin A19 against resistant *Candida tropicalis* and *Pythium irregular* with high antimicrobial and cytotoxic activity (Nafis *et al*., 2018 ; Baranova *et al*., 2020).

Isolate NK08203 tend to be *Streptomyces hydrojenans* with similarity 100*%. Streptomyces hydrojenans* was reported to be biocontrol agents to control *Alternaria brassicicola* caused black leaf spot of crucifers (Manhas and Kaur, 2016).

Isolate NK21201 tend to be *Streptomyces corchorusii* with similarity 99.86*%.* Tamreihao and colleague, (20160 reported that *Streptomyces corchorusii* showed antifungal activities against rice fungal pathogens.

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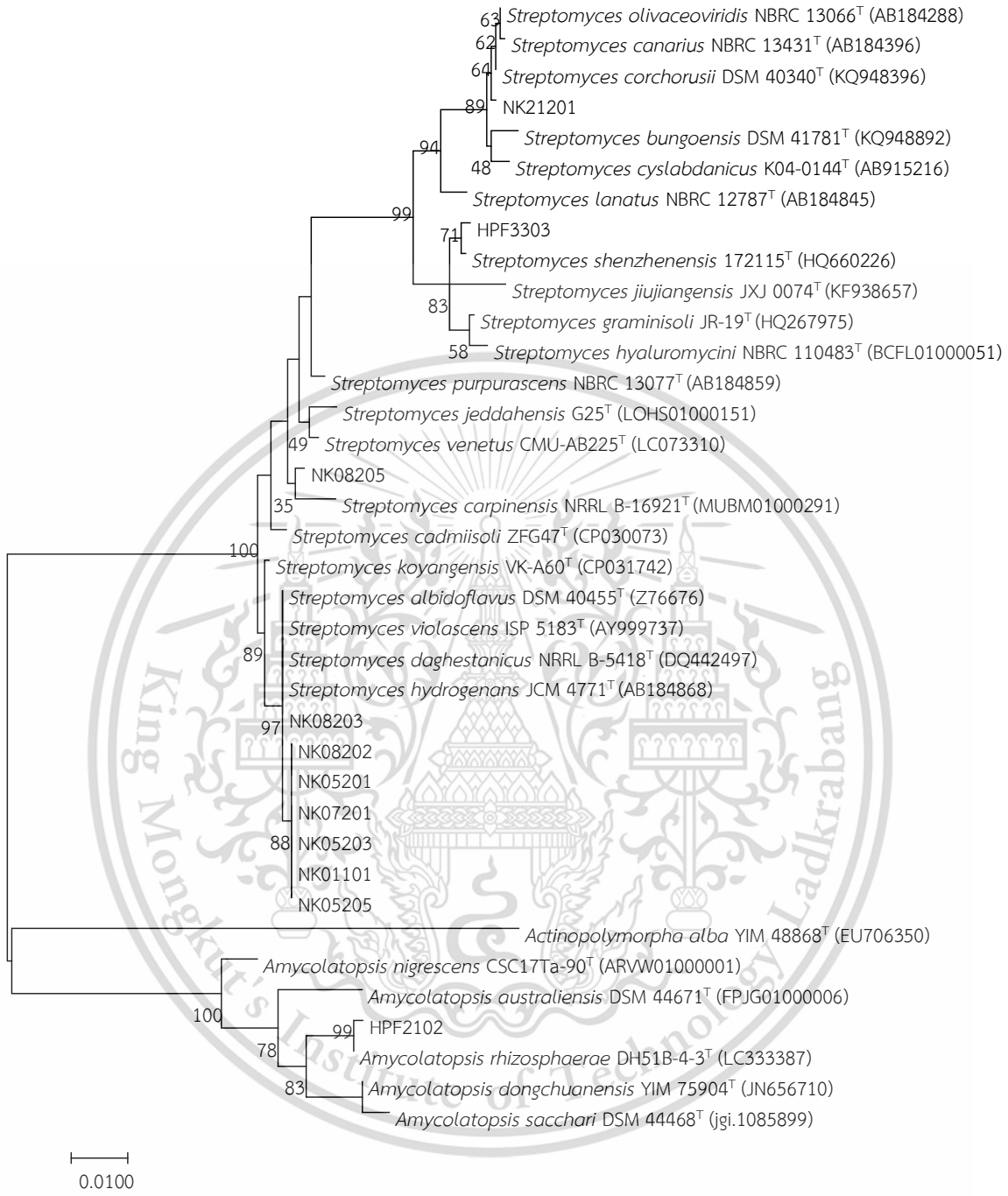

Figure 4.8 Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method for eleven isolates that showed antifungal activity and their closely related type strains.

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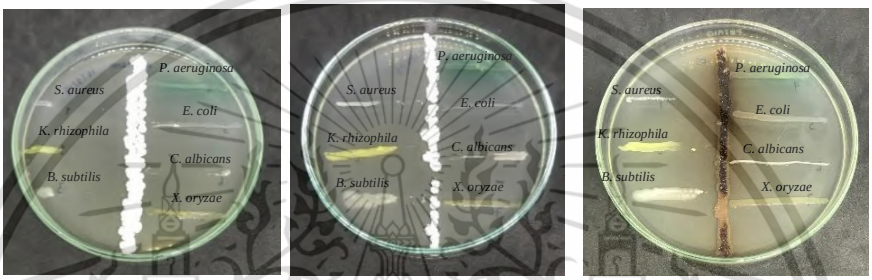
4.3 Antimicrobial activities

4.3.1 Preliminary screening for antibacterial activities

Preliminary screening for antimicrobial activity of one hundred isolates were tested against , *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Xanthomonas oryzae* X3 using T'streak method. The isolates that gave positive result (Figure 4.9) were used for secondary metabolite screen using agar disc diffusion method.



(1) **(2)** **(3)**

Figure 4.9. Some of actinomycetes showed antimicrobial activities against test microorganisms as evaluated by T' streak method. The central major streak corresponds to the actinomycetes isolates NK21200 (1), NK15203 (2) and NK01105 (3) and minor streaks with different microorganisms.

4.3.2 Crude Extracts Preparation and Screening for Secondary Metabolite Using Agar Disc Diffusion method

The results of partially purified extracellular and intracellular crude extracts showed that five actinomycetes isolates were able to inhibit growth of test microorganisms. The extracellular crude extract of NK13102 at concentration 50 mg/mL gave the highest inhibition zone against *B. subtilis* ATCC 6633 (26 mm), *S. aureus* ATCC 25923 (27 mm), *K. rhizophila* ATCC 9341 (29 mm), and *X. oryzae* X3 (9 mm) as shown in Table 4.11 and Figure 4.10, respectively.

Almost all of the purified extracellular and intracellular crude extracts had ability to inhibit gram positive bacteria rather than gram negative as presented in Table 4.11 and 4.12, respectively.

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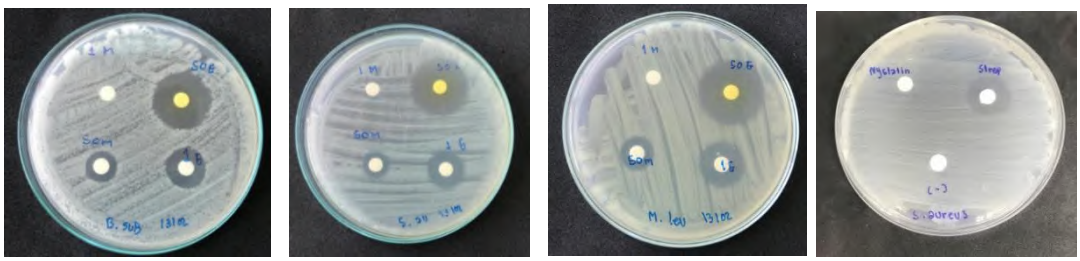

(1) (2) (3) (4)

Figure 4.10. Antibacterial activity test of the crude extract at 50 mg/ml and 1 mg/ml of isolate NK13102 against *B. subtilis* ATCC 6633 (1), *S. aureus* ATCC 25923 (2), *K. rhizophila* ATCC 9341 (3), positive control 10 µg/mL streptomycin and negative control as methanol (4) using disc diffusion method.

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Table 4.11. Antibacterial activity test of partially purified extracellular crude extracts produced by selected actinomycete isolates.

Isolate No.	Disc Concentration (mg/mL)	Diameter of Inhibition zone (mm.)					
		X. oryzae X3	*E. coli* ATCC 25922	*K. rhizophila* ATCC 9341	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*B. subtilis* ATCC 6633
NK01105	1	-	-	-	-	-	-
	50	-	-	13	-	11	15
NK08204	1	-	-	-	-	6	2
	50	-	-	19	-	25	17
NK13102	1	-	-	20	-	16	17
	50	9	-	29	-	27	26
NK14101	1	-	-	8	-	-	7
	50	-	-	18	-	12	10
NK17205	1	-	-	-	-	-	-
	50	-	-	-	-	-	-

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Table 4.12. Antibacterial activity test of partially purified intracellular crude extracts produced by selected actinomycete isolates.

Isolate No.	Disc Concentration (mg/mL)	Diameter of Inhibition zone (mm.)					
		X. oryzae X3	*E. coli* ATCC 25922	*K. rhizophila* ATCC 9341	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*B. subtilis* ATCC 6633
NK01105	1	-	-	-	-	-	-
	50	-	-	10	-	8	10
NK08204	1	-	-	-	-	-	-
	50	-	-	9	-	9	8
NK13102	1	-	-	-	-	-	-
	50	-	-	14	-	10	13
NK14101	1	-	-	8	-	-	8
	50	-	-	17	-	12	10
NK17205	1	-	-	-	-	-	-
	50	7	-	12	-	10	9

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An ability of antibacterial activity against *Xanthomonas oryzae* X3 was determined because of *Xanthomonas oryzae* is one of the most important and serious disease of rice that caused bacterial leaf blight disease. Several crop losses of yield 10 to 50% (Ou, 1985 ; Mew *et al*., 1993). If rice is infected at booting stage, bacterial blight does not affect yield production but reduced the quality of grains. Another most important and vary serious disease is blast disease. Valent (2004) reported that blast disease is the world's chief disease of rice. Blast disease is caused *Pyricularia oryzae* (*Magnaporthe oryzae*). The fungus could be infected all part of rice (Sesma and Osbourn, 2004). The symptoms were observed frequently in an infected rice field are the leaf blast, neck blast and the panicle blast. The blast fungus also causes the collar rot and nodal blast symptoms which are less common than the leaf and neck blast symptoms. Leaf blast lesion could be performed 20,000 conidia and an infected rice spikelet can produce up to 60,000 conidia in a night to keep the leaf blast infection cycle going (Kato, 2001). Approximately 5% of leaf blast infection could be induced neck blast incidence in the same crop. Neck blast is the most serious phase of holocaust as the broken necks lead to dead panicles and chaffy grains. These findings have significant implications for focused on rice blast disease and searching for the potential strain of actinomycete that could be control rice blast disease.

4.3.3 Antifungal activities

Screening for antifungal activity of one hundred isolates were tested against *Pyricularia* sp. CRI60007. The results were expressed in radial growth inhibition percentage (RGI%). Antifungal activity was evaluated by following range (Yacharone, 2009):

Weak inhibition	<50 %
Moderate inhibition	51-60%
High inhibition	61-75%
Strong inhibition	>75%

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The results of antifungal activity showed that eleven actinomycete strains were able to inhibit growth of *Pyricularia* sp. CRI60007 more than 50% including of NK05201 (100 ± 0.00%), 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%) as presented in Figure 4.11 and 4.12.

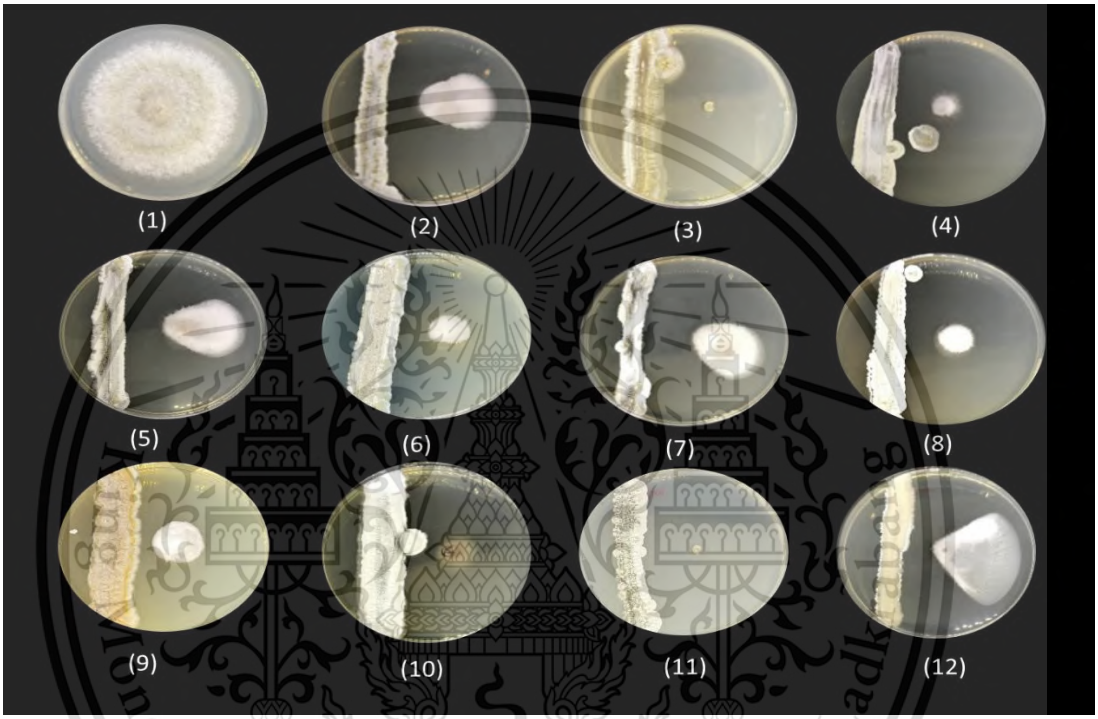

Figure 4.11 Antifungal activity of actinomycetes against *Pyricularia* sp. CRI60007 by using dual culture method ; control (1), NK01101 (2), NK05201(3), NK05203 (4), NK05205 (5), NK07201 (6), NK08202 (7), NK08203 (8), NK08205 (9), NK21201 (10), HPF2102 (11), and HPF3303 (12).

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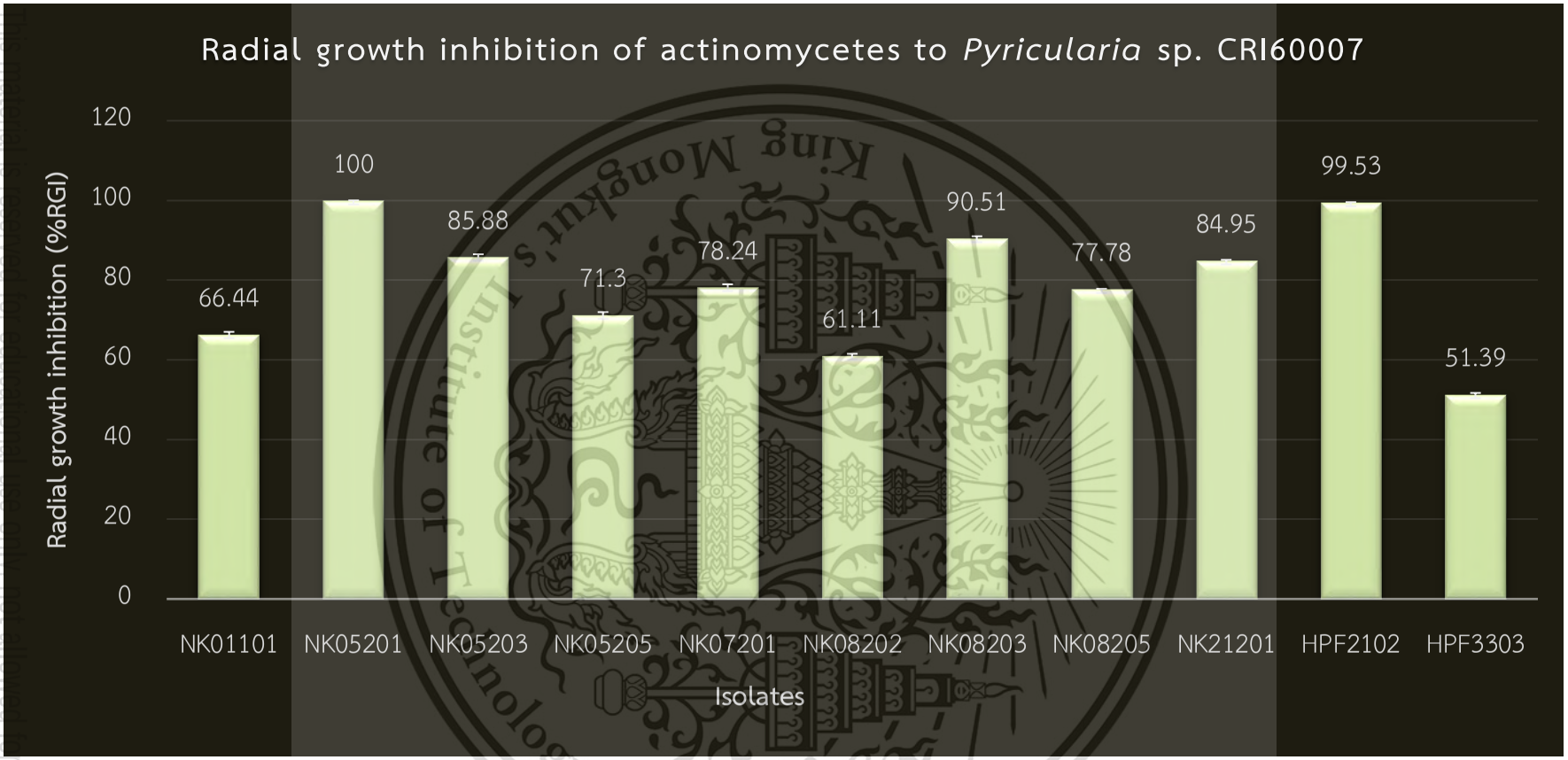

Figure 4.12 Averaged radial growth inhibition and standardisation of actinomycetes isolates against *Pyricularia* **sp. CRI60007**

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4.4 In *vitro* screening of degrading enzyme production to fungal cell wall

Actinomycete strains that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50% were studied of hydrolytic enzyme production to fungal cell wall. In this study, screening of hydrolytic enzyme production to fungal cell wall was determined enzyme protease, lipase, and chitinase production (Table 4.13).

Protease production was determined peptonisation, coagulation, and gelatinisation as presented in Figure 4.13. Isolate NK01101, NK05201, NK05203, NK05205, NK07201, NK08202, NK08203, and NK21201 showed the positive result of peptonisation by determining clear zone around the colony (Figure 4.13 (1)). Isolate NK01101, NK05205, NK07201, NK08202, NK08203, and NK21201 showed the positive result of producing coagulation (Figure 4.13 (2)). NK01101, NK05205, NK07201, NK08202, NK08203, and NK21201 could be liquefactive gelatin. (Figure 4.13 (3)).

(1) (2) (3)

Figure 4.13 Protease production: peptonisation (1), coagulation (2), and gelatinisation (3).

Lipase production was determined by using Tween 80 agar plate and phenol red plate. Screening of lipase production on Tween 80 agar was used for the detection lipase that contain esters of oleic acid (Kumar *et al*., 2012). The positive result was determined by producing turbidity around the colony (Figure 4.14 (1)). The result showed that almost the selected isolates could be produced lipase on Tween 80 agar excepted from isolate NK08205, HPF2102, and HPF3303. Screening of lipase production on phenol red agar plate, the positive result was determined by changing colour red to yellow (Figure 4.14 (2)). This method cloud be separated between esterase and lipase by using tributyrin and oil (Singh *et al*., 2006). In this study, phenol red agar

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plates were used oil as substrate. The result showed that isolate NK08202 and NK21201 have ability to produce enzyme lipase.

(1) (2)

Figure 4.14 Lipase production tested on Tween 80 agar plate (1) and phenol red agar plate (2).

Chitinase production was prepared by following method of Nawani and colleague (2002) by using chitin agar plate. All of the selected actinomycete strains showed negative result to produce enzyme chitinase.

Table 4.13 In *vitro* screening of the selected actinomycete strains for hydrolytic enzyme production against fungal cell wall.

Characteristics	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
Protease Production											
Peptonisation	+	+	+	+	+	+	+	-	w	-	-
Coagulation	+	-	-	+	+	+	+	-	w	-	-
Gelatinisation	+	-	-	+	+	+	+	-	W	-	-
Lipase production											
Tween80	+	+	+	+	+	+	+	-	+	+	+
Phenol red	-	-	-	-	-	+	-	-	+	-	+
Chitinase	-	-	-	-	-	-	-	-	-	-	-

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

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4.5 In *vitro* screening of plant growth-promoting (PGP) activities

Actinomycete strains that showed the radial growth inhibition percentage of the growth of the fungi (%RGI) more than 50% were studied of siderophore production, phosphate solubilisation, hydrogen cyanide (HCN) production, and indole acetic acid (IAA) as presented in Table 4.14.

Production of siderophore was determined yellow colour around the growth spot as presented in Figure 4.15 (1). Isolate NK05205, NK07201, NK08203, and NK08205 showed positive result. Iron (Fe) is one of an essential micronutrient for plants and microorganisms growth. All strains that showed positive result can produce siderophores to overcome the nutritional iron limitation (Schwyn and Neilands, 1987 ; Krewulak and Vogel, 2008).

Ammonia production was determined brown colour after added Nessler 's reagent as presented in Figure 4.15 (2) (Cappucino and Sherman, 1992). Isolate NK05201, NK05203, NK05205, NK07201, NK08202, and NK08203 showed positive result of ammonia production. Ammonia production cloud increase nitrogen to plants (Hayat *et al*., 2010).

Phosphate solubilisation was determined clear zone around the colony after growing the selected strains on Pikovskaya's agar plate as presented in Figure 4.15 (3). The result showed that isolate NK21201 and HPF2102. Phosphate solubilisation ability of rhizosphere actinomycete plays an important role to traits associate with plant phosphate nutrition (Chen *et al*., 2006).

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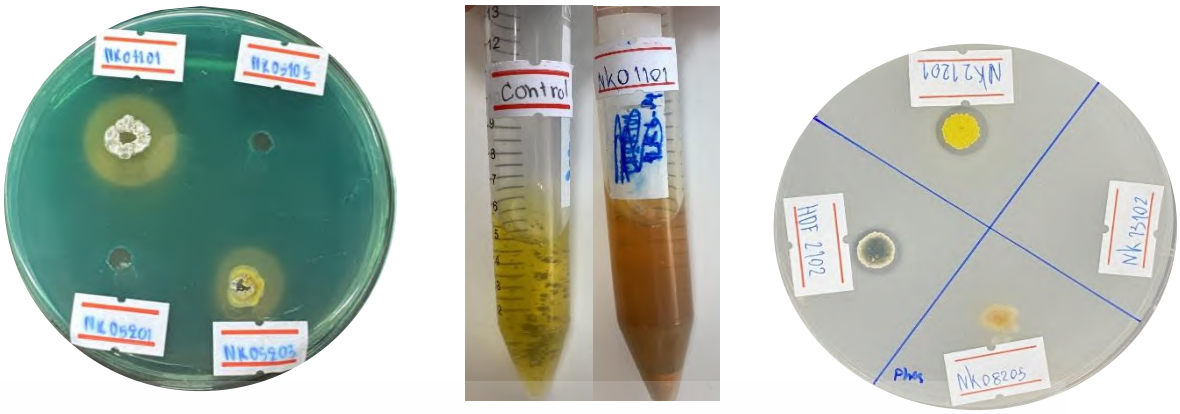

(1) (2) (3)

Figure 4.15 Screening of plant growth-promoting (PGP) activities: siderophore production (1), ammonia production (2), and phosphate solubilisation (3).

Table 4.14 In vitro screening of the selected actinomycete strains for plant growth-promoting (PGP) activities.

Characteristics	Isolate no.										
	1	2	3	4	5	6	7	8	9	10	11
Production of											
Siderophore	-	-	-	+	+	-	+	+	-	-	-
Ammonia	-	+	+	+	+	+	+	-	-	-	-
HCN	-	-	-	-	-	-	-	-	-	-	-
Phosphate solubilisation	-	-	-	-	-	-	-	-	+	+	-

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

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Indole acetic acid (IAA) production was performed by growing the selected actinomycetes strains with 0.2% L-tryptophane in YM broth medium (Kaur *et al*., 2012). The result indicated IAA production in range 4.743 -19.874 µg/mL as presented in Table 4.15. Isolate NK08203 showed the highest IAA production with 19.874 ± 0.028 µg/mL. Indole acetic acid plays an importance role to produce active auxins that controls various plant growth stage including cell division, cell elongation, gravitational and light responses, and regulation of leaf fall and fruit maturing (Meena *et al*., 2015).

Table 4.15 IAA production of the selected actinomycete strains.

Isolate no.	IAA production (µg/mL)	Isolate no.	IAA production (µg/mL)
NK01101	9.270 ± 0.012	**NK08203**	19.874 ± 0.028
NK05201	10.838 ± 0.00	**NK08205**	15.789 ± 0.062
NK05203	7.832 ± 0.010	**NK21201**	19.629 ± 0.003
NK05205	7.227 ± 0.001	**HPF2102**	4.743 ± 0.006
NK07201	15.250 ± 0.006	**HPF3303**	Non-producing IAA
NK08202	13.306 ± 0.036		

4.6 Study of potential antifungal strain *Streptomyces* sp. NK08203

Potential strain was selected based on antifungal activity, biochemical, physiological characterization, 16S rRNA gene analysis and whole genome sequencing analysis. In this study, *Streptomyces* sp. NK08203 was selected to be a potential strain. This strain showed strong antifungal activity (90.51 %) to *Pyricularia* sp. CRI60007, produced enzyme protease and lipase that could be degraded fungal cell wall, growth on different type of carbon source and tolerance to temperature 80 °C for 15 mins. 16S rRNA gene analysis of *Streptomyces* sp. NK08203 tend to be *Streptomyces hydrojenans* JCM 4771^{T} with similarity 100%. However, the phylogenetic tree of *Streptomyces* sp. NK08203 grouped with four strains in the same cluster that contained *Streptomyces violascens* ISP 5183^{T}, *Streptomyces albidoflavus* DSM 40455^{T},

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Streptomyces daghestanicus NRRL B-5418^{T} and *Streptomyces hydrogenans* JCM 4771^{T} as showed in Figure 4.16.

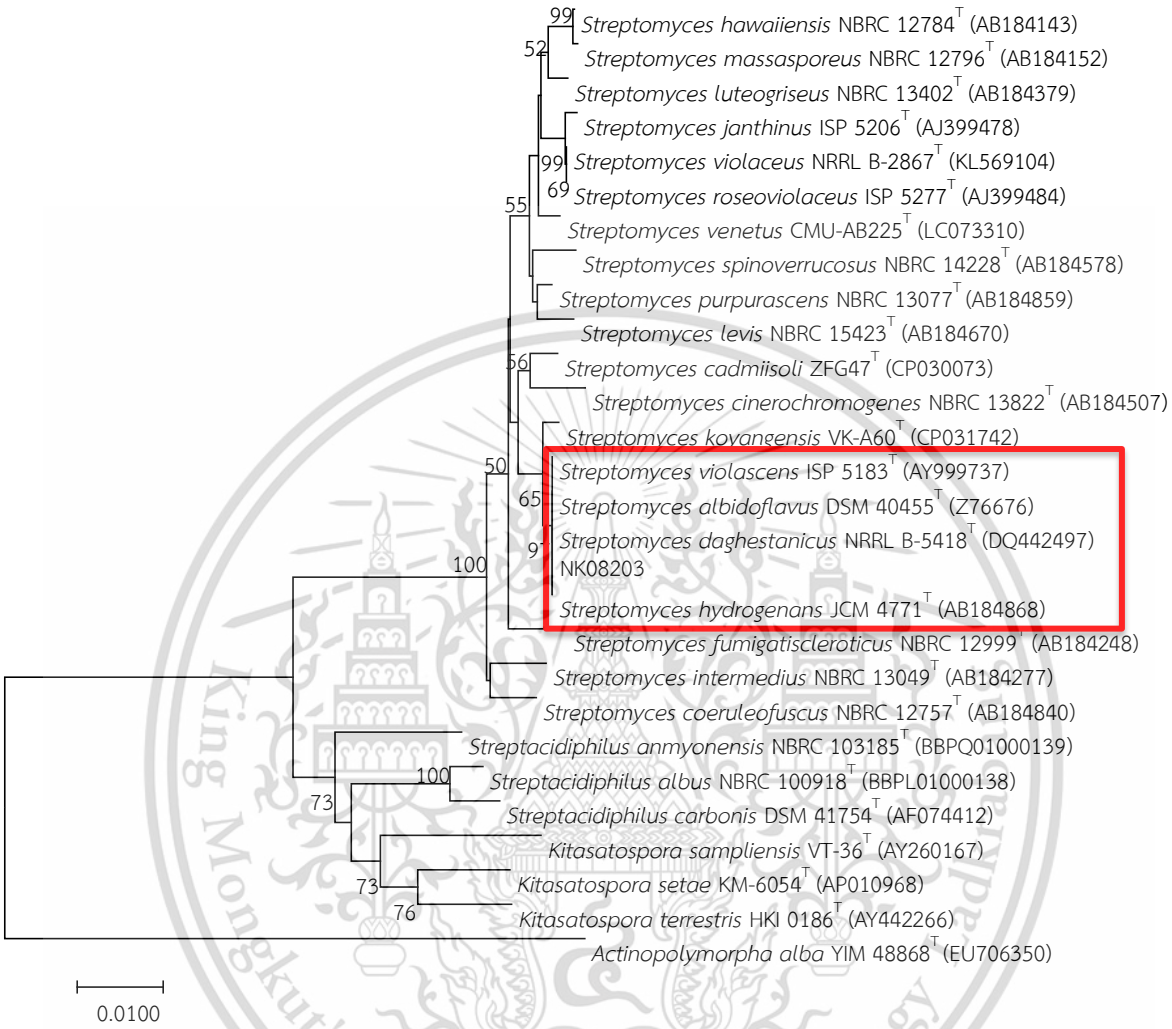

Figure 4.16 Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of isolate no. NK08203 that selected to be a potential antifungal strains and their closely related type strains.

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The whole genome sequencing of strain NK08203 similarity tend to be *Streptomyces albidoflavus* with ANIb 98.35%, ANIm 98.88%, and DNA-DNA hybidisation 89.9%. However, comprising whole genome sequencing between strain NK08203 and related type strains that grouped in the same cluster (phylogenetic tree as Figure 4.16). The results of strain NK08203 could be indicated that strain NK08203 is different species from *Streptomyces violascens* ISP 5183^{T}, *Streptomyces daghestanicus* NRRL B-5418^{T} and *Streptomyces hydrogenans* JCM 4771^{T} by determining nucleotide identity values as presented in Table 4.16 – 4.18. The ANI value is equal or more than 95% and DNA-DNA hybidisation is equal or more than 70% (Raina *et al*., 2019) as presented in Table 4.16. Phylogenomic tree of strain NK08203 was conducted by using TYGS server as presented in Figure 4.17. To summarised, based on genome-based taxonomy of strain NK08203 was identified as *Streptomyces albidoflavus*.

Several reported of *Streptomyces albidoflavus* presented ability to control plant pathogen with plant growth promoting and biocontrol potential. Kunova and collaboration. (2021) reported *Streptomyces albidoflavus* strain DEF1AK against *Fusarium gramineaum* on wheat seedings and could be induced seed germination of maize, rocket, savoy cabbage but reduced seed germination of lamb lettuce. Brzezinska and collaboration (2013) studied of chitinase production of *Streptomyces albidoflavus* to inhibit the growth of *Alternaria alternata, Fusarium culmorum, Fusarium oxysporum, Botrytis cinerea* and the crude chitinase inhibited the growth of *Fusarium solani*. Isaram and collaboration (2009) reported *Streptomyces albidoflavus* C24 presented 60.27% inhibition percentage of the mycelial growth of *Rhizoctonia solani* AG2-2(IV) that caused sheets blight disease. Gao and collaboration (2019) reported *Streptomyces albidoflavus* OsiLf-2 against rice blast disease and reduced 18.0% and 19.6% disease index in greenhouse and field conditions, respectively.

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Table 4.16 Average nucleotide identity values (ANIm).

Strain	*Streptomyces* sp. NK08203	*S. albidoflavus* DSM 40455^{T}	*S. hydrogenans* JCM 4771^{T}	*S. daghestanicus* NRRL B-5418^{T}	*S. violascens* ISP 5183^{T}
Streptomyces sp. NK08203	*	98.88	85.45	85.46	85.33
S. albidoflavus DSM 40455^{T}	98.88	*	85.44	85.50	85.33
S. hydrogenans JCM 4771^{T}	85.45	85.43	*	85.43	85.22
S. daghestanicus NRRL B-5418^{T}	85.46	85.49	85.43	*	85.65
S. violascens ISP 5183^{T}	85.32	85.33	85.23	85.64	*

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Table 4.17 Average nucleotide identity values (ANIb).

Strain	*Streptomyces* sp. NK08203	*S. albidoflavus* DSM 40455^{T}	*S. hydrogenans* JCM 4771^{T}	*S. daghestanicus* NRRL B-5418^{T}	*S. violascens* ISP 5183^{T}
Streptomyces sp. NK08203	*	98.35	77.86	77.66	77.03
S. albidoflavus DSM 40455^{T}	98.72	*	78.28	78.03	77.20
S. hydrogenans JCM 4771^{T}	77.93	77.96	*	77.83	77.47
S. daghestanicus NRRL B-5418^{T}	77.38	77.48	77.49	*	77.9
S. violascens ISP 5183^{T}	76.61	76.58	77.02	77.82	*

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Table 4.18 Digital DNA-DNA hybidisation.

Strain	*Streptomyces* sp. NK08203	*S. albidoflavus* DSM 40455^{T}	*S. hydrogenans* JCM 4771^{T}	*S. daghestanicus* NRRL B-5418^{T}	*S. violascens* ISP 5183^{T}
Streptomyces sp. NK08203	*	89.9	22.9	22.9	22.5
S. albidoflavus DSM 40455^{T}	89.9	*	23.2	23	22.4
S. hydrogenans JCM 4771^{T}	22.9	23.2	*	22.9	23.1
S. daghestanicus NRRL B-5418^{T}	22.9	23.0	22.9	*	22.5
S. violascens ISP 5183^{T}	22.4	22.5	23.1	22.5	*

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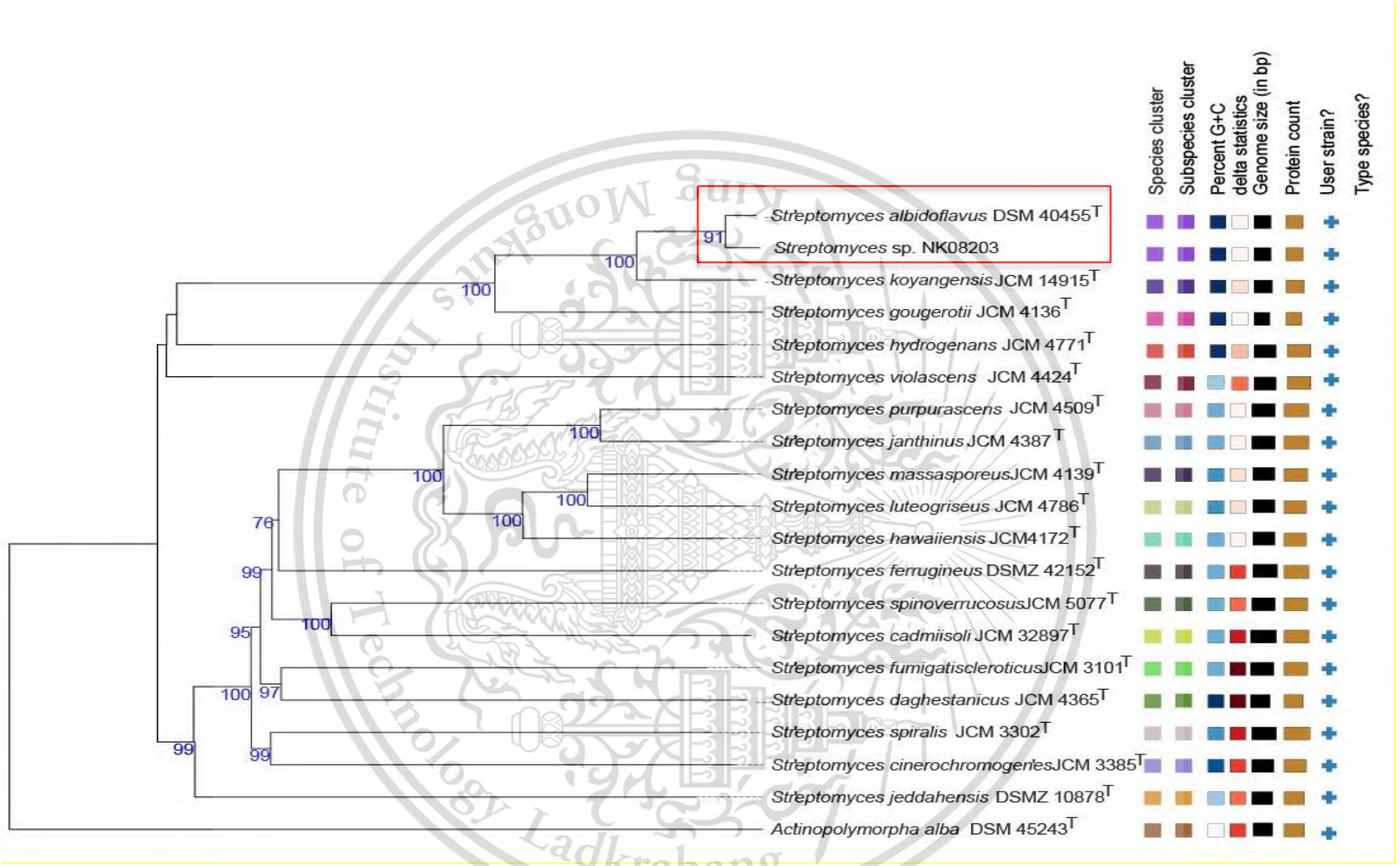

Figure 4.17 Phylogenomic tree based on whole genome sequences using greedy-with-trimming algorithm of isolate no. NK08203 that selected to be a potential antifungal strains and their closely related type strains.

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4.6.1 Secondary metabolite gene cluster of *Streptomyces* sp. NK08203

The genome of *Streptomyces* sp. NK08203 was deposited at NCBI database with accession number PRJNA718162. Whole genome sequencing of *Streptomyces* sp. NK08203 contained 7,005,868 bp, 33 contigs, and showed GC content 73.49%. The whole genome sequencing was analysed secondary metabolite gene cluster by using antiSMASH platform (Bin *et al*., 2019). The results showed that the genome of *Streptomyces* sp. NK08203 contained type I polyketide synthase (T1PKS), type II polyketide synthase (T2PKS), type III polyketide synthase (T3PKS), non-ribosomal peptide synthetase (NRPS), siderophore gene clusters as presented in Table 4.19.

Polyketides are major group of secondary metabolites that defined to be natural products from bacteria, fungi, plant, or animal. Polyketide could be classified into 3 types of polyketide synthase (PKS) enzyme include type I PKS, type II PKS, and type III PKS (Gokulan *et al*., 2014). The genome of *Streptomyces* sp. NK08203 was also found to have the polyketide gene clusters revealing a high similarity percentage included 100% to antimycin (T1PKS), 96% to fredericamycin A (T2PKS), and 90% to candicidin (T1PKS). Antimycin and candicidin was reported to be secondary metabolism gene cluster for antifungal metabolites (Viegelmann *et al*., 2014 ; Nafis *et al*., 2018 ; Baranova *et al*., 2020). Fredericamycin A exhibits antifungal, antibacterial, and antitumor activities (Warnick *et al*., 1981).

Not only detected polyketide gene clusters in the genome of *Streptomyces* sp. NK08203 but also found non-ribosomal peptide synthetase (NRPS) gene clusters with high similarity percentage of surugamide A/ surugamide D (95%). Surugamide A exhibited good antifungal activity with an IC50 of 3.5 μM against *Saccharomyces cerevisiae* (Xu *et al*., 2017).

Besides, the genome of *Streptomyces* sp. NK08203 showed low similarity percentage contained hopene (76%), melidamycin (60%), julichrome Q3-3 (25%), lobophorin(13%), herboxidiene (12%), mycotrienin (7%), ficellomycin (5%), and fluostatins M-Q (4%). This genomic data showed that strain NK08203 showed various gene clusters to synthesis antifungal that can be considered as a potential strain to control fungal disease.

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Table 4.19 Secondary metabolite gene cluster of *Streptomyces* sp. NK08203.

Cluster	Type	From (bp)	To (bp)	% Similarity	Most similar known cluster
Cluster 1	Siderophore	312,195	324,015	100%	Desferrioxamin B
Cluster 2	Ecotine	1,210,248	1,220,646	100%	Ectoine biosynthetic gene cluster
Cluster 3	Terpene	357,592	378,566	25 %	Julichrome Q3-3
Cluster 4	T1PKS	2	100,319	60%	Meridamycin
Cluster 5	T3PKS	106,710	147,807	12%	Herboxidiene
Cluster 6	Terpene, RiPP-like	292,477	321,200	75%	Isorenieratene
Cluster 7	NRPS-like	341,239	385,0659	80%	Indigoidine
Cluster 8	T1PKS	430,726	487,458	13%	Lobophorin A
Cluster 9	Terpene	473,960	500,524	76 %	Hopene
Cluster 10	Lanthipeptide-class-iii	69,893	92,478	80 %	AmfS
Cluster 11	T2PKS	318,613	391,086	96 %	Fredericamycin
Cluster 12	Thiopeptide, LAP	230,020	262,492	4%	Fluostatins M-Q

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Table 4.19 Secondary metabolite gene cluster of *Streptomyces* sp. NK08203 (continued).

Cluster	Type	From (bp)	To (bp)	% Similarity	Most similar known cluster
Cluster 13	T1PKS, NRPS	30,980	80,390	100%	SGR PTMs
Cluster 14	NRPS, NRPS-like, terpene	240,366	317,772	13 %	Valinomycin/ montanastatin
Cluster 15	NRPS	341,492	370,746	66 %	Diisonitrile antibiotic SF2768
Cluster 16	NRPS	53,661	116,779	16 %	Dechlorocuracomycin
Cluster 17	RiPP-like	344,712	356,040	7%	Mycotrienin
Cluster 18	RRE-containing, NRPS	42,601	151,253	95%	Surugamide A/ surugamide D
Cluster 19	Terpene	183,749	206,034	100 %	Geosmin
Cluster 20	NRPS-like, T1PKS	2	41,572	90 %	Candicidin
Cluster 21	T1PKS, NRPS, lanthipeptide-class-ii, NRPS-like	42,045	157,716	100 %	Antimycin
Cluster 22	NRPS	1	36,850	7 %	WS9326

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Table 4.19 Secondary metabolite gene cluster of *Streptomyces* sp. NK08203 (continued).

Cluster	Type	From (bp)	To (bp)	% Similarity	Most similar known cluster
Cluster 23	Siderophore	15,431	30,462	5%	Ficellomycin
Cluster 24	T1PKS	1	4,247	-	-
Cluster 25	T1PKS	1	4,138	-	-

4.6.2 Determined of tolerance to commercial chemicals and biological products using for agriculture

Nowadays various agriculture chemicals were used for control rice pest and disease. For instance, (1) fungicide product to control rice disease such as amigo (sheath blight disease) , greendrive (sheath blight disease), carbendazim (rice blast disease), beam75 (rice blast disease), or omycin (sheath blight disease) (Suwannual and Sunyo, 2014), (2) herbicide product such as paraquat or quinclorac (Widowati and Ginting, 2020), (3) insecticide product such as provado. The aims of this experiment to determine sensitivity or resistance to commercial chemicals and biological products because of some farmer still used pesticide, herbicide, or fungicide products in field or used with biocontrol product. If the potential strain cannot tolerance to these products, growth of the potential strain would be inhibited and loss of potential properties. In this study, *Streptomyces* sp. NK08203 was determined resistance to commercial chemicals and biological products that using in agriculture by paper strip diffusion method that contained the commercial product with usage rate in the middle of petri dish plate as Figure 4.18. The results showed that strain NK08203 was tolerance to the commercial products with usage rate as present in Table 4.20.

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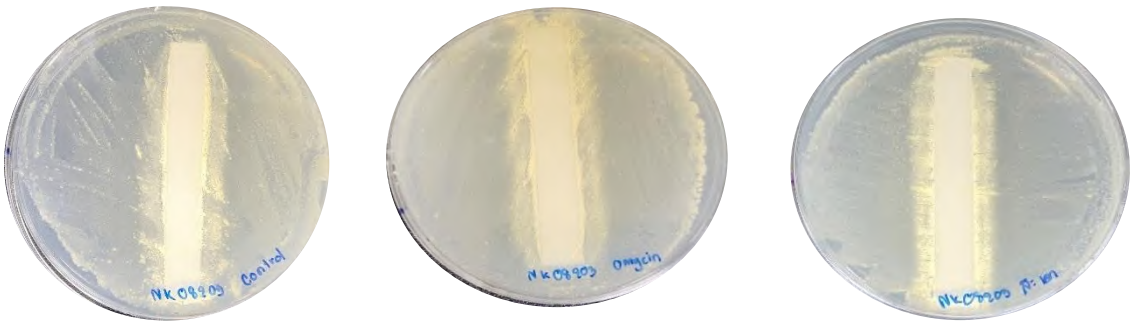

(1) (2) (3)

Figure 4.18 Determined *Streptomyces* sp. NK08203 tolerance to commercial chemical and biological product using for agriculture ; control (1), omycin (2), and siamese neem (3).

Table 4.20 Growth of Streptomyces sp. NK08203 with commercial chemical and biological product using for agriculture.

Commercial name	Final concentration (Usage rate)	NK08203
Amigo	0.25%	+
Thaibact	3%	+
Greendive	0.05%	+
Carbendazim	0.05%	+
Beam 75	0.075%	+
Omycin	0.2%	+
Paraquat	1%	+
Quinclorac	0.25%	+
Provado	0.0075%	+
Trichoderma spp.	0.4%	+
Beauverin	0.4%	+
Metarhzan	1%	+
TAB-1	0.25%	+
Siamese neem	1.5%	+

Note: +, positive result.

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4.6.3 Study of spore formation in submerged cultivation

Streptomyces sp. NK08203 was formed pellets and clumps in ISP2 liquid culture and absence of spore in the liquid medium as Figure 4.19 (1). In this experiment, the liquid culture was modified based on using ISP2 culture medium to increase spore formation in submerged cultivation. 20 mM of calcium chloride was added to the liquid medium and sonicated at 50% amplitude for 2 mins was treated followed by the experiment of Daza and colleague (1989). To study the effects of calcium chloride and resistance to sonication on the sporulation of *Streptomyces* sp. NK08203 in submerged cultivation. The result of presented calcium chloride in the modified ISP2 culture medium, *Streptomyces* sp. NK08203 could be induced spore formation in submerged cultivation with long chain form of spores as presented in Figure 4.19 (2). After sonicating at 50% amplitude for 2 mins was treated, spores presented almost uniformly in liquid culture as showed in Figure 4.19 (3). Sporulation in submerged cultivation was studied and determined to be spore preparation approach for microencapsulated formulation.

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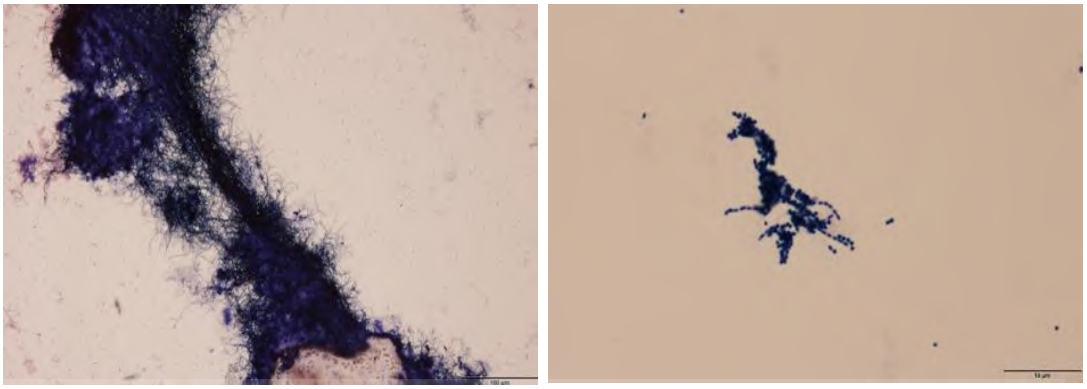

(1) (2)

(3)

Figure 4.19 Spore in harvested pellet of strain NK08203 ISP2 at bars 100 µM (1), Spores in liquid medium with 20mM $CaCl_2$ (2), Spores in liquid medium with 20mM $CaCl_2$ and sonicated at 50 % amplitude for 2 min at bars 10 µM (3). Photographs were taken after growth for 96 hrs.

Growth of *Streptomyces* sp. NK08203 in modified ISP2 showed the maximum biomass yield and the maximum colony forming unit were estimated 6.25 g/L at 24 hrs and 4.44 $x10^{13}$ CFU/mL at 96 hrs with sonication, respectively. Colony forming unit was determined using cell suspension by non-sonicated and sonicated, the results showed that sonicated cell suspension was increasing number of colony forming unit approximately 10^2 to 10^3 cfu/mL after 24 hrs as showed in Table 4.21. Kendrick and Ensign (1983) reported that spores were resistance to sonication while mycelia were eliminated after 2 mins sonication. The report related to the previous result, spores presented almost uniformly in liquid culture after sonication, and it would be the reason of colony forming unit increased. Also, the result of modified ISP2 (added 20 mM calcium chloride) induced submerged spores related to Mandelstam and Dunn

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(1977) and Salas *et al*. (1983) researched that Ca^{+} ion plays an importance role to induce sporulation in *Bacillus* and *Streptomyces* genus.

Table 4.21 Growth of *Streptomyces* sp. NK08203 in submerged cultivation.

Time (hrs)	CFU/mL (non-sonicate)	CFU/mL (Sonicated)	Dry biomass g/L	Total Glucose g/L	pH
0	6.70×10^4	7.20×10^4	0.35	5.30	6.538
24	1.03×10^7	2.56×10^9	6.25	5.18	7.263
48	6.67×10^7	1.15×10^{10}	5.75	4.59	5.854
72	7.10×10^{10}	2.50×10^{12}	4.80	2.62	6.117
96	3.96×10^{11}	4.44×10^{13}	2.50	1.92	6.240

4.6.4 Minimum inhibitory concentration (MIC): Minimum fungicidal concentration (MFC)

Minimum inhibitory concentration (MIC) determination was used broth dilution method. The spore suspension of actinomycete was prepared (as 3.6.2.4) and enumerated the CFU/mL by spread plate technique. The actinomycete suspension was serially diluted with water up to 10^{-12}. The spore suspension of *Pyricularia* sp. was determined by using hemacytometer (10^6 conidial /mL). Normally, conidial suspension of *Pyricularia* spp. could be infected to rice leaves and showed the blast disease symptoms at 10^4 to 10^6 conidial /mL . For this reason, the experiment was control initial concentration of *Pyricularia* sp. at 10^6 conidial /mL (Immanuel *et al*., 2011). After growing *Pyricularia* sp. and *Streptomyces* sp. NK08203, the test bottles were stand still at room temperature for 1 day. MIC was determined by observing the mycelium of *Streptomyces* sp. NK08203 at the top of culture medium. The result showed that MIC of *Streptomyces* sp. NK08203 against *Pyricularia* sp. is 10^5 CFU/mL as presented in Figure 4.20.

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Figure 4.20 Determination minimum inhibitory concentration (MIC) of *Streptomyces* sp. NK08203 against *Pyricularia* sp. CRI60007.

After incubation, each bottle was spread on PDA medium for minimum fungicidal concentration (MFC) determination. MFC determination was examined by observing fungal colony on PDA medium. MFC was determined the lowest concentration of *Streptomyces* sp. NK08203 could be killed all colonies of *Pyricularia* sp. CRI60007. The minimum fungicidal concentration result showed that *Streptomyces* sp. NK08203 could be killed *Pyricularia* sp. is 10^7 CFU/mL as presented in Figure 4.21. These results used for determination the lowest colony forming unit of *Streptomyces* sp. NK08203 to inhibit rice blast fungal disease.

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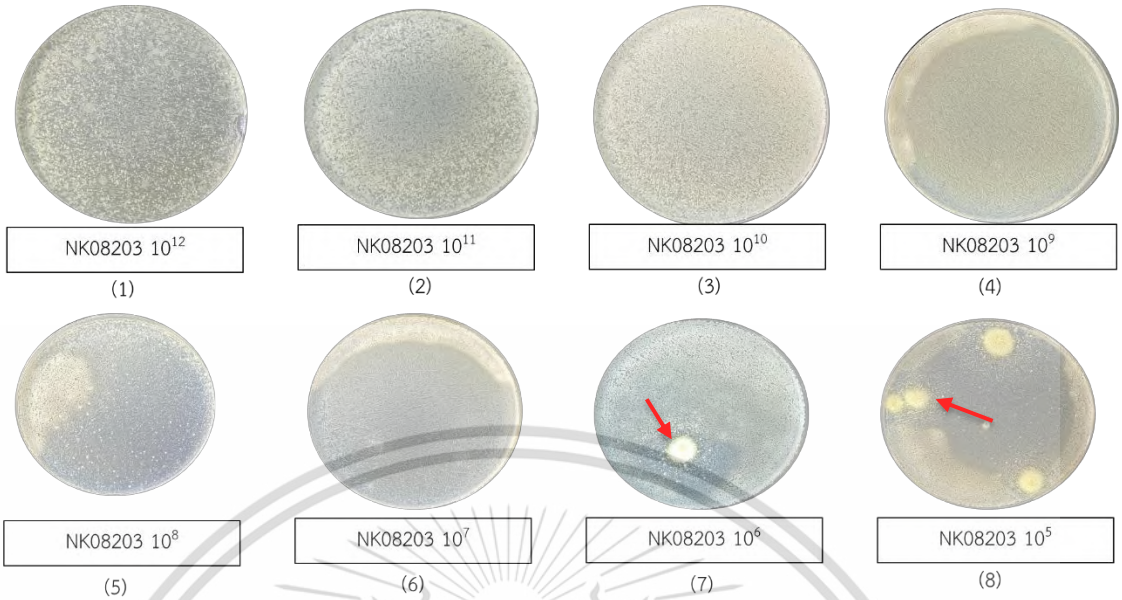

Figure 4.21 Determination minimum fungicidal concentration (MFC) of *Streptomyces* sp. NK08203 against *Pyricularia* sp. CRI60007: showed only *Streptomyces* sp. NK08203 (1-6) and present of *Pyricularia* sp. CRI60007 (7-8).

4.6.5 Powder formulation

Powder formulation was conducted by spray-dryer (Mini Spray-Dryer BÜCHI B-290, BÜCHI, Switzerland). Spray-drying powder was determined spray-dried powder yield, moisture content, and viable cell after spray-drying. Following the required information for biocontrol agent registration, the maximum moisture content of bacterial formulation not exceeded 12% and the minimum colony forming unit of antagonistic bacteria is $1x10^8$ CFU/mL (Sigh, 2012). Wall material of microencapsulation contained substance A, C, and D. Component A is a substance used to encapsulate the properties of film and performed the small size of the microparticle. B is a carbohydrate with high solubility and low viscosity that used as a component of wall material and increases the weight of powder products. Component D is a substance that performed smooth surface of the microparticle. Component B was used to treat spores in long term storage and this substance could be reduced the swelling of water that will increase the moisture content of the powder product. The results of microencapsulation yield back percentage showed that spray-drying additives composed of 10% of C and 0.05% of D gave the highest microencapsulated yield

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(20.43%) as formulation no. 10 and 15% of C and 0.1% of D gave the lowest microencapsulated yield (13.55%) as formulation no. 17. The results of moisture content (%M) of the spray-dried powders were showed the highest moisture content composed of 10% of C and 0.3% of D and the lowest consisted of 5% of C and 0.05% of D. The initial cell before spry-drying process approximately 10^{13} CFU/mL. The results of cell survival after spray-drying with differential concentration of C and D. The spray-drying additives that showed the highest cell survival after spray-drying were composed of 5% of C and 0.3% of D and the lowest were composed of 15% of C and 0.05% of D as shown in Table 4.22. Viable cell was decreased 3.0 - 4.0 log loss after spray drying process. Formulation no. 10 was selected for future study based on the highest spray-dried powder yield and the powder formulation are under condition of general required information for biocontrol agent registration.

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Table 4.22 The results of spray dried powder yield (% yield), moisture content (%M), and viable cell (CFU/mL) of powder formulation

Formulation No.	1% Wall material (A)	2% Preserved spore (B)	Wall material (C)			Wall material (D)			20% Spore	% yield	%M	Viable cell (CFU/mL)
			5%	10%	15%	0.05%	0.1%	0.3%				
1	✓	✓	✓	-	-	✓	-	-	-	16.28 %	4.35 %	-
2	✓	✓	✓	-	-	-	✓	-	-	16.38 %	6.90 %	-
3	✓	✓	✓	-	-	-	-	✓	-	16.78 %	4.76 %	-
4	✓	✓	✓	-	-	✓	-	-	✓	17.44 %	2.95 %	$4.59 \times 10^{9} \pm 1.67$
5	✓	✓	✓	-	-	-	✓	-	✓	17.22 %	6.21 %	$5.27 \times 10^{9} \pm 1.01$
6	✓	✓	✓	-	-	-	-	✓	✓	10.94 %	6.53 %	$1.22 \times 10^{10} \pm 0.27$
7	✓	✓	-	✓	-	✓	-	-	-	27.88 %	3.57 %	-
8	✓	✓	-	✓	-	-	✓	-	-	32.70 %	9.52 %	-

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Table 4.22 The results of spray dried powder yield (% yield), moisture content (%M), and viable cell (CFU/mL) of powder formulation (continued).

Formulation No.	1% Wall material (A)	2% Preserved spore (B)	Wall material (C)			Wall material (D)			20% Spore	% yield	%M	Viable cell (CFU/mL)
			5%	10%	15%	0.05%	0.1%	0.3%				
9	✓	✓	-	✓	-	-	-	✓	-	23.64 %	9.09 %	-
10	✓	✓	-	✓	-	✓	-	-	✓	20.43 %	4.17 %	$4.73 \times 10^9 \pm 1.57$
11	✓	✓	-	✓	-	-	✓	-	✓	14.71 %	4.35 %	$4.59 \times 10^9 \pm 1.67$
12	✓	✓	-	✓	-	-	-	✓	✓	17.25 %	8.00 %	$4.00 \times 10^9 \pm 0.14$
13	✓	✓	-	-	✓	✓	-	-	-	9.67 %	5.00 %	-
14	✓	✓	-	-	✓	-	✓	-	-	8.16 %	4.55 %	-
15	✓	✓	-	-	✓	-	-	✓	-	8.65 %	4.76 %	-
16	✓	✓	-	-	✓	✓	-	-	✓	17.56 %	7.14 %	$1.15 \times 10^9 \pm 1.95$

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Table 4.22 the results of spray dried powder yield (% yield), moisture content (%M), and viable cell (CFU/mL) of powder formulation (continued).

Formulation No.	1% Wall material (A)	2% Preserved spore (B)	Wall material (C)			Wall material (D)			20% Spore	% yield	%M	Viable cell (CFU/mL)
			5%	10%	15%	0.05%	0.1%	0.3%				
17	✓	✓	-	-	✓	-	✓	-	✓	13.55 %	8.82 %	$1.85 \times 10^9 \pm 0.35$
18	✓	✓	-	-	✓	-	-	✓	✓	16.07 %	3.51 %	$2.00 \times 10^9 \pm 0.14$

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Chapter 5
Conclusions and suggestions

5.1 Conclusion

In the present study, one hundred actinomycetes were isolated from rice field soil at Nong Bua, Nakonsawan, Thailand and "Happy Rice Organic Farm" at Nong sua, Pathumthani, Thailand. Fourteen categories of aerial mycelium colour were characterised based on ISCC-NBS colour system and four types of spore were observed under light microscopic with long working distance lens.

All actinomyces strains were screened antibacterial to gram-positive, gram-negative bacteria. Five strains include NK01105, NK08204, NK13102, NK14101, and NK17205 showed antibacterial activity to microorganism test. The extracellular crude extract of NK13102 at 50 mg/mL gave the highest inhibition zone against *B. subtilis* ATCC 6633 (26 mm), *S. aureus* ATCC 25923 (27 mm), *K. rhizophila* ATCC 9341 (29 mm) and *X. oryzae* X3 (9 mm), respectively. An ability of antibacterial activity against *Xanthomonas oryzae* X3 was determined because of *Xanthomonas oryzae* is one of the most important and serious disease of rice that caused bacterial leaf blight disease but bacterial blight does not affect yield production but reduced the quality of grains. These findings have significant implications for focused on rice blast disease and searching for the potential strain of actinomycete that could be control rice blast disease.

All actinomyces strains were screened antifungal activity against *Pyricularia* sp. Eleven strains showed inhibition percentage more than 50% include NK05201 (100 %), HPF2102 (99.53 %), NK08203 (90.51 %), NK05203 (85.88 %), NK21201 (84.95 %), NK07201 (78.24 %), NK08205 (77.78 %), NK05205 (71.30 %), NK01101 (66.44 %), NK08202 (61.11 %), and HPF3303 (51.39 %) were studied biochemical, physiological characteristics, screening of degrading enzyme production to fungal cell wall, screening of PGP activities to search for potential strain.

The selected strains were identified by analysing 16s rRNA gene. NK01105 and NK08204 tend to be *Streptomyces echinntus* with similarity 99.42 and 99.16%, respectively. NK13102 tend to be *Streptomyces avellaneus* (99.51%). NK14101 tend

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to be *Streptomyces bungoensis* (99.86%). NK17205 tend to be *Streptomyces laurentii* (99.37%). NK01101, NK05201, NK05203, NK05205, NK07201, and NK08202 tend to be *Streptomyces albidoflavus* with similarity 99.56-99.79%. NK08203 tend to be *Streptomyces hydrojenans* (100%). NK08205 tend to be *Streptomyces jeddahensis* (99.17%). NK21201 tend to be *Streptomyces corchorusii* (99.86%). HPF2102 tend to be*Amycolatopsis rhizosphaerae* (99.86%). HPF3303 tend to be *Streptomyces shenzhenensis* (99.72%).

The potential strain was selected based on antifungal activity, biochemical, physiological characterisation, 16s rRNA gene analysis and whole genome sequencing analysis. In this study, *Streptomyces* sp. NK08203 was selected to be a potential strain. This strain showed strong antifungal activity (90.51 %) to *Pyricularia* sp. CRI60007, produced enzyme protease and lipase that could be degraded fungal cell wall, and tolerance to temperature 80 °C for 15 mins. *Streptomyces* sp. NK08203 was selected to be a potential strain and whole genome sequencing similarity tend to be *Streptomyces albidoflavus* with ANIb 98.35%, ANIm 98.88%, and DNA-DNA hybidisation 89.9%. Secondary metabolite gene cluster analysis showed *Streptomyces* sp. NK08203 contained gene clusters revealing a high similarity percentage included 100% to antimycin (T1PKS), 96% to fredericamycin A (T2PKS), 90% to candicidin (T1PKS), and NRPS gene clusters with high similarity percentage of surugamide A/ surugamide D (95%). *Streptomyces* sp. NK08203 was tested other properties include tolerance to the commercial chemical products that used in rice field and strain NK08203 showed ability to tolerance to the commercial pesticide products, MIC showed the lowest inhibition concentration at 10^5, and MFC showed the lowest concentration to kill *Pyricularia* sp. at 10^7.

In this study, spore formation in submerged cultivation was conducted for powder formulation by adding calcium chloride to the medium and treating with sonication. Surprisingly, the result of presented calcium chloride in the modified ISP2 culture medium, *Streptomyces* sp. NK08203 could be induced spore formation in submerged cultivation. After sonicating at 50% amplitude for 2 mins was treated, spores presented almost uniformly in liquid culture. *Streptomyces* sp. NK08203 was produced biomass 6.25 g/L at 24 hrs and colony forming unit 4.44 x10^{13} CFU/mL at 96 hrs. This study successfully to performed sporulation in submerged cultivation and determined to be spore preparation approach for microencapsulated formulation.

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Powder formulation was conducted by spray-dryer. The results of microencapsulation yield percentage showed that spray-drying additives composed of 10% of C and 0.05% of D gave the highest microencapsulated yield (20.43%) as formulation no. 10, and presented viable cell 4.73 x 10^9 CFU/mL after spray-drying.

5.2 Suggestions

5.2.1 *Streptomyces* sp. NK08203 should be studied of antifungal activities under pot and field condition to fulfill the potential of this strain.

5.2.2 *Streptomyces* sp. NK08203 should be screened of antimicrobial activities to other plant pathogens.

5.2.3 Toxicity of the potential strain would be screen for side effects to other plants, environment and human health for the future work.

5.2.4 Powder formulation should be determined the potential of antifungal activity *in vitro* and *in vivo*, the result should be showed strong antifungal activity against rice blast disease as well as fresh cell activities.

5.2.5 Shelf life of powder formulation should be determined viable cell after stored after 6 to 12 monts of storage or in accelerated condition.

5.2.6 Storage condition of powder formulation should be examined and the powder poduct should be kept at room temperature condition.

5.2.7 Actinomycete strains that showed antibacterial activity to microorganism test should be study of chemical structure that their produced because of it might be novel chemical or antibiotic compound.

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Appendix

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Appendix A

Culture medium

1. Modified Zhang's Starch Soil Extract agar (ZSSE) (modified from Zhang, 2011)

Soluble starch	5.0	g
KNO_3	1.0	g
Soil extracts	500	mL
Agar	18.0	g
Distilled water	500	mL
pH 7.0		
Autoclaving at 121 °C for 15 mins.		
Nalidixic acid (dissolving in 0.2N NaOH)	0.025	g
Soil extract		
Humic soil	1.0	kg
Distilled water	1	L
Autoclaving at 121 °C for 15 mins.		
Centrifuge at 8000 RPM for 10 mins		
Autoclaving at 121 °C or 15 mins.		

2. International *Streptomyces* Project No.2 (ISP2)

Glucose	4.0	g
Yeast extract	4.0	g
Malt extract	10.0	g
Agar	18.0	g
Distilled water	1	L
pH 7.0-7.3		
Autoclaving at 121 °C or 15 mins.		

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3. Modified International *Streptomyces* Project No.2 (ISP2)

Glucose	4.0	g
Yeast extract	4.0	g
Malt extract	10.0	g
Agar	18.0	g
Distilled water	919	mL
Trace salts element	1	mL
3.68% $CaCl_2 \cdot 2H_2O$	80	mL

Autoclaving at 121 °C or 15 mins.

Trace salts element

$FeSO_4 \cdot 7H_2O$	0.1	g
$MnCl_2 \cdot 4H_2O$	0.1	g
$ZnSO_4 \cdot 7H_2O$	0.1	g

4. International *Streptomyces* Project No.4 (ISP4)

Soluble starch	10.0	g
$CaCO_3$	2.0	g
$(NH_4)_2SO_4$	2.0	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	1.0	g
NaCl	1.0	g
Trace salts element	1	mL
Agar	20.0	g
Distilled water	1	L

pH 7.0-7.3

Autoclaving at 121 °C or 15 mins.

5. International *Streptomyces* Project No.6 (ISP6)

Peptic digest of animal tissue	15.0	g
Proteose peptone	5.0	g

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Yeast extract	1.0	g
Ferric ammonium citrate	0.5	g
Dipotassium phosphate	1.0	g
Sodium thiosulphate	0.08	g
Distilled water	1	L

pH 7.0-7.2

Autoclaving at 121 °C for 15 mins.

6. International *Streptomyces* Project No.9 (ISP9)

Basal mineral salts agar

$(NH_4)_2SO_4$	2.64	g
KH_2PO_4·anhydrous	2.38	g
$K_2HPO_4 \cdot 3H_2O$	5.65	g
$MgSO_4 \cdot 7H_2O$	1.0	g
Pridham and Gottlieb trace salts	1.0	mL
Distilled water	900	mL
Agar	15.0	g

pH 6.8 – 7.0

Autoclaving at 121 °C for 15 mins.

Pridham and Gottlieb trace salts

$CuSO_4 \cdot 5H_2O$	0.64	g
$FeSO_4 \cdot 7H_2O$	0.11	g
$MnCl_2 \cdot 4H_2O$	0.79	g
$ZnSO_4 \cdot 7H_2O$	0.15	g
Distilled water	100	mL

Carbon sources

Carbohydrate	10.0	g
Distilled water	100	mL

Filtrated by using 0.2 µm.

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7. Muller's Hinton agar (MHA)

Beef extract	2.0	g
Acid hydrolysate of casein	17.50	g
Soluble starch	1.5	g
Agar	18.0	g
Distilled water	1	L

pH 7.2-7.4

Autoclaving at 121 °C or 15 mins.

8. Potato Dextrose Agar (PDA)

Infusion from potatoes	200.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1	L

Autoclaving at 121 °C or 15 mins.

9. Peptonization and Coagulation test medium

Skim milk	10.0	g
Distilled water	100	mL

Autoclaving at 110 °C for 10 mins.

10. Peptonization test medium

Solution A

Skim milk	5.0	g
Distilled water	50	mL

Autoclaving at 110 °C for 10 mins.

Solution B

Agar	1.0	g
Distilled water	50	mL

Autoclaving at 110 °C for 10 mins.

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11. Boullion's gelatin broth

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15.0	g
Distilled water	100	mL

pH 7.0 – 7.2

Autoclaving at 121 °C for 15 mins.

12. Peptone KNO_3 broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g
Distilled water	100	mL

pH 7.0

Autoclaving at 121 °C for 15 mins.

13. Tween 80 (lipase test) (Akhila *et al*., 2011)

Peptone	10.0	g
NaCl	5.0	g
$CaCl_2 \cdot 2H_2O$	0.1	g
Agar	10.0	g
Tween 80	10.0	g
Distilled water	1	L

Autoclaving at 121 °C for 15 mins.

14. Phenol red (lipase test) (Akhila *et al*., 2011)

Phenol red	0.01	g
Lipidic substrate (tributyrin/ olive oil)	1.0	g
$CaCl_2 \cdot 2H_2O$	10	mM
Agar	2.0	g

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Distilled water	100	mL
pH 7.3-7.4		
Autoclaving at 121 °C for 15 mins.		

15. Chitin agar (Hsu and Lockwood, 1975)

Colloidal chitin	4.0	g
K_2HPO_4	0.7	g
KH_2PO_4	0.3	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$FeSO_4 \cdot 7H_2O$	0.01	g
$MnCl_2 \cdot 4H_2O$	0.01	g
$ZnSO_4 \cdot 7H_2O$	0.001	g
Agar	20.0	g
Distilled water	1	L
pH 7.3-7.4		
Autoclaving at 121 °C for 15 mins.		

16. Pikovskaya's agar (Gaur, 1990)

Yeast extract	0.50	g
Dextrose	10.0	g
$Ca_3(PO_4)_2$	5.0	g
$(NH_4)_2SO_4$	0.5	g
KCl	0.2	g
$MgSO_4 \cdot 7H_2O$	0.1	g
$FeSO_4 \cdot 7H_2O$	0.0001	g
$MnSO_4 \cdot 4H_2O$	0.0001	g
Distilled water	1	L
pH 7.3-7.4		
Autoclaving at 121 °C for 15 mins.		

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17. Peptone water

Peptone	10.0	g
NaCl	5.0	g
Distilled water	1	L

pH 7.3-7.4

Autoclaving at 121 °C for 15 mins.

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Appendix B

Reagent and buffer

1. 1% Iodine solution

Potassium iodide (KI)	10.0	g
Iodine	5.0	g
Distilled water	100	mL

2. 5N Acetic acid

Acetic acid	30	mL
Distilled water	70	mL

3. Sulfanilic acid solution (Nitrate reagent A)

4-aminobenzene sulfonic acid	0.8	g
5N acetic acid	100	mL

4. Alpha-Naphthylamine solution (Nitrate reagent B)

N,N-dimethyl-1 naphthylamine	0.6	g
5N acetic acid	100	mL

5. 1mM Saline-EDTA (Na_2-EDTA) pH 8

EDTA	29.224	g
Distilled water	700	mL

29.224 g of EDTA was dissolved in distilled water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets. The volume was adjusted to 1 litter and sterilized by autoclaving at 121 °C for 15 mins.

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6. Lysozyme (100 mg/mL)

Lysozyme	100	mg
0.1M SEDTA	1	mL

7. 0.1M Tris-HCl pH 7.5

Tris	12.11	g
Distilled water	900	mL

12.11 g of Tris was dissolved in distilled water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 7.5 with conc. HCl. The volume was adjusted to 1 litter and sterilized by autoclaving at 121 ℃ for 15 mins.

8. 50 mM Tris-HCl pH 7.5

0.1M Tris-HCl pH 7.5	250	mL
Distilled water	250	mL

9. Proteinase K (20 mg/mL)

Proteinase K	0.002	g
50 mM Tris-HCl pH 7.5	100	µL

10. 10% Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate	10.0	g
Distilled water	100	mL

11. 5M NaCl

NaCl	29.22	g
Distilled water	100	mL

Autoclaving at 121 ℃ for 15 mins.

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12. 0.7M NaCl

NaCl	4.09	g
Distilled water	100	mL

13. 10% Cetyltrimethylammonium bromide (CTAB)

CTAB	10.0	g
0.7M NaCl	100	mL

Autoclaving at 121 °C for 15 mins.

14. Phenol: Chloroform (1 : 1 v/v)

Crystalline phenol was dissolved at 65 °C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle at 4 °C.

15. RNase A solution (concentration 100mg/mL)

RNase A	100	mg
0.15 M NaCl	10	mL

Dissolve 20 mg of RNase A in 10 ml 0.15 M NaCl and heat at 95 °C for 5-10 minutes. Keep RNase A solution at -20 °C.

16. TE buffer

10 mM Tris-HCl (pH 8)	10	mL
1 mM Na_2-EDTA (pH 8)	10	mL
Distilled water	980	mL

Autoclaving at 121 °C for 15 mins.

17. 0.8% Agarose gel

Agarose	0.8	g
TE buffer	100	mL

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18. 1X TAE buffer

50X TAE	20	mL
Distilled water	980	mL

19. 6M Hydrochloric acid

Conc. HCl	60	mL
Distilled water	60	mL

20. Salkowski's reagent

0.5M $FeCl_3$	1	mL
35% $HClO_4$	50	mL

21. Picric acid solution

Sodium carbonate	2	g
Picric acid solution	0.5	g
Distilled water	100	mL

22. Nessler 's reagent

Potassium iodide	2	g
Mercury (II) iodide	3	g
Potassium hydroxide	40	g
Distilled water	100	mL

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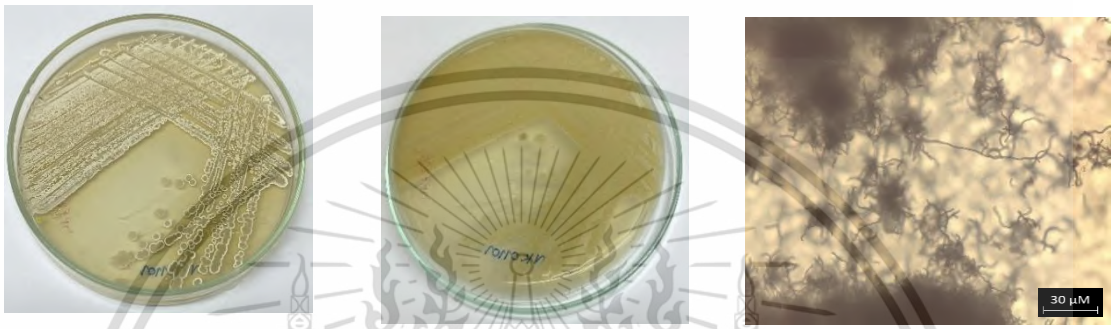
Appendix C

Morphological and cultural characteristics

Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores and non-producing soluble pigment. Sporulation showed rectiflexible chains of NK01101.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK01102.

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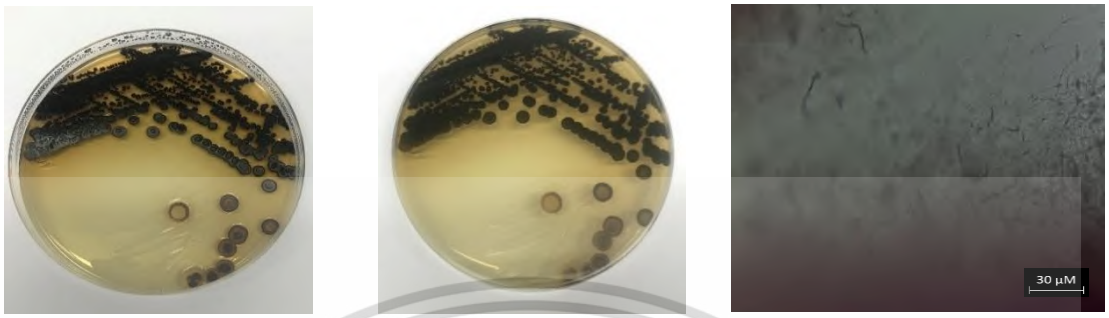
Blackish purple aerial mycelium, blackish red substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK01103.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, and non-producing soluble pigment, and sporulation showed verticillati chains of NK01104 .

Non-producing aerial mycelium, strong reddish brown substrate mycelium, non-producing spores, and light olive brown soluble pigment of NK01105.

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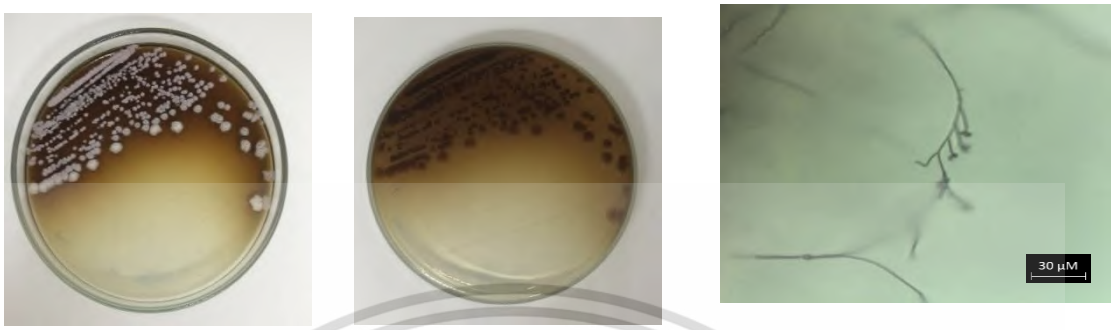
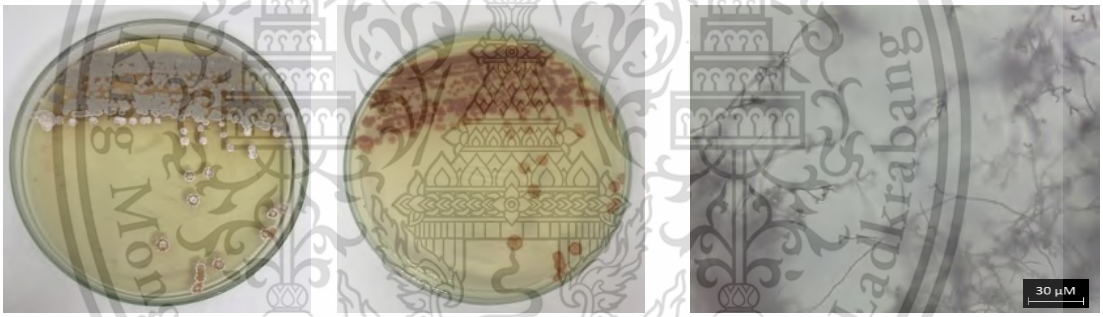
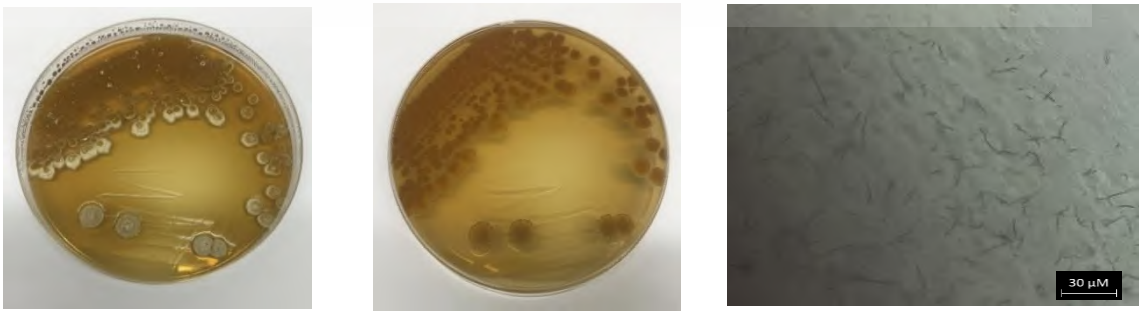
Pale purple aerial mycelium, moderate olive brown substrate mycelium, white mass of spores, light olive brown soluble pigment, and sporulation showed spiral chains of NK03101.



Pale yellowish pink aerial mycelium, deep reddish orange substrate mycelium, brownish gray mass of spores, and sporulation showed spiral type of spore chains of NK03201.



Pale greenish yellow aerial mycelium, light olive brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK03202.



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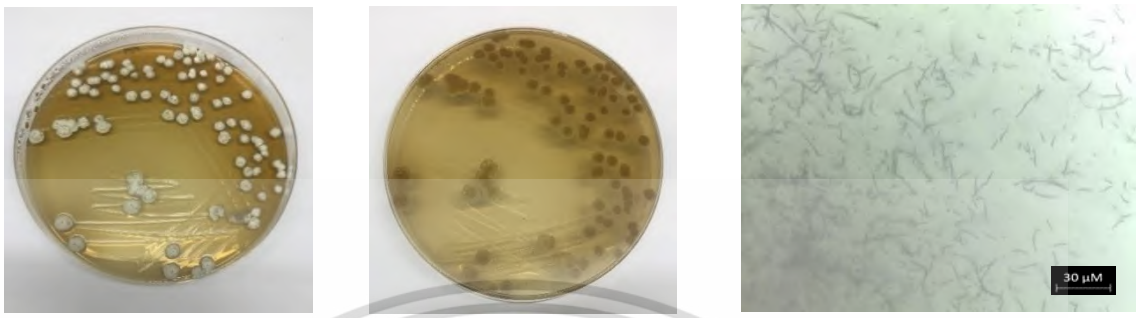
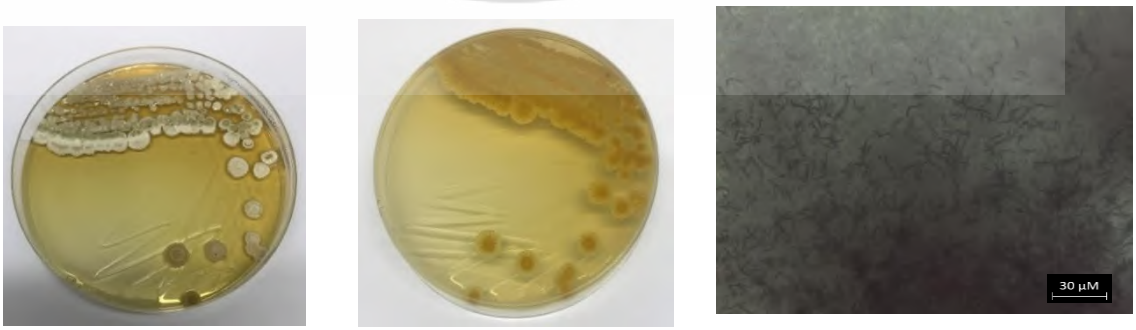
Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK04101.



Bluish gray aerial mycelium, dark grayish yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK04103.

Pale greenish yellow aerial mycelium, light olive brown substrate mycelium, white mass of spores, and non-producing soluble pigment, and sporulation showed rectiflexible chains of NK05101.



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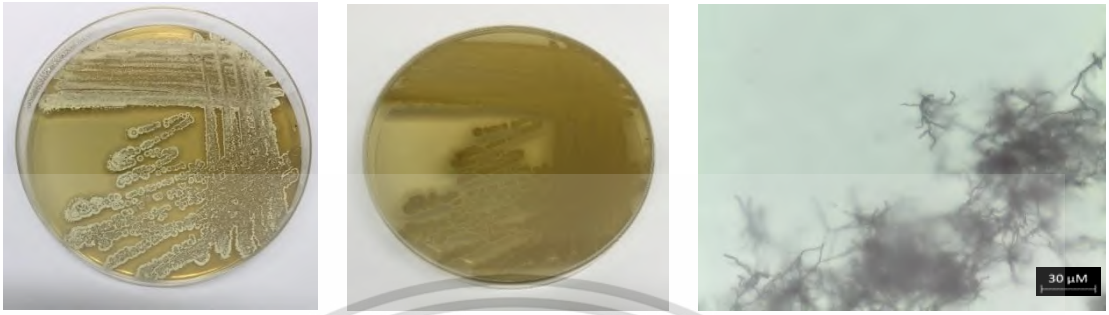
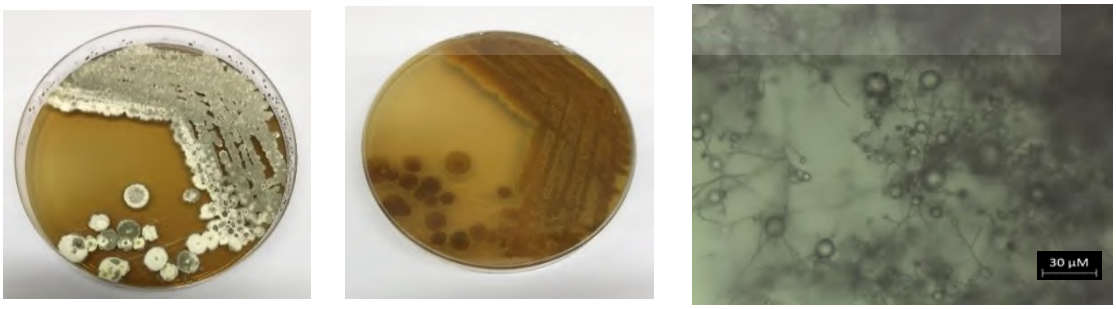
Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK05104.



Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, vivid yellow soluble pigment, and sporulation showed rectiflexible chains of NK05105.

Yellowish white aerial mycelium, vivid yellow substrate mycelium, greenish gray mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK05202.



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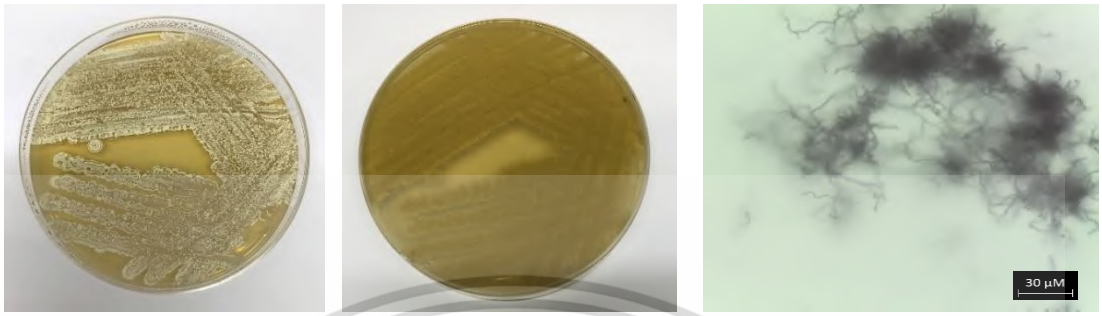
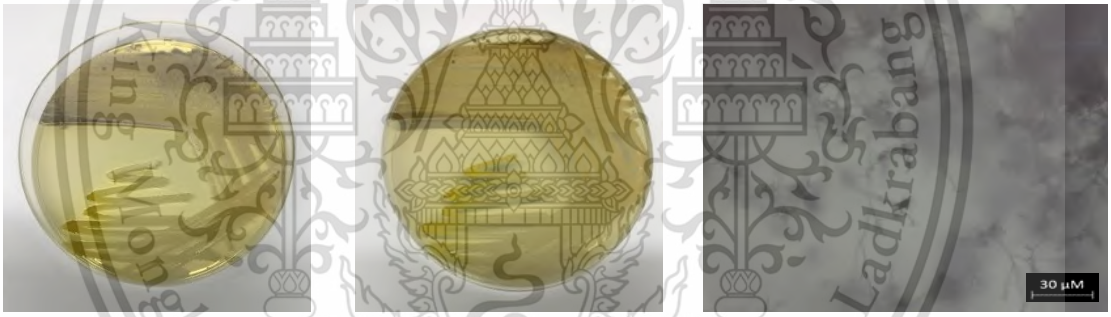
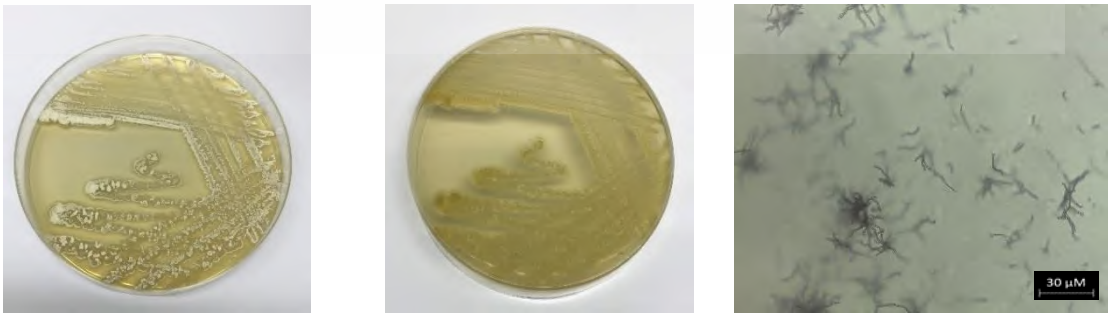
Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK05203.



Yellowish white aerial mycelium, vivid greenish yellowish white aerial mycelium, greenish gray mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK05206.



Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK05207.



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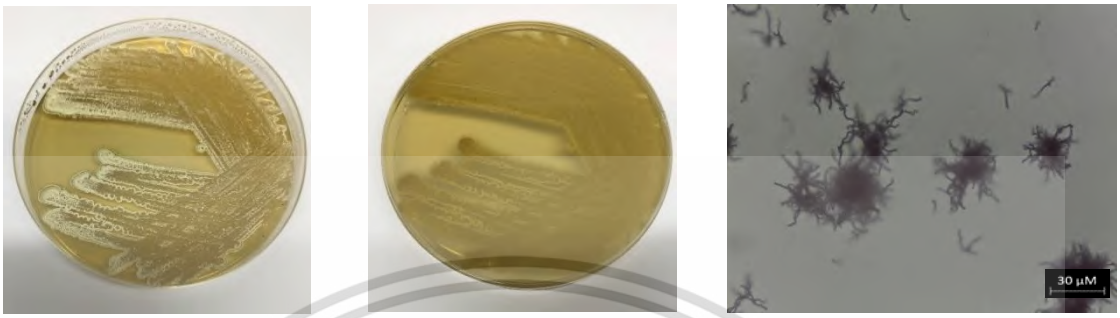
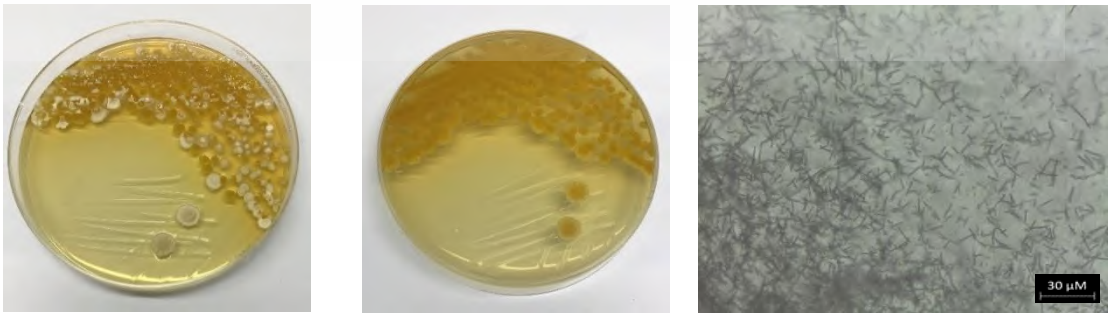
Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK07201.



Dark grayish green aerial mycelium, very dark green substrate mycelium, greenish gray mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK07203.

Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK08201.



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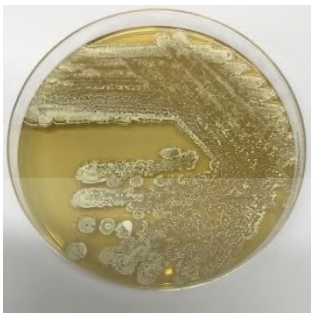
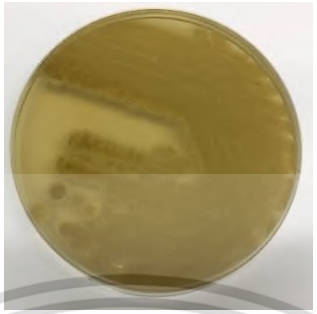
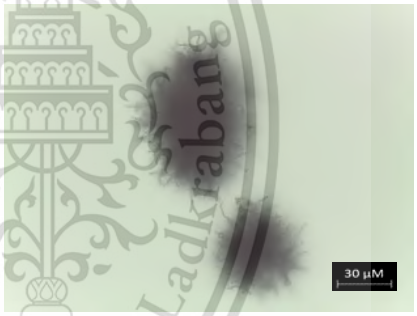
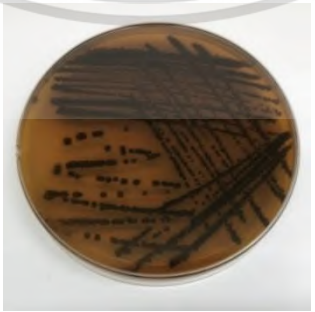
Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK08202.

Yellowish white aerial mycelium, strong yellowish brown substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK08203.



Dark olive brown substrate mycelium, light olive brown soluble pigment, non-producing spores, and aerial mycelium of NK08204.



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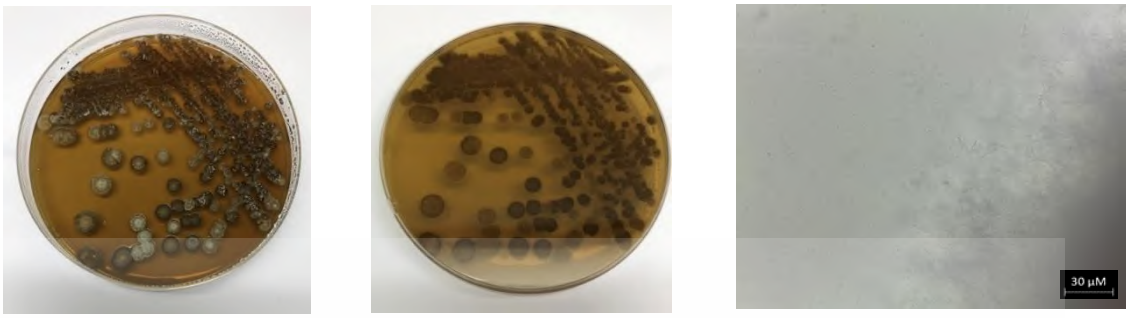
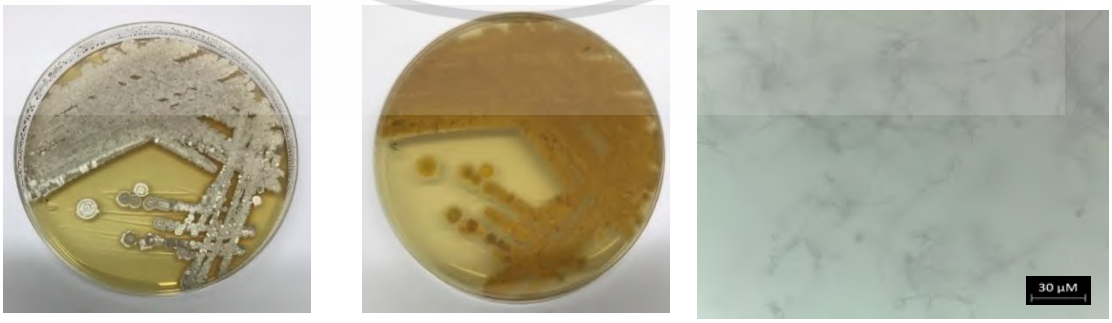
Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK08205.



Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, vivid yellow soluble pigment, and sporulation showed rectiflexible chains of NK08209.

Yellowish white aerial mycelium, vivid yellow substrate mycelium, greenish gray mass of spores, non-producing soluble pigment, and sporulation showed spiral type of type of spore chains of NK09102.



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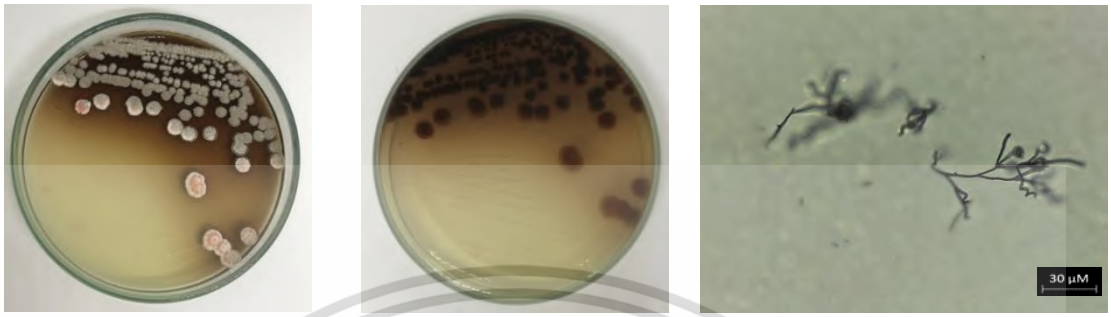
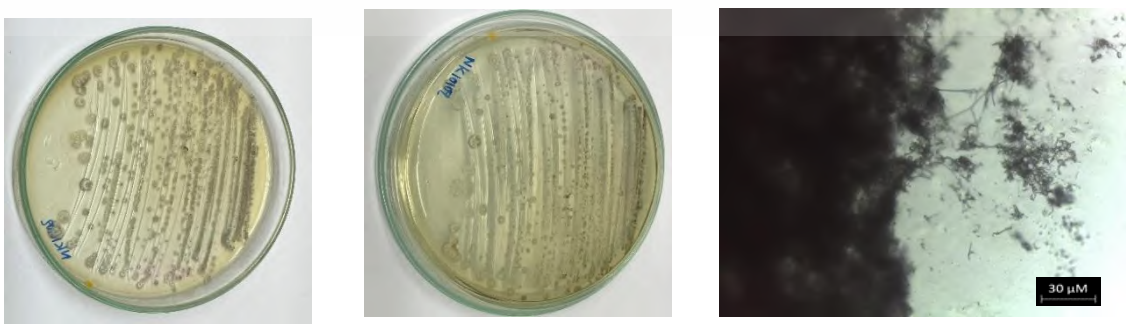
Pale yellowish pink aerial mycelium, dark yellowish brown substrate mycelium, brownish gray mass of spores, dark olive brown soluble pigment and sporulation showed spiral chains of NK09201.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK10101.

Pale greenish yellow aerial mycelium, greenish white substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK10102.



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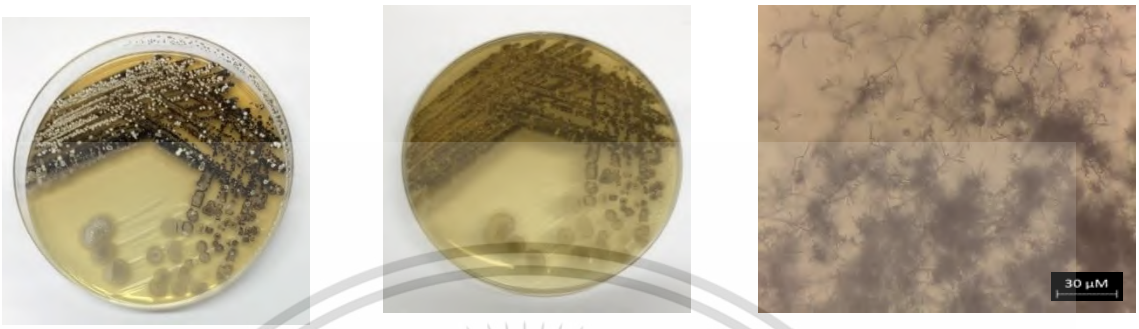
Bluish gray aerial mycelium, dark grayish yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK10103.



Dark grayish green aerial mycelium, very dark green substrate mycelium, greenish gray mass of spores, vivid greenish yellow soluble pigment, and sporulation showed spiral chains of NK10105.

Dark grayish yellow substrate mycelium, non-producing aerial mycelium, soluble pigment, and spores of NK10106 .

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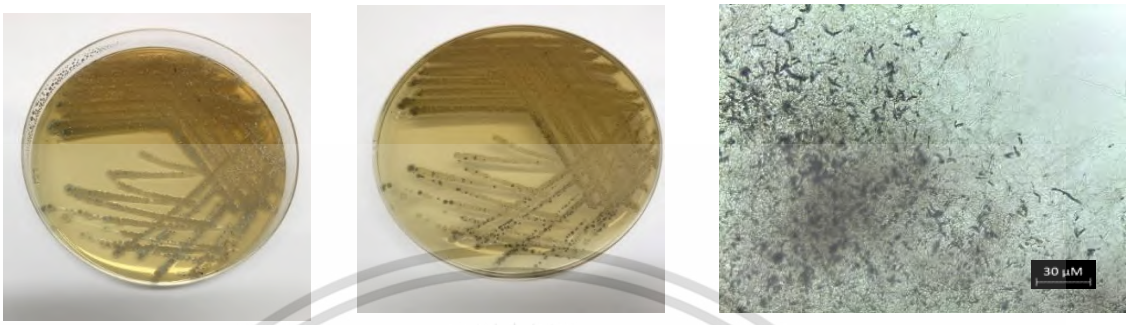
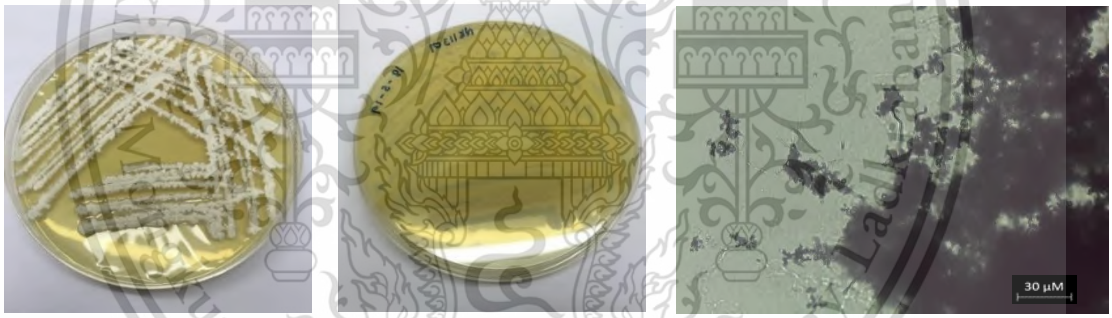
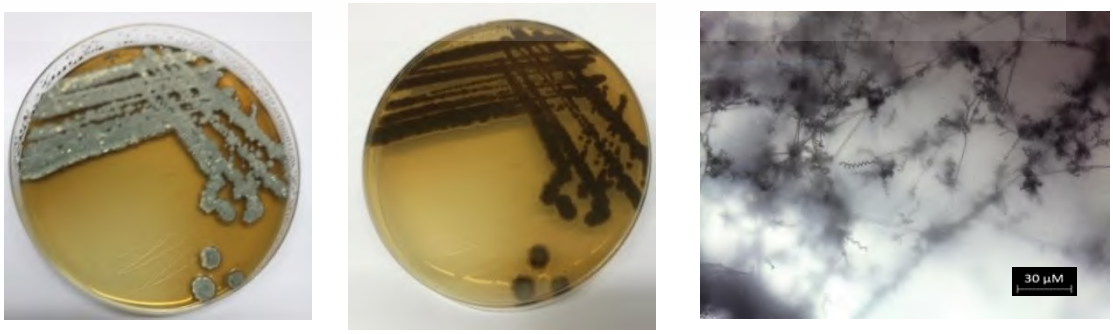
Dark grayish green aerial mycelium, very dark green substrate mycelium, greenish gray mass of spores, non-producing soluble pigment, sporulation showed rectiflexible chains of NK10107.



Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment and sporulation showed verticillati chains of NK11301.



Dark grayish green aerial mycelium, very dark green substrate mycelium, greenish gray mass of spores, and non-producing soluble pigment, and sporulation showed spiral chains of NK11302.



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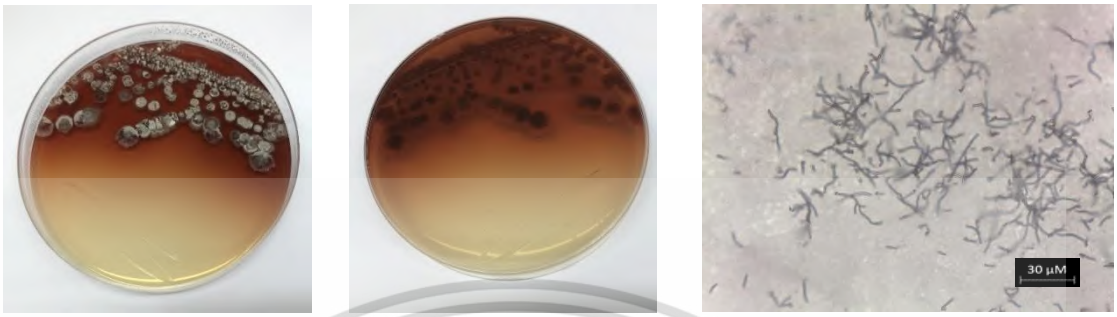
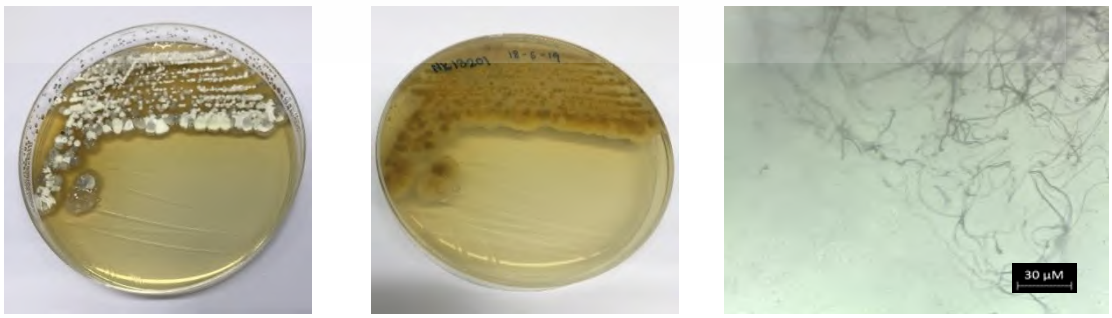
Light grayish reddish brown aerial mycelium, dark reddish brown substrate mycelium, greenish gray mass of spores, strong reddish brown, and sporulation showed rectiflexible chains of NK13101.



Blackish purple aerial mycelium, blackish red substrate mycelium, white mass of spores, and moderate olive brown soluble pigment, and sporulation showed rectiflexible type of spore chains of NK13102.

Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK13201.



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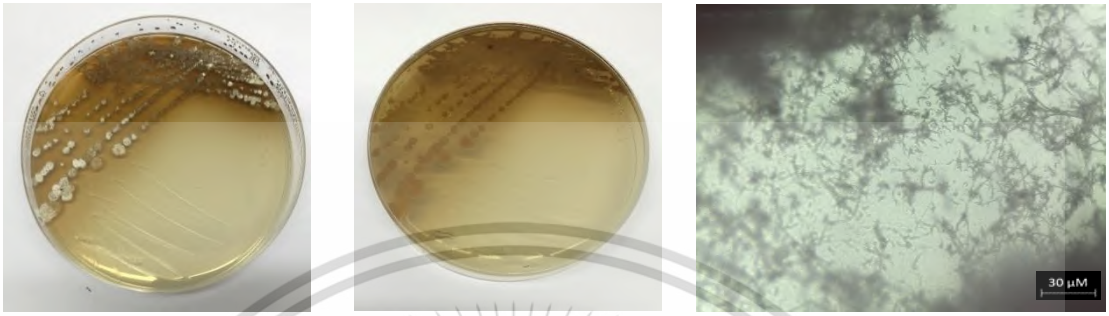
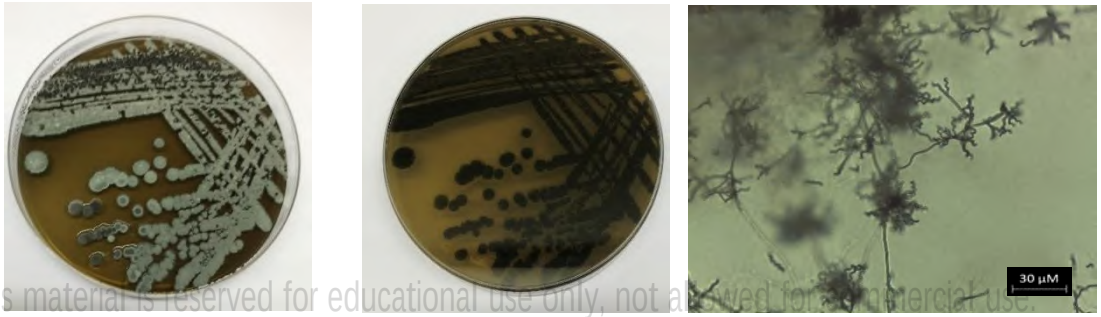
Yellowish white aerial mycelium, very dark green substrate mycelium, greenish gray mass of spores, moderate yellowish brown soluble pigment, and sporulation showed rectiflexible chains of NK14101.



Yellowish white aerial mycelium, blackish red substrate mycelium, white mass of spores, and moderate olive brown soluble pigment, and sporulation showed verticillati chains of NK15101.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral chains of NK15103.



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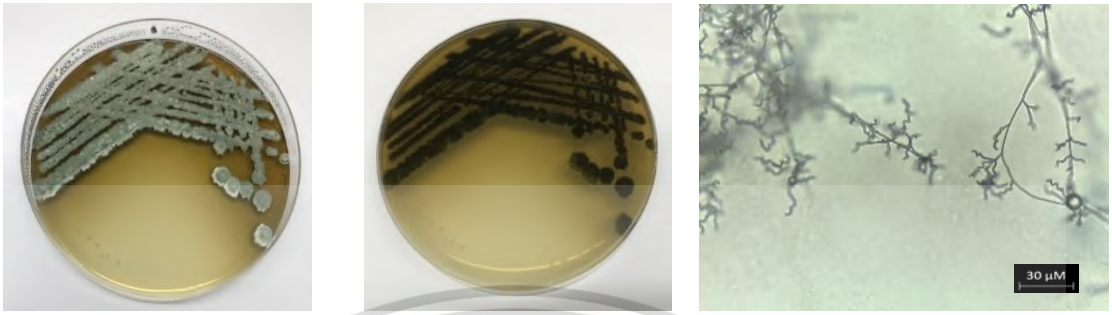
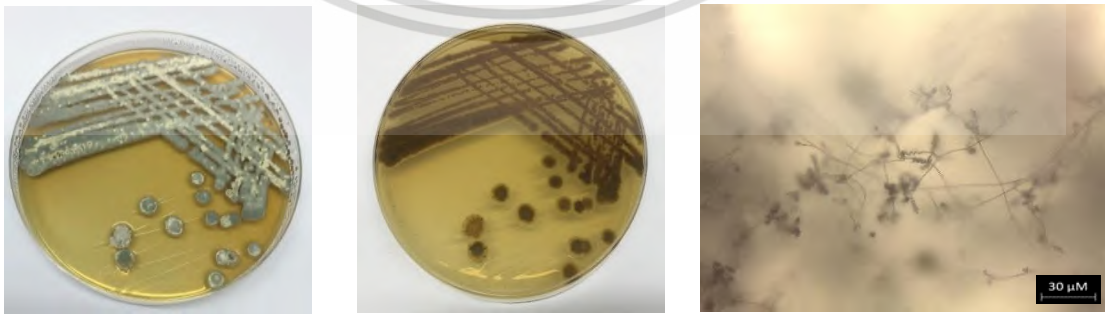
Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral chains of NK15104.



Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK15201.

Grayish green aerial mycelium, light olive substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK15202.



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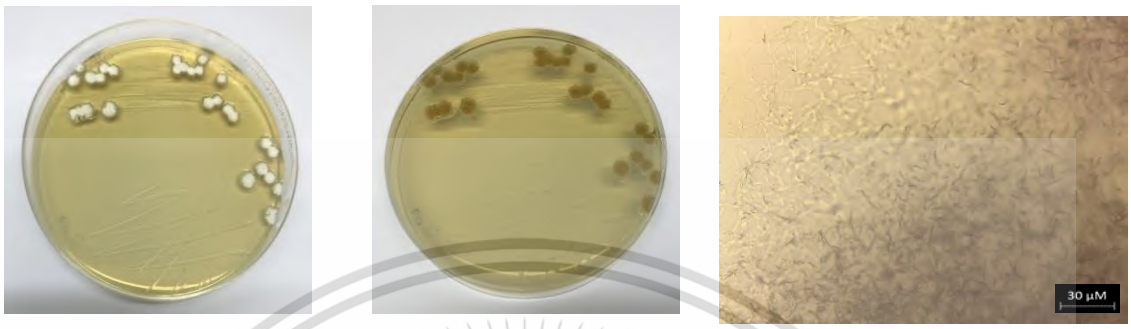
Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of NK15203.



Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK15301.

Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK15302.

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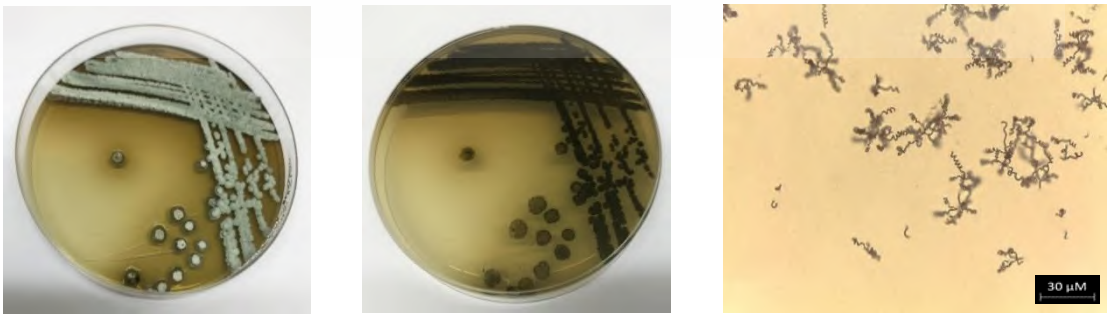
Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK15303.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, and moderate olive brown soluble pigment, and sporulation showed spiral chains of NK16101.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral chains of NK16102.



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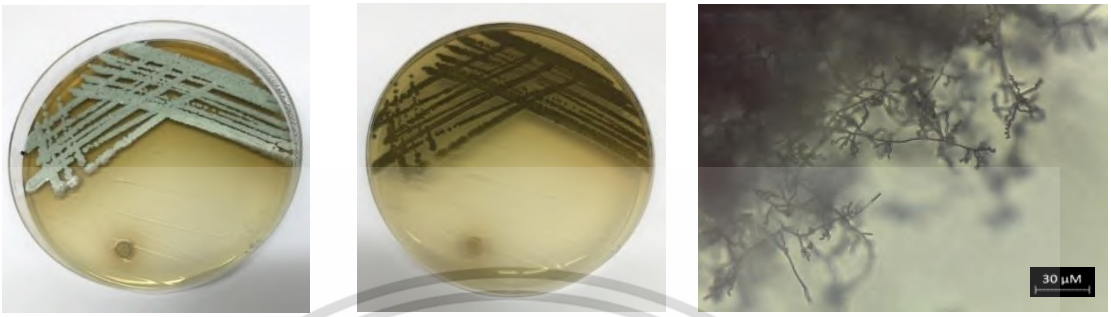
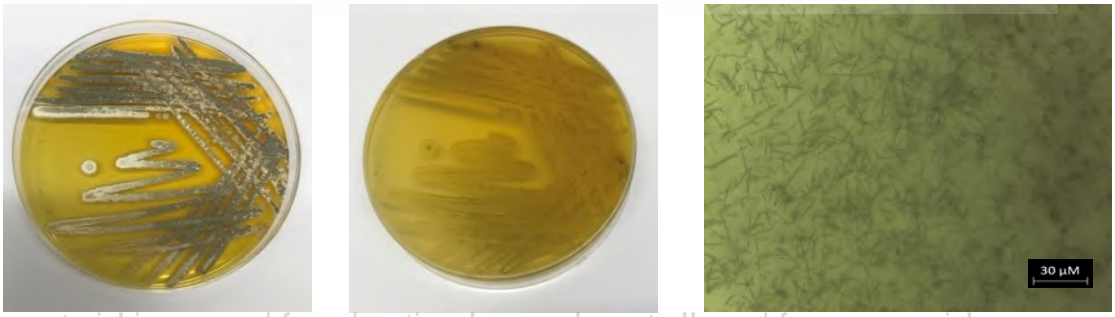
Grayish green aerial mycelium, light olive substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral chains of NK16103.



Grayish green aerial mycelium, moderate yellow green substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed verticillati chains of NK16104.

Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, vivid yellow soluble pigment, and sporulation showed rectiflexible chains of NK16105.



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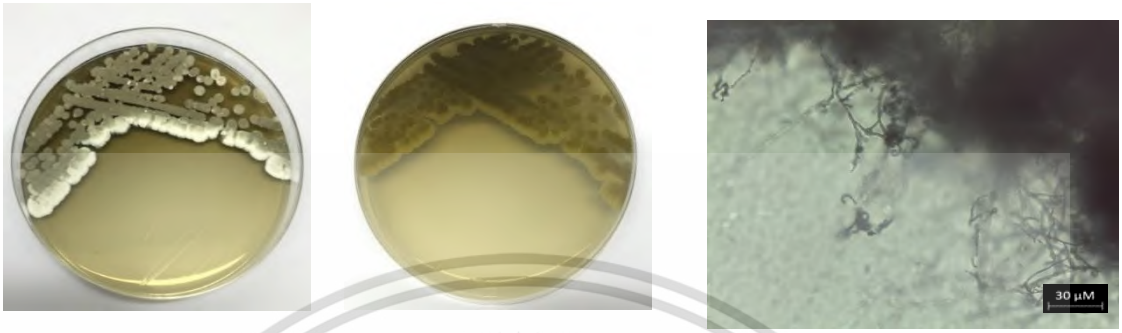
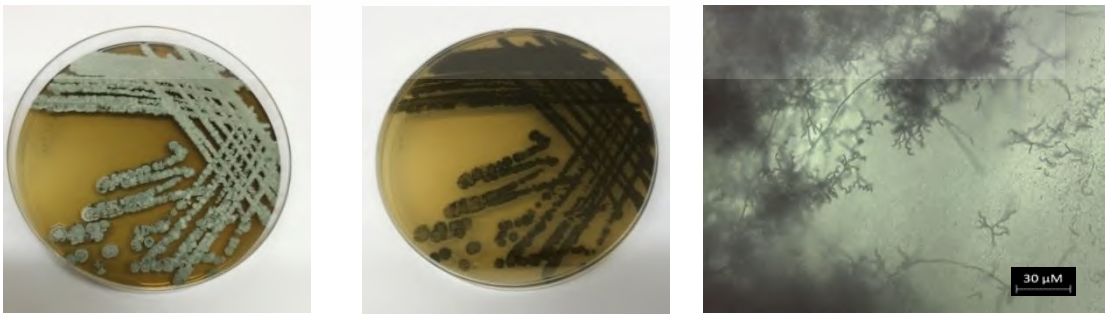
Bluish gray aerial mycelium, moderate olive substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectinaculiaperti chains of NK16201.



Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral type of spore chains of NK16202.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed verticillati chains of NK16203.



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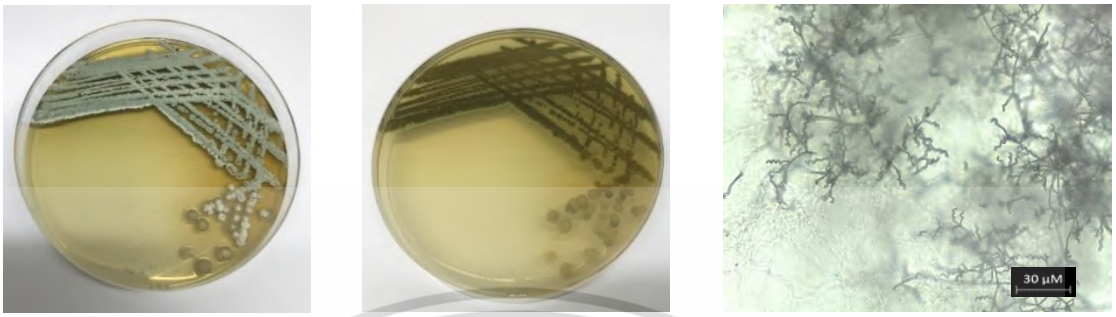
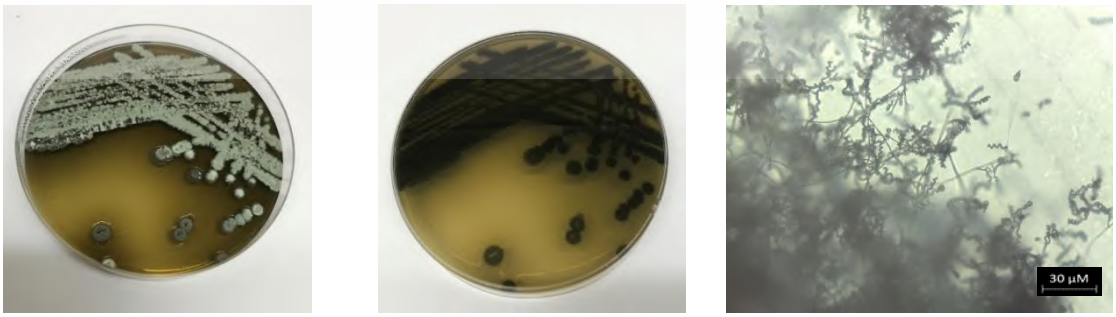
Grayish green aerial mycelium, light olive substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK16204.



Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed spiral type of spore chains of NK16205.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, dark grayish olive green soluble pigment, and sporulation showed spiral chains of NK16206.



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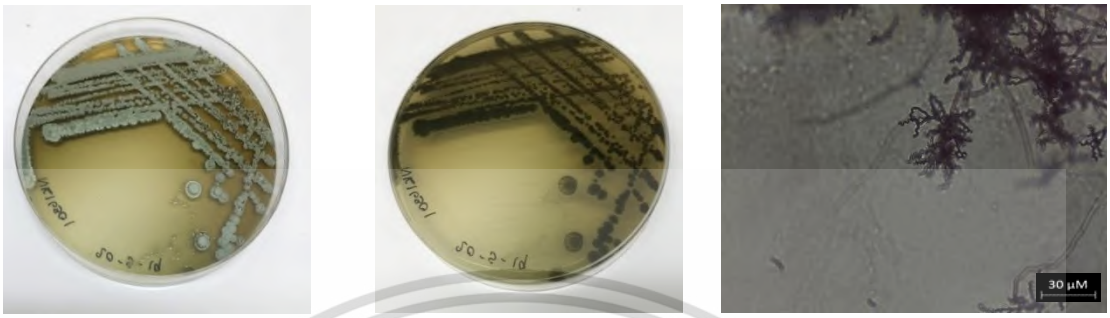
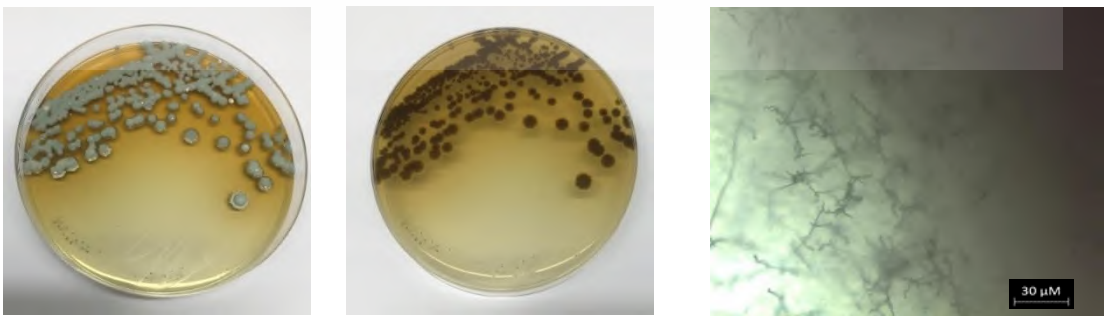
Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed verticillati chains of NK16301.



Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, vivid yellow soluble pigment, and sporulation showed rectinaculiaperti chains of NK16302.

Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, moderate olive brown soluble pigment, and sporulation showed rectinaculiaperti type of spore chains of NK16303.



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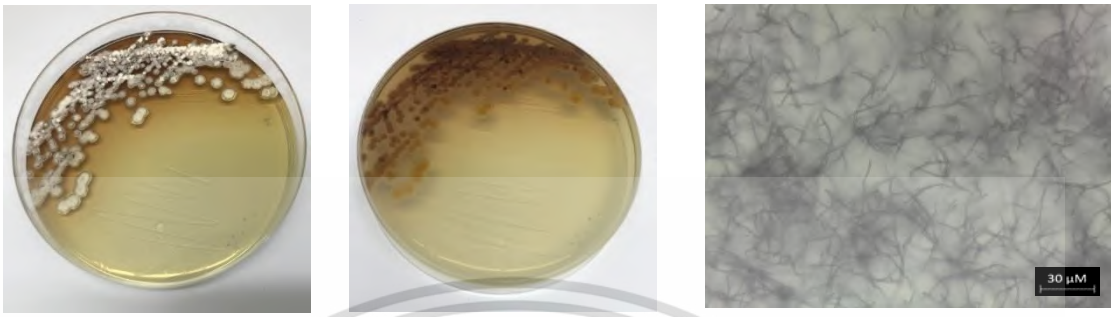
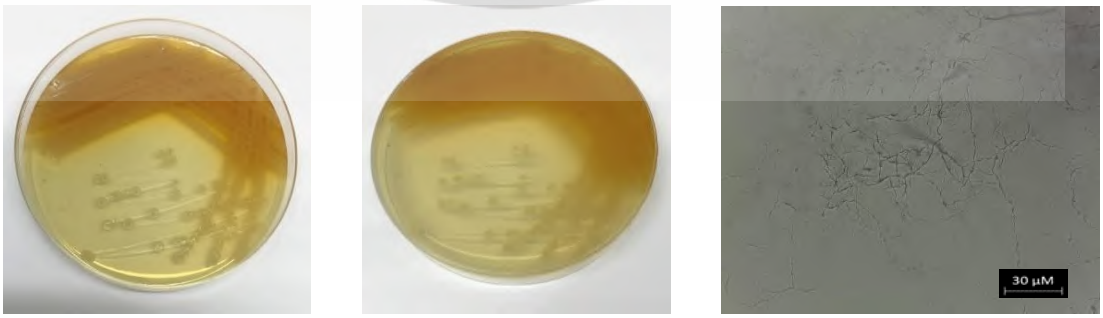
Pinkish gray aerial mycelium, grayish red substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK17101.



Moderate pink aerial mycelium, deep red substrate mycelium, pinkish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK17200.

Vivid yellow substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK17201.



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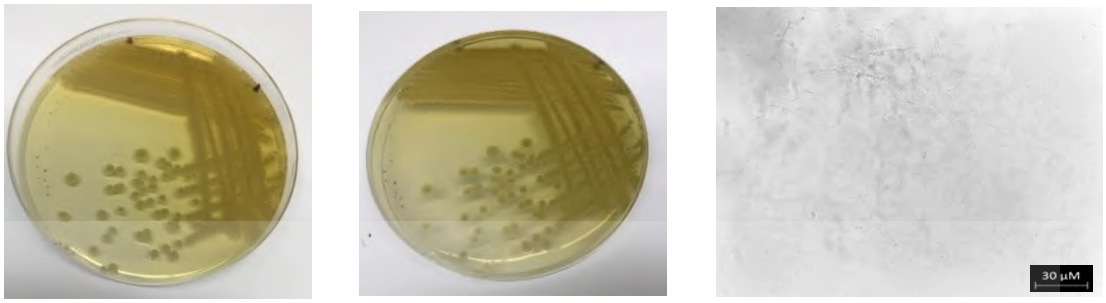
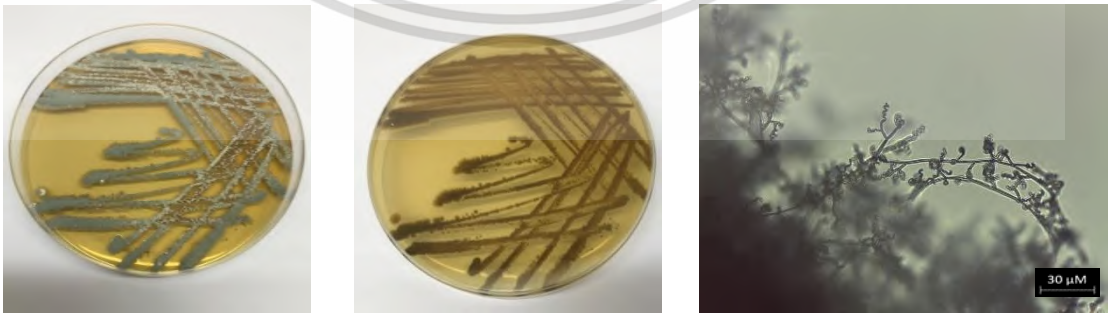
Brilliant greenish substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK17202.



Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, dark grayish olive green soluble pigment, and sporulation showed spiral type of spore chains of NK17203.

Bluish gray aerial mycelium, blackish green substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK17204.



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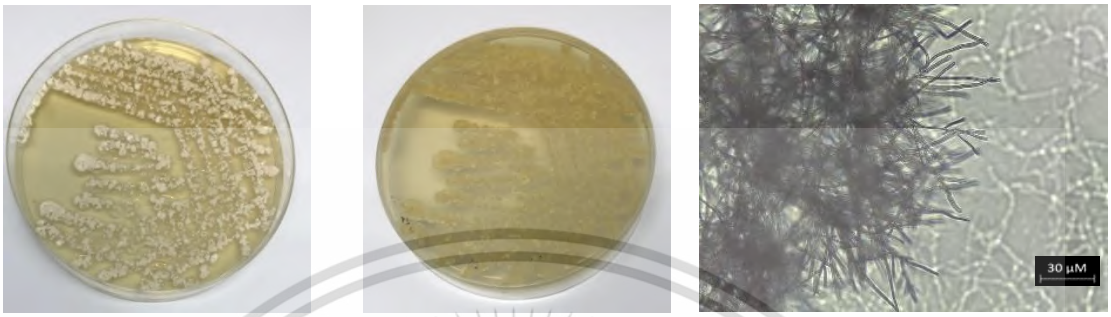
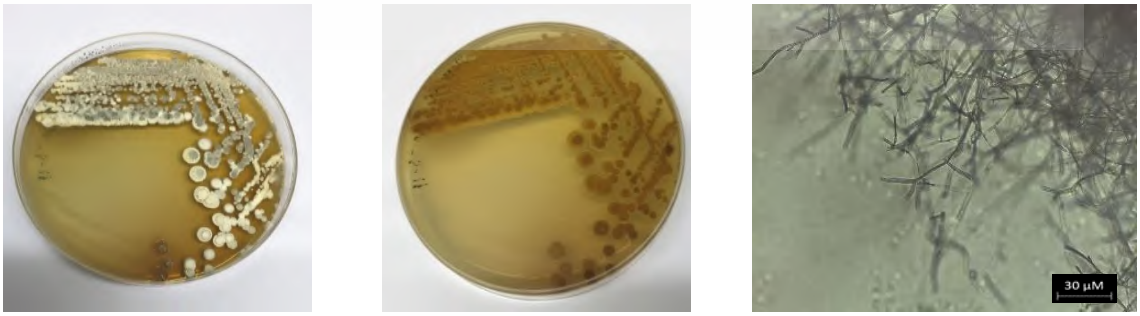
Pale pink aerial mycelium pale yellow substrate mycelium, pinkish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK17205.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK18101.

Light greenish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK18201.



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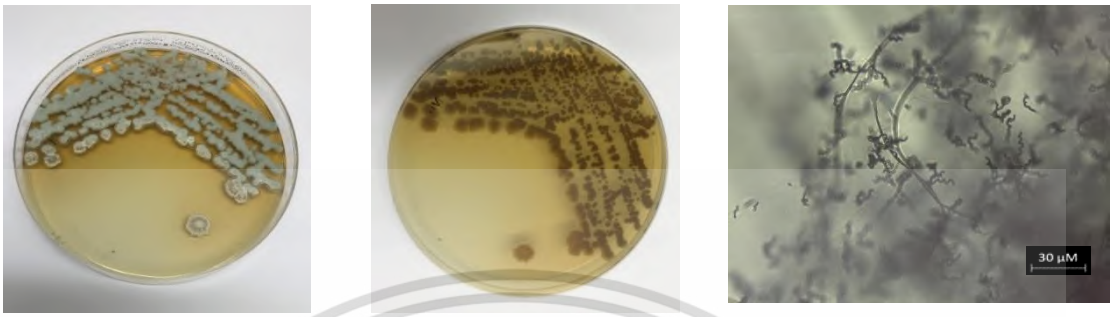
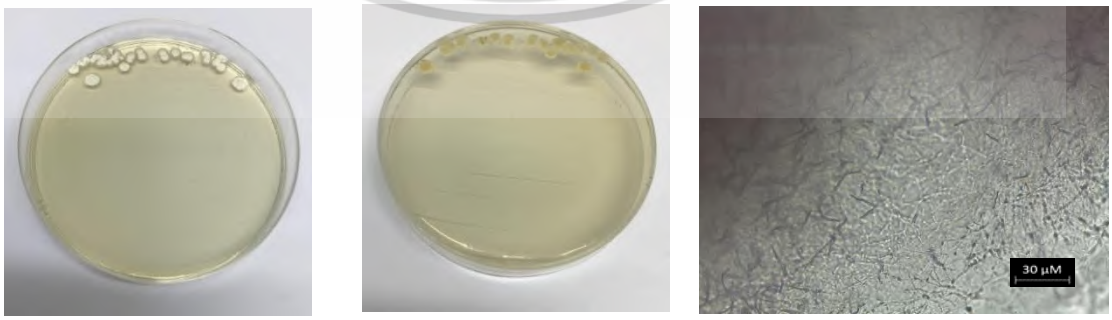
Grayish green aerial mycelium, blackish green substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK19301.



Light olive brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of NK20201.

Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment and sporulation showed rectiflexible chains of NK21200.



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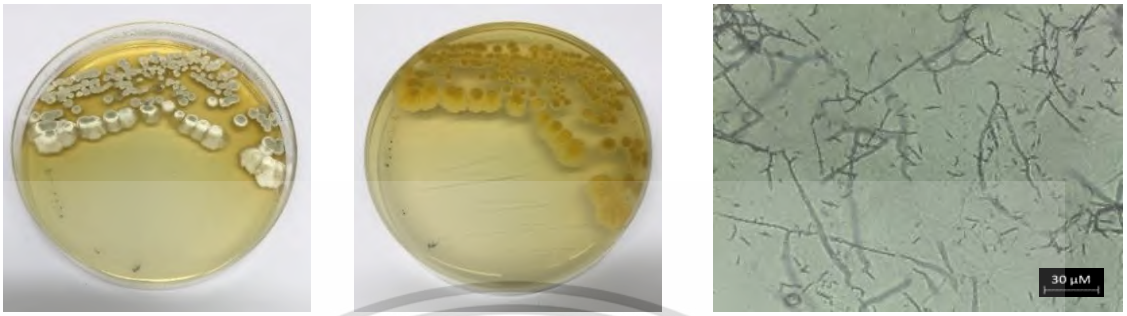
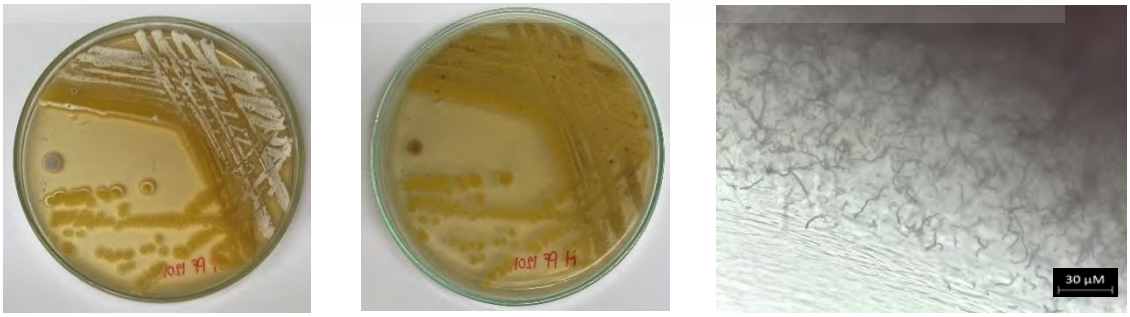
Light gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of NK21201.



Bluish gray aerial mycelium, blackish green substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of NK 21202.

Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, non-producing soluble pigment and sporulation showed rectiflexible chains of HPF1201.



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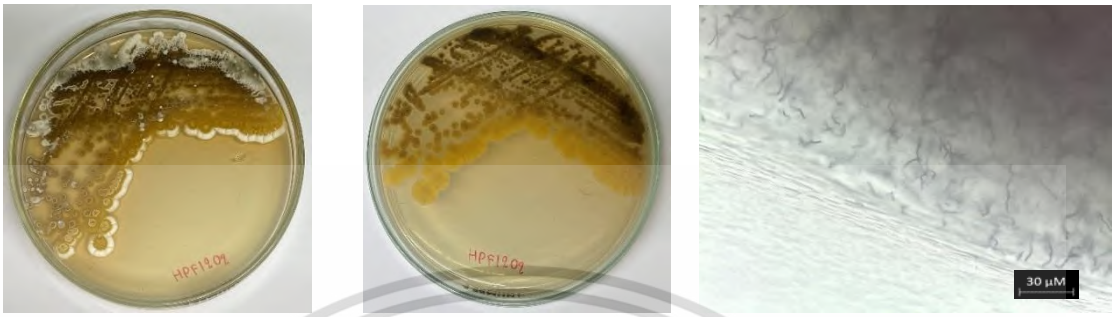
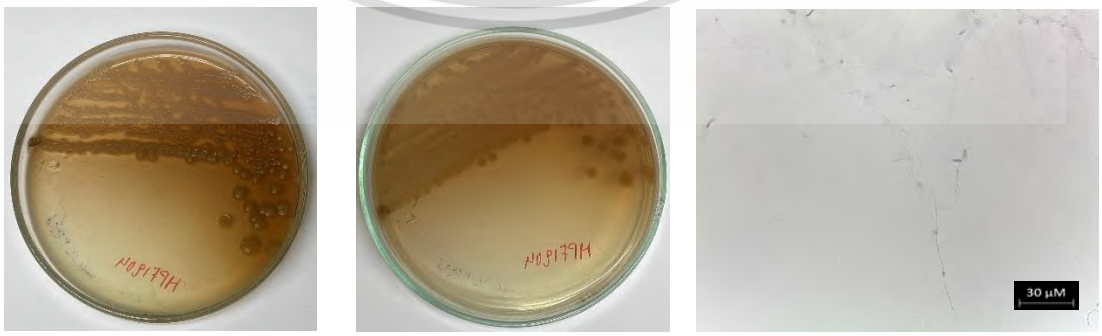
Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of HPF1202.



Light gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible chains of HPF1203.

Vivid greenish yellow substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF1204.



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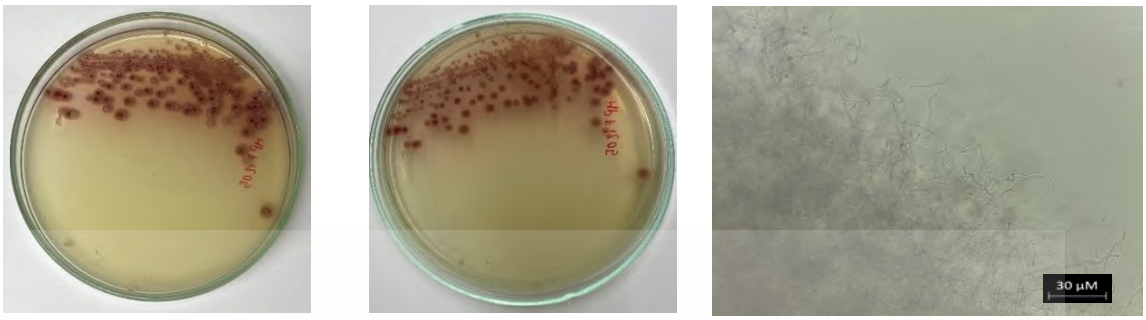
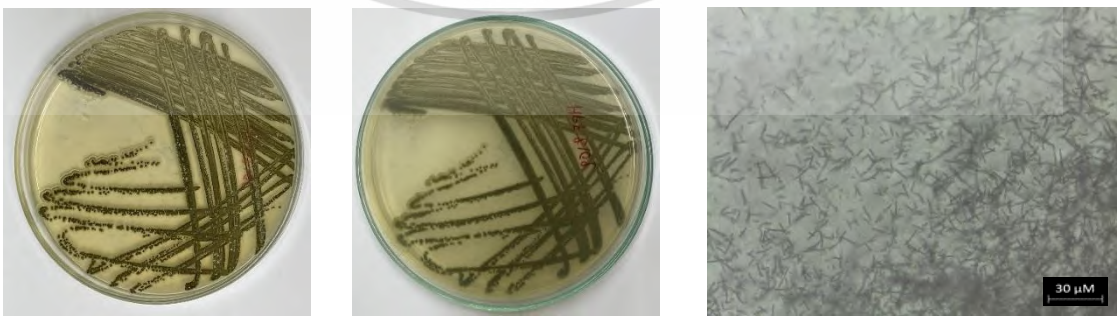
Deep red substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF1205.



Bluish gray aerial mycelium, dark grayish yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment and sporulation showed spiral chains of HPF1301.

Yellowish white aerial mycelium, moderate olive-green substrate mycelium, light grey mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of HPF2102.



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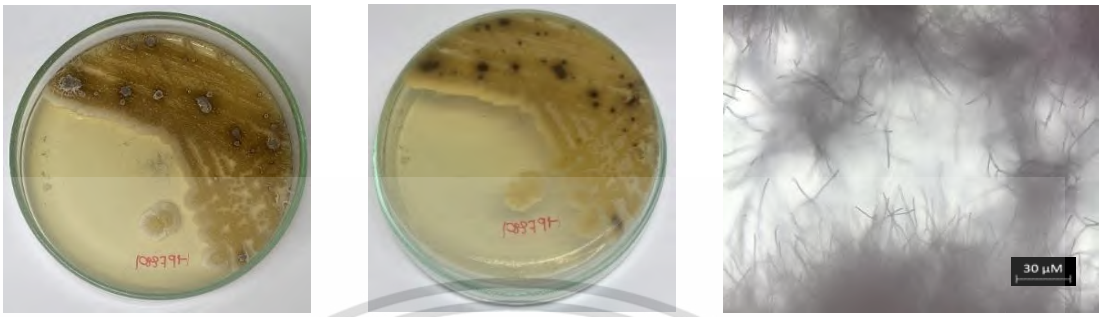
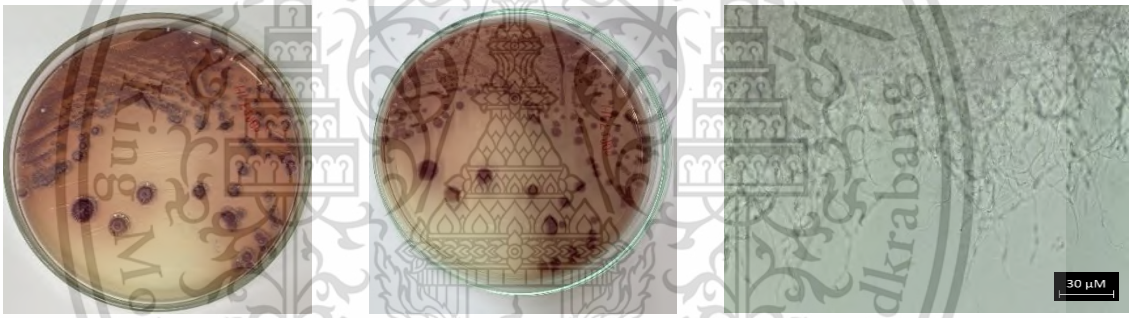
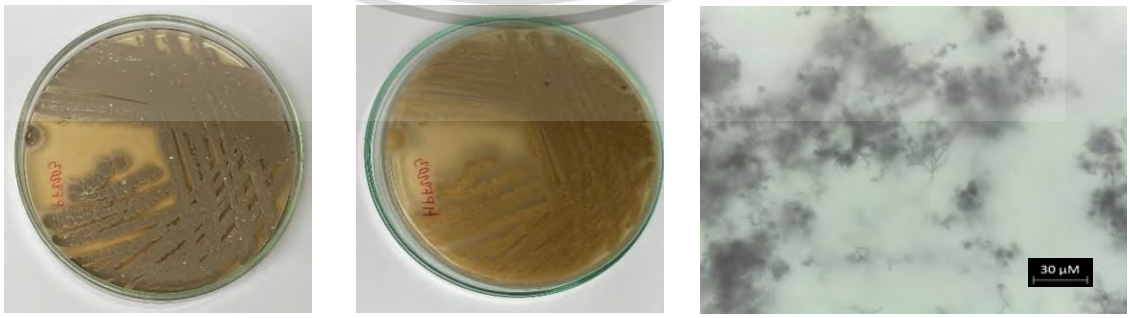
Yellowish white aerial mycelium, vivid greenish yellow substrate mycelium, yellowish white mass, non-producing soluble pigment of spores, and sporulation showed rectiflexible chains of HPF2201.



Deep red substrate mycelium, deep red soluble pigment, non-producing spores, and aerial mycelium of HPF2202.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed verticillati chains of HPF2203.



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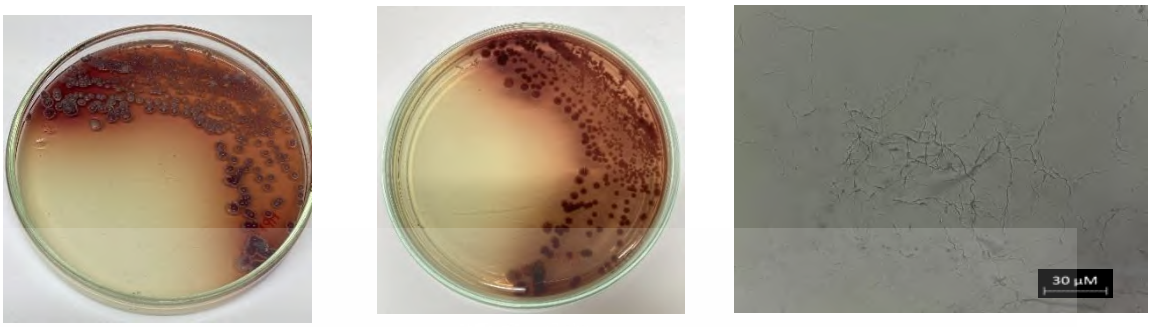
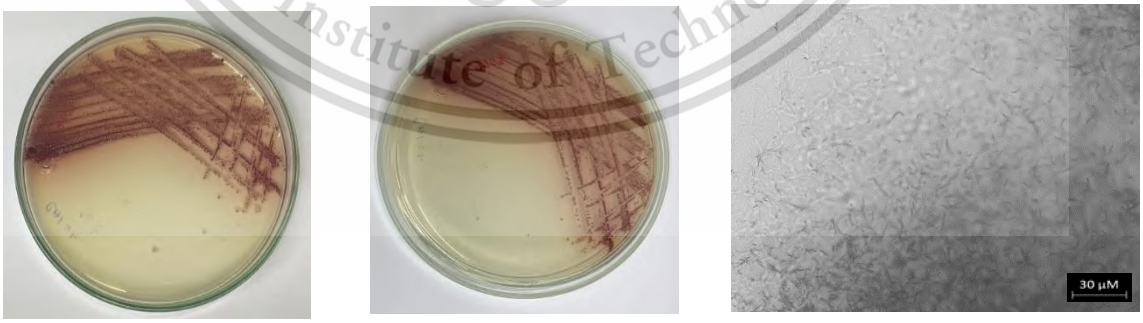
Deep red substrate mycelium, deep red soluble pigment, non-producing spores, and aerial mycelium of HPF2204.



Moderate yellowish brown substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF2206.

Deep red substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF2207.



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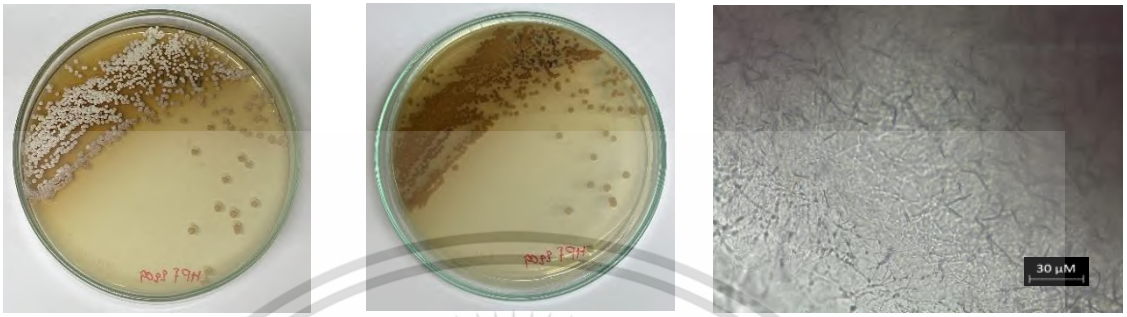
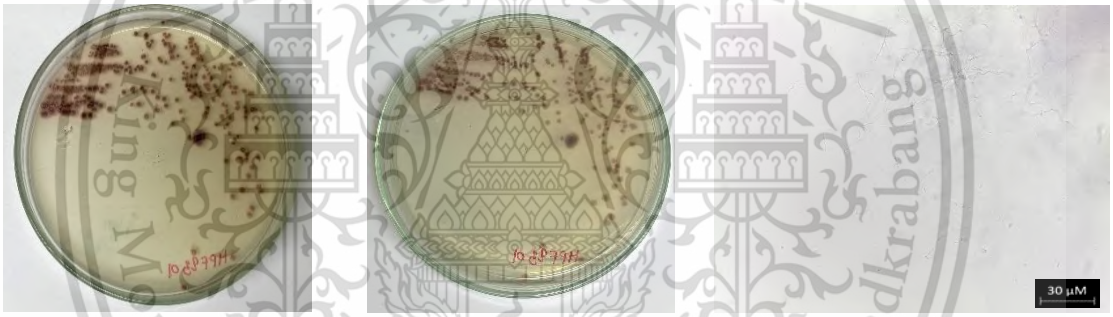
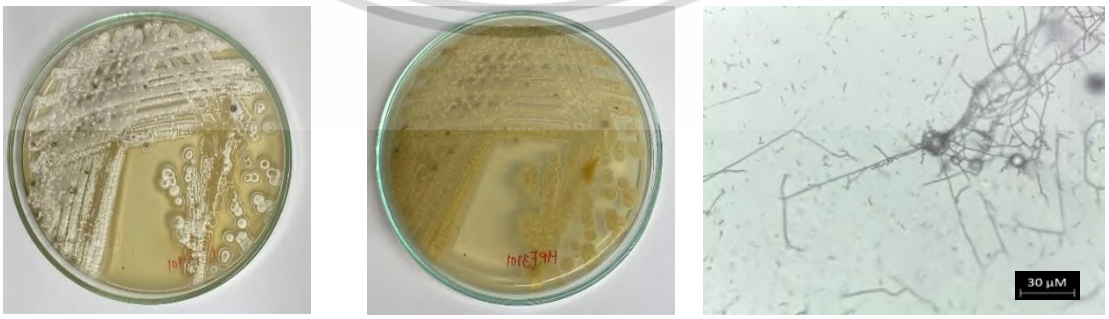
Light gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, and dark grayish olive green soluble pigment, and sporulation showed rectiflexible chains of HPF2209.



Deep red substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF2301.



Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectinaculiaperti chains of HPF3101.



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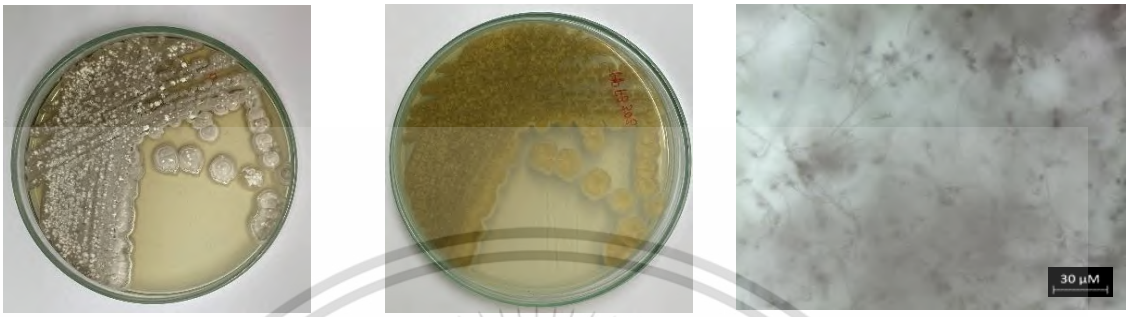
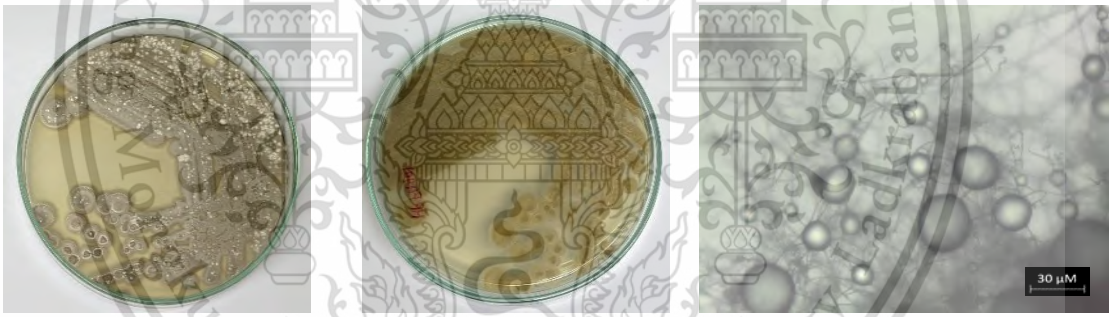
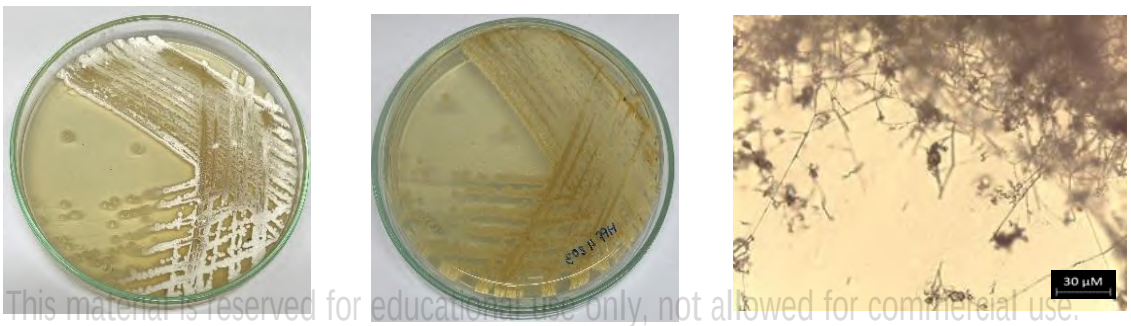
Bluish gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, and non-producing soluble pigment on ISP2 medium. Sporulation showed rectinaculiaperti chains of HPF3303.



Bluish gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectinaculiaperti type of spore chains of HPF4201.



Yellowish white aerial mycelium, light yellow substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed spiral chains of HPF4203.



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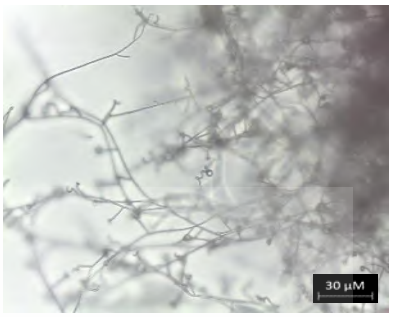
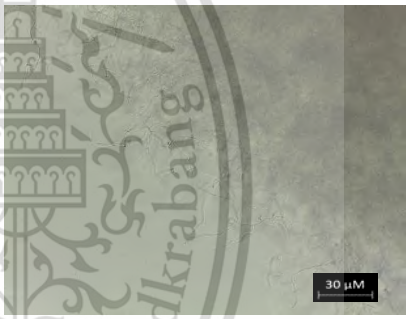
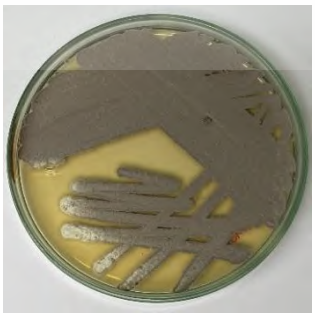
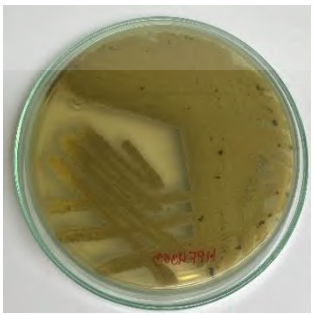
Bluish gray aerial mycelium, brilliant orange substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectinaculiaperti chains of HPF4301.



Light yellow substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF4302.



Bluish gray aerial mycelium, moderate yellowish brown substrate mycelium, white mass of spores, non-producing soluble pigment, and sporulation showed rectiflexible type of spore chains of HPF4303.

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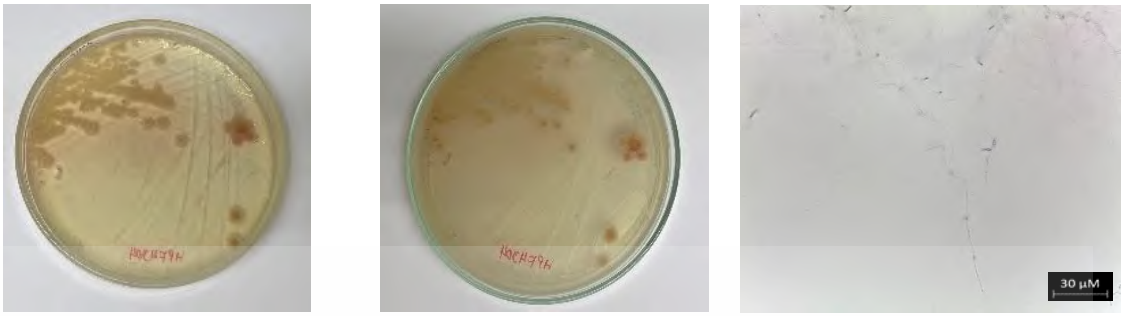
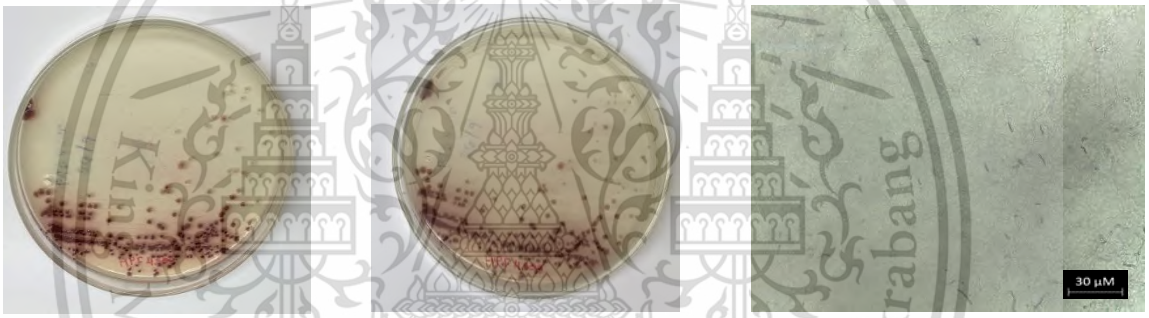
Light yellow substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF4304.



Deep red substrate mycelium, non-producing spores, soluble pigment, and aerial mycelium of HPF4304.



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Appendix D

Results of 16S rRNA gene

1. NK01101 (1,406 bp)

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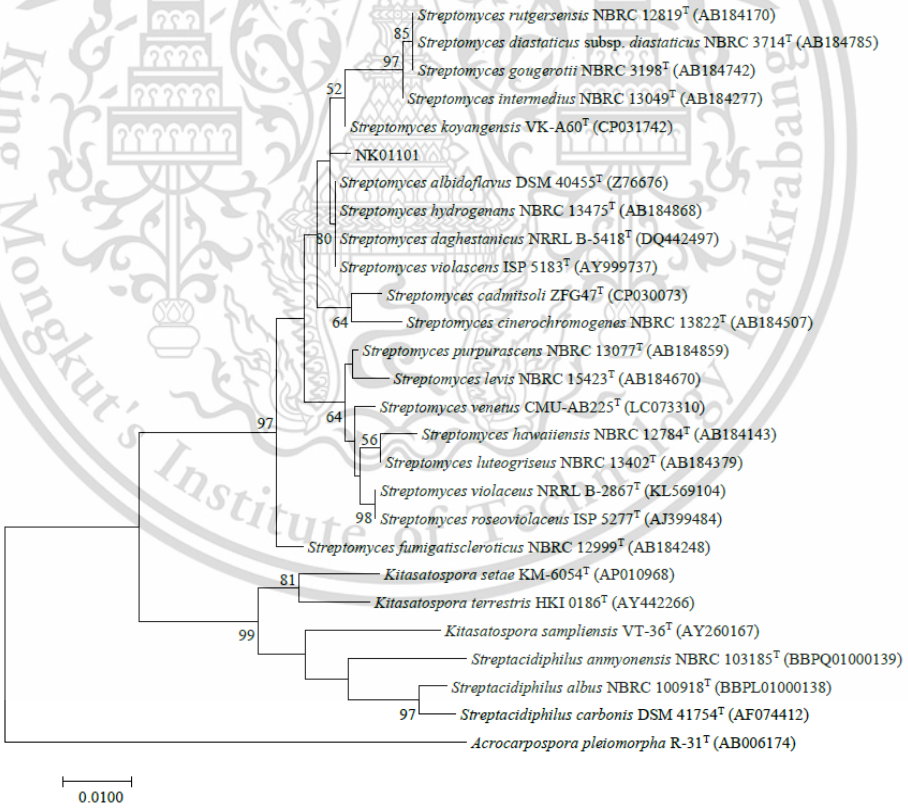
ATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGG
GCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCG
GGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAA
GGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGT
GTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTG
GTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCC
CTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGT
GCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAG
TCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCGGGGCCTTGTACACCCCGCCCGTCACGTCACGAAAGTCGGC



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK01101 that showed antifungal activity and their closely related type strains.

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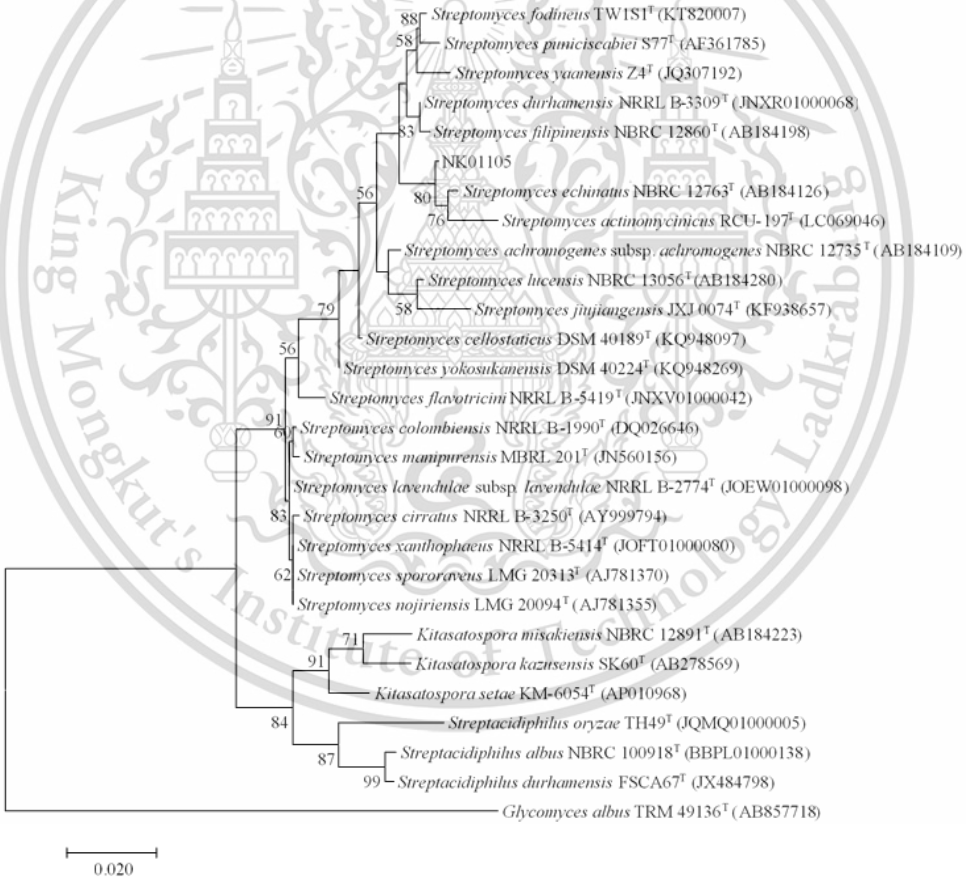
2. NK01105 (1,408 bp)

CTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGT
GGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGAGCCGGGACCGCATGGTCCTGGCTGTAAAGCTCCGGCGGTGAA
GGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTT
GTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTCGATACGGGCTAGC
TAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGG
TGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGC
TTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAG
GAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAG
CTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA
TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTTGGTAACACCCGAAGCCGGTGGCCCAACC



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK01105 that showed antibacterial activity and their closely related type strains.

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3. NK05201 (1,405 bp)

CTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCG
AACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGGTGGATGGTG
TAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTG
CAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG
ATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGG
CAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGT
GGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGA
GTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK05201 that showed antifungal activity and their closely related type strains.

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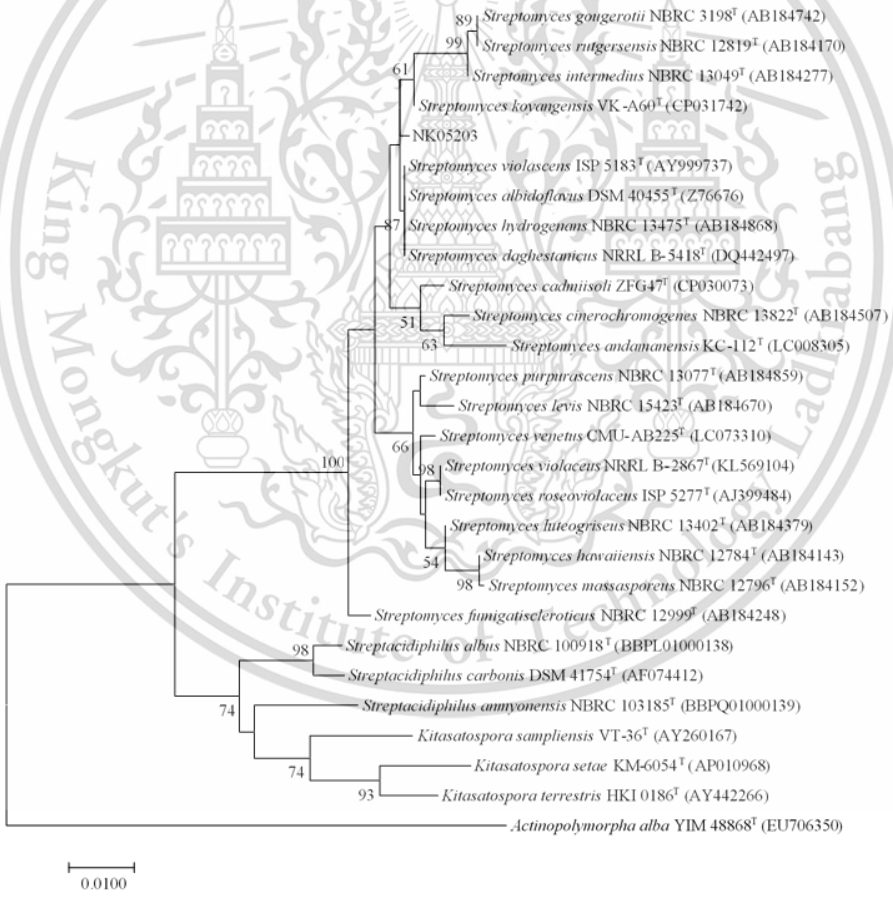
4. NK05203 (1,435 bp)

CTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCG
AACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGGTGGATGGTG
TAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTG
CAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG
ATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGG
CAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGT
GGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGA
GTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGT
GGCCCAACCCCTTGTGGGA



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK05203 that showed antifungal activity and their closely related type strains.

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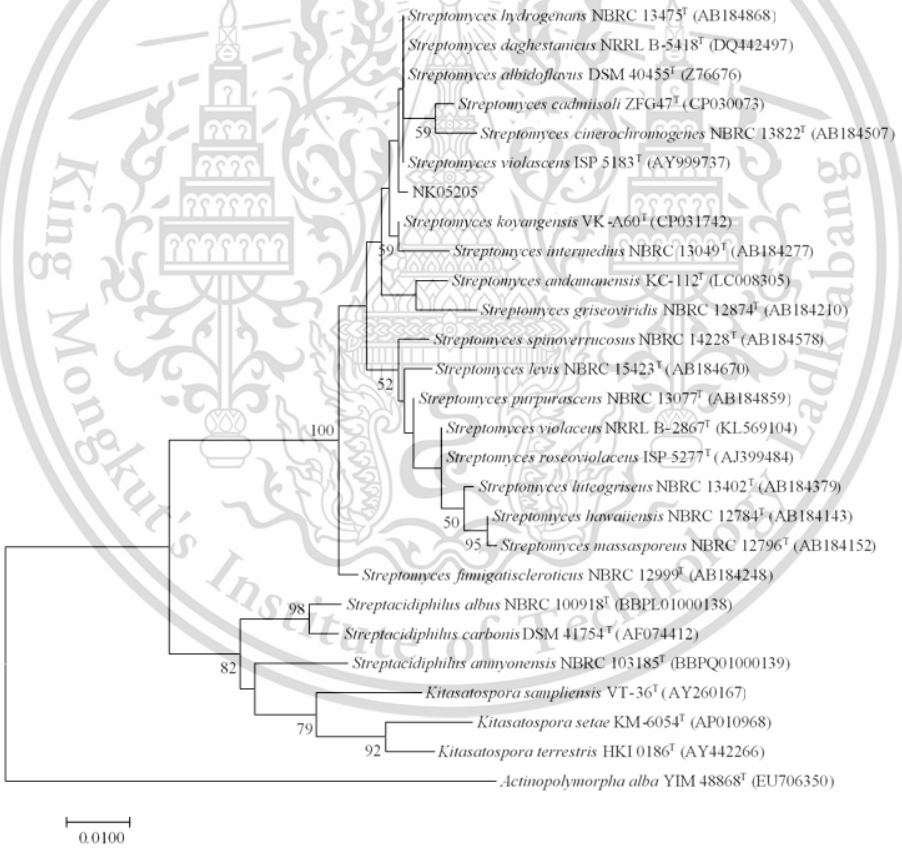
5. NK05205 (1,453 bp)

CTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAACG
GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGGTGGAT
GGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA
TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCG
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCAC
GTCGGGTTGTGAAAGCCCGGGGCTTAACCCCGGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTA
GCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCG
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTA
AGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGAC
GCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACT
CACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC
GGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC
CCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGAT



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK05205 that showed antifungal activity and their closely related type strains.

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6. NK07201 (1,413 bp)

CCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAAC
GGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGGTGGA
TGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA
TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCG
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCAC
GTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCG
GTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGT
GCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCA
ACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCAC
GGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCG
GTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGA
GTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCG
AAGCCGGTGGCCCAAC

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK07201 that showed antifungal activity and their closely related type strains.

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7. NK08202 (1,424)

TTTGAATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAACG
GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCGCCGCATGGTGGATGGTGTAAA
GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATG
ACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGT
CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAA
CATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGT
GCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
GGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTC
GCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGC
CCAACCCCT

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK08202 that showed antifungal activity and their closely related type strains.

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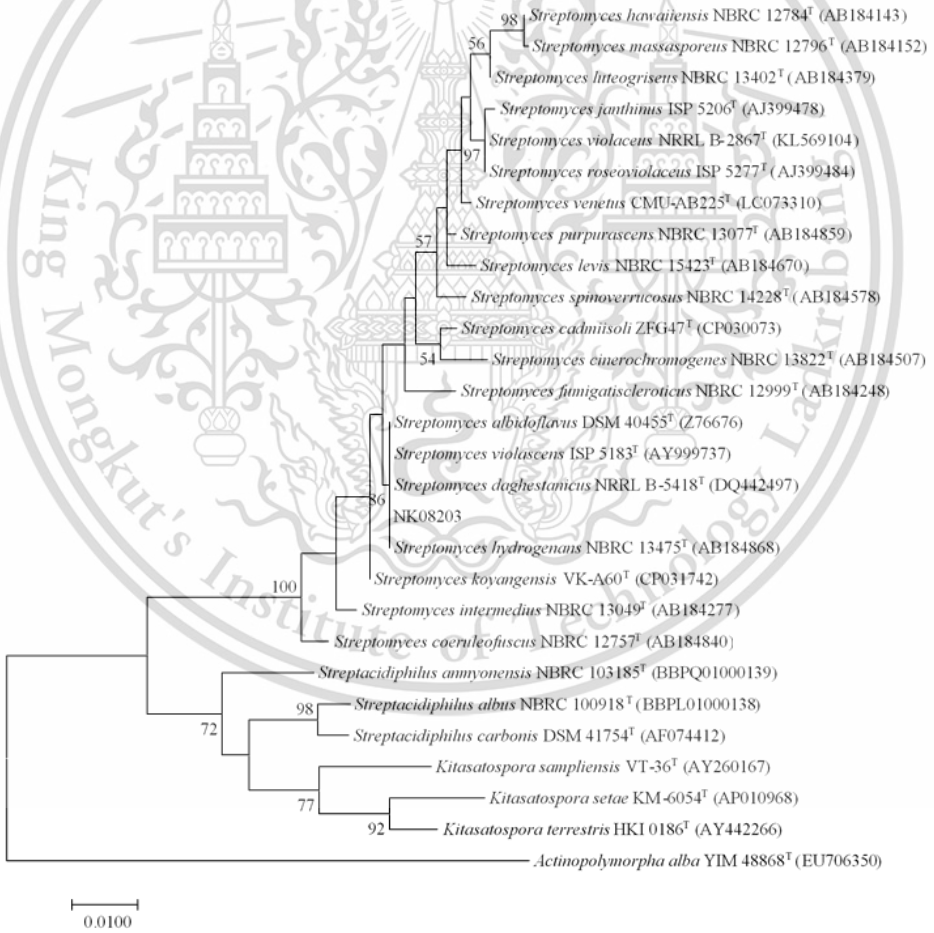
8. NK08203 (1,452)

CTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTTTCGGGCGGGGATT
AGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCATC
GCATGGTGGATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGG
GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTG
CAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATT
CCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCT
AACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCT
TAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTG
CTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTG
CTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCC
CATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGT
CGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTG



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK08203 that showed antifungal activity and their closely related type strains.

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9. NK08204 (1,423 bp)

GCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTT
CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGAGCCGGGACCGCATGGTCCTGGCTGTAAAGCTCCGGCGGTGAAGGATGA
GCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGG
CCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCCCGAGGCTTAACCT
CGGGTCTGCAGTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACA
CCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGG
CTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACA
TACACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGG
AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGTGAGGT
GGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTG
CTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTTGGTAACACCCGAAGCCGGTGGCCCAACCCGTAAGGGAG
GGAGCTGTCGAAGGTGGGACTAGCGATT

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK08204 that showed antifungal activity and their closely related type strains.

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10. NK08205 (1,421 bp)

CAGGCTGGATCACCTCCTTAGCTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCT
TCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCGTC
TTGGGCATCCTTGATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG
CAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGG
GGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
GTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAG
GCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTG
TTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
GGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACT
CGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAGTGCTGCAGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCG
GTAC

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK08205 that showed antifungal activity and their closely related type strains.

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11. NK13102 (1,467 bp)

CTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAGCCCTTCGGGGTGGATCAGTGGCGAACGGGTGAGTAAC
ACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCTTCCTCCGCATGGGGGTTGGTGGAAAGCTCCGGCGG
TGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCG
GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
GAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGC
AGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCG
TCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATG
TGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATGCCGGAAACGTCTAGAGATAGGCGCCCCCTTGTGGTCGGTATACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCGAGTAATGTCGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGTCGGTACAAAGGGCTG
CGATGCCGTGAGGCGGAGCGAATCCCAAAAAGCCGGCCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGTAATCGCAGATCA
GCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCGTAAGGGGAGGA
GCCGTCGAAGGTGGGACCAGCGATTGGGACGAAGTCGTAACAAGGTAGCC

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK13102 that showed antibacterial activity and their closely related type strains.

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12. NK14101 (1,440 bp)

GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGC
AATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATCACTCCCGCAGGCATCTGCGGGGGTCGAAAGCTCCGGCGGTGAAGGAT
GAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGA
GTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCC
GCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAA
TTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGA
CCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGC
GATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAG
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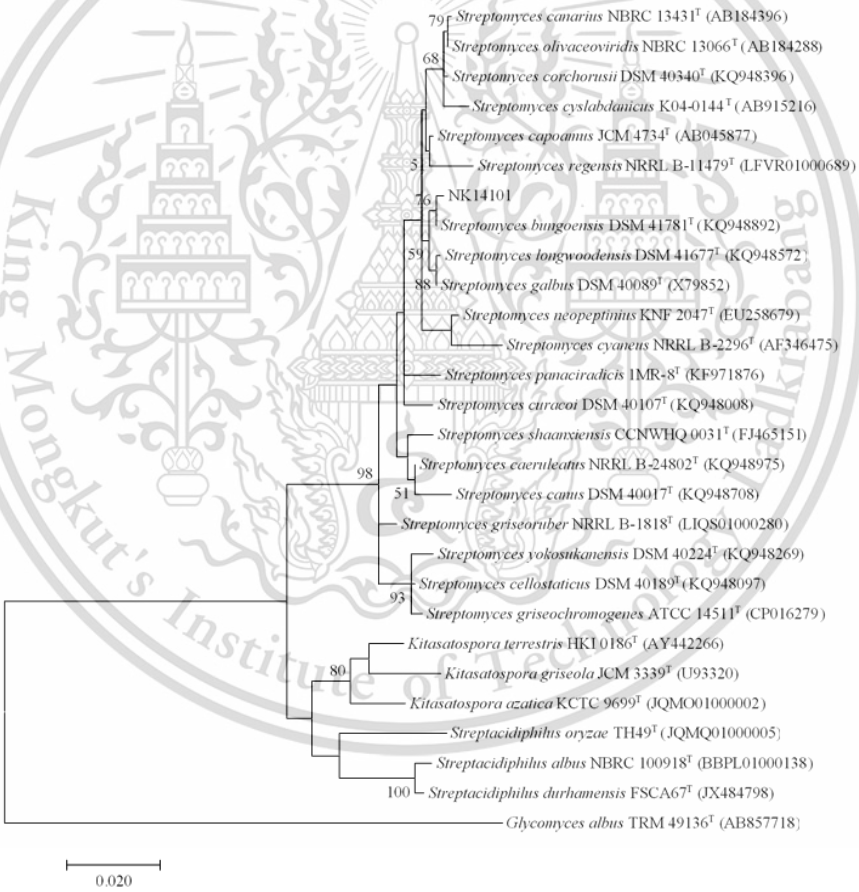
AGCTGTCGAAGGTGGGACTAGCG



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK14101 that showed antibacterial activity and their closely related type strains.

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13. NK17205 (1,422 bp)

AGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAA
CACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACTGCGGGAGGCATCTCCTGCGGTGGAAAG
CTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGAC
GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGT
GAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATG
CGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCC
TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAG
AACCTTACCAAGGCTTGACATATACCGGAAACACCCAGAGATGGGTGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGAC
CGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAA
GAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTA
GTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGG
TGGCCCAACCCCTTGTGGGAGGGAGCT

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK17205 that showed antibacterial activity and their closely related type strains.

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14. NK21201(1,415 bp)

CTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAA
CGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACCACTCTCGCAGGCATCTGTGAGGGTTGA
AAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCA
TTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATC
TCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGA
CATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGG
TGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAA
TGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGT
TGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTTGGTAACACCCGAAGCCGGT

Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate NK21201 that showed antifungal activity and their closely related type strains.

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15. HPF2102 (1,420 bp)

CTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGCTGAAGCTACTTCGGTGGTGGATG
AGTGGCGAACGGGTGAGTAACACGTGGGTAATCTGTCCTGCACTCTGGGATAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTCTGCACC
GCATGGTGTGGGGTGGAAAGTTCCGGCGGTGCAGGGTGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGG
GTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCGCCAGGGACGAAGCGCAAGTGACGGTACCTG
GAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGTTTGTTGCGTCGGCCGTGAAAACTGGAGGCTTAACCTTCAGCTTGCGGTCGATACGGGCAGACTTGAGTTCGGTAGGGGAGACTGGAATT
CCTGGTGTAGCGGTGAAATGCGCACATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGGCGACATCCACGTTGTCCGTGCCGTAGCTA
ACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATT
AATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGCATCAGACAGCCGTAGAGATACGGTTTCCCTTGTGGTTGGTGTACAGGTGGTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCGCGTAATGGCGGGGA
CTCGCGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAAT
GGCTGGTACAGAGGGCTGCGATACCGCGAGGTGGAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG
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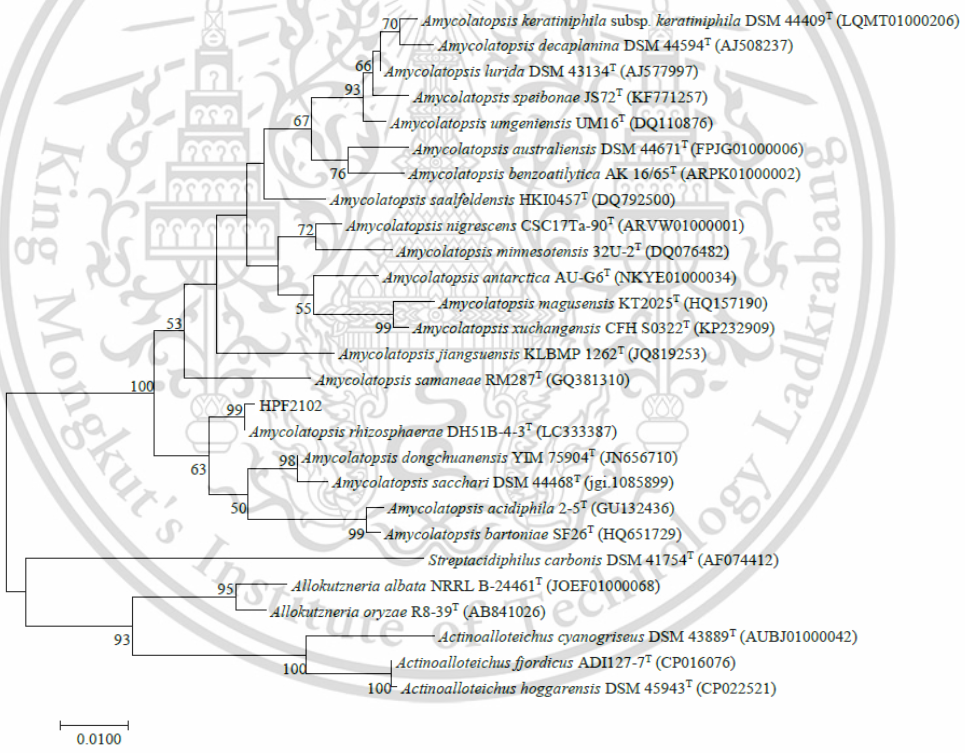
ACCCGAAGCCCACGGCCCAACCCG



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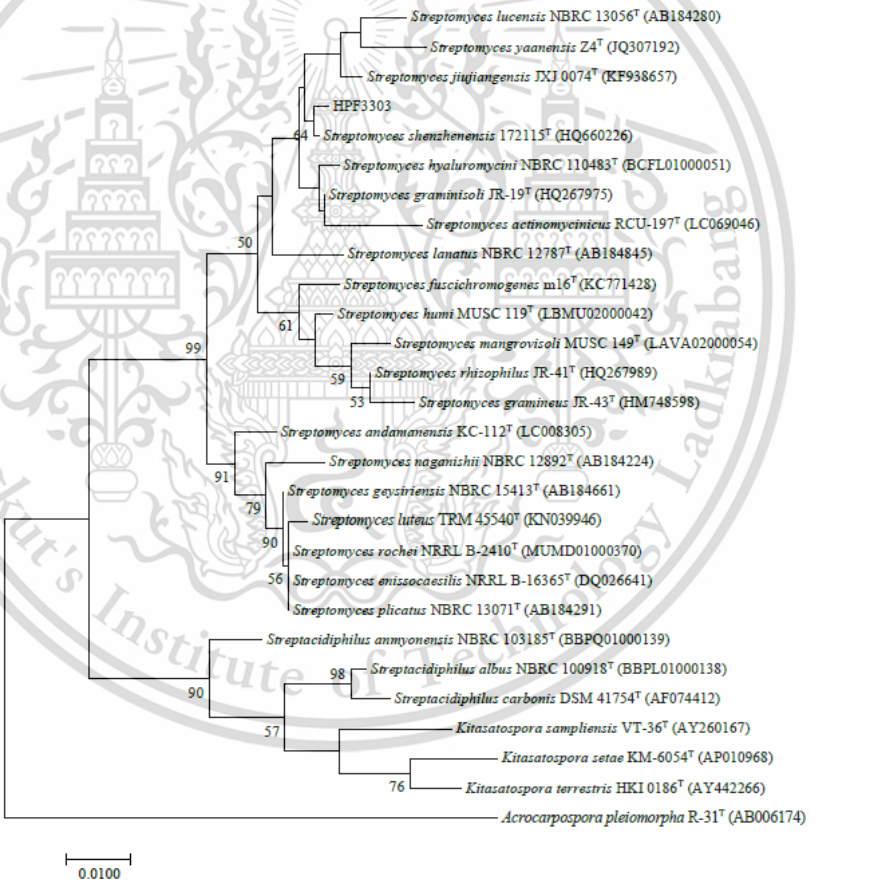
16. HPF3303 (1,512 bp)

CTCAGGCTGGATCACCTCCCTAGCTGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCA
CTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGAGCC
TGGGAGGCATCTCCCGGGTTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTA
GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA
TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCCC
GAGGCTTAACCTCGGGTCTGCAGTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGACA
GGGTCCCTCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGT
GTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT
TGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAAC
TCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTC
GGTAACACCCGAAGCCGGTGGCCCAACCTCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGT



Phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood method of actinomycete isolate HPF3303 that showed antifungal activity and their closely related type strains.

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Academic Publications

1. Rattanakavil, T., Hawanit, P., Boonchai, S., Kaewaram, S., Kumlung, T., and Klanbut, K. 2018. Screening and antimicrobial of Actinomycetes isolated from hot spring soil sample, Boekleng Suan Phueng and Potential Microbes Selection to Control Mushroom Pathogens in Mushroom Farm." The 30*th* Annual Meeting of the Thai Society for Biotechnology and International Conference. November 22 - 23, 2018 Ambassador Hotel, Bangkok, Thailand.

2. Rattanakavil, T., and Klanbut, K. 2019. "Actinomycetes from rice field soil, Nakonsawan and their antimicrobial activity." The 31st Annual Meeting of the Thai Society for Biotechnology and International Conference. November 10 - 12, 2019 Duangjitt Resort & Spa, Patong, Phuket, Thailand.

3. Rattanakavil, T., Kumlung, T., and Klanbut, K. 2020. "Isolation and Molecular Characterization of Antifungal Production from Rice Fields Rhizosphere soil, Thailand." *International Jounal of Science and Innovative Technology*. 3 (1) : 41-52.

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