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by Erna Prawita Setyowati

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Antimicrobial activity and Identification of fungus associated *Stylissa flabelliformis* sponge collected from Menjangan Island West Bali National Park, Indonesia

Erna Prawita Setyowati[1]*, Sylvia Utami Tunjung Pratiwi[1], Purwantiningsih[1], Putu Oka Samirana[2]

[1]Faculty of Pharmacy, Universitas Gadjah Mada (UGM). Jl. Sekip Utara 55281, Yogyakarta, Indonesia
[2]Dept of Pharmacy, Faculty of Math and Natural Sciences, Udayana University, Bali, Indonesia

*Corresponding author : Erna Prawita Setyowati
Email: erna_prawita@ugm.ac.id

ABSTRACT

The Fungus is a very important microorganism as a producer of bioactive secondary metabolites. Active substances of microbial origin have been sought through the process of screening methods to obtain antimicrobial compounds. The purpose of this study was to isolate fungi associated with sponge taken from Menjangan Island National Park West Bali (Indonesia) and identify fungi that have antimicrobial activity. Isolation of fungus from sponge was carried out by spread plate method using Saboroud Saline Agar medium. Each fungi will be tested to *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. Identification of fungi is based on the observation of macroscopic, microscopic and also using 16rRNA/ITS phylogeny tree. The results showed that *S. flabelliformis* sponge had 10 fungal isolates. Most of them have antimicrobial activity. The name associated with a sponge fungus is These 10 fungus are *Aspergillus flavus* strain UPMZ02, *Aspergillus fumigatus* strain CD1621, *Trichoderma reesei* strain JCM 2267, *Aspergillus nomius* strain KUB105, *Aspergillus* sp. strain TLWK-09, *Aspergillus flavus* strain MC-10-L, *Penicillium* sp. strain RMA-2, *Aspergillus* sp. strain TLWK-09, *Aspergillus fumigatus* and *Trichoderma reesei* strain TV221

Keyword: Sponge-associated fungi, *Stylissa flabelliformis*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*

INTRODUCTION

Sponges are the subject of interesting antibiotic development studies because the sponges form associations with various microbes and are rich in bioactive compounds. Sponges are the largest source of new bioactive compounds than other marine products (Mehbub, *et al.*, 2014). Bioactive compounds obtained from sponges are known to have various activities such as cytotoxic, antimicrobial, anti-inflammatory, antiparasite and others (Proksch, *et al.*, 2002; Setyowati *et al*, 2016). The bioactive compound on the sponge acts as a self-protection mechanism against the environment. It was surprising to find that many secondary metabolites reported from marine invertebrate are produced by their microorganism symbionts (Thakur and Muller, 2004).

Marine sponges often contain diverse and abundant microbial communities, including bacteria, archaea, microalgae, and fungi. In some cases, these microbial associates comprise as much as 40% of the sponge volume and can contribute significantly to host metabolism (Taylor, *et al.*, 2007). Correspondingly, the studies of natural products from sponge-associated microorganisms, particularly bacteria and fungi, are also plenty. Secondary metabolites produced by fungi associated with sponges are much larger when compared with secondary metabolites produced by bacteria that associate with sponges. In more detail, Ascomycota dominates the proportion of fungal producer by division (Kim, *et al.*, 2013). The main focus on secondary metabolites produced by fungi is due to their diverse biological activities having both toxic and curative effects in maintaining human and veterinary health. More intense studies on marine fungi have been done in the past three

decades. These continuous studies revealed that marine fungi are rich sources of bioactive natural products (Ebada and Prosch, 2010; Bills and Gloer, 2016).

Previous study conducted by Setyowati *et al.* (2004) showed that *Stylissa flabelliformis* sponge taken from Menjangan Island's waters of the West Bali National Park could produce jaspamide compounds. This compound is highly potent against *C. albicans* and more active than Miconazole nitrate as a comparison, this compound also has cytotoxic power equivalent to vincristine (leukemia cancer drug) in myeloma cells. This compound is usually found from the genus *Jaspis* sp and has never been found from the species *S. flabelliformis.* Often metabolites with compound structures similar to those produced from sponges with different taxonomies (Mehbub *et al.*, 2014). This suggests that the structural characteristics of compound are strongly thought to originate from sponge microorganisms and are responsible for the production of various bioactive compounds (Koopmans, *et al.*, 2009). Therefore it is estimated that some sponge metabolites are produced by symbiotic microorganisms (Thomas, *et al.*, 2010). Antimicrobial assay against fungi associated with *S. flabelliformis* sponge and molecular identification of the sponge fungus was performed in this study.

MATERIAL AND METHODS

Sampling and Isolation of the fungal association sponge.

Specimens of the marine sponge *S. flabelliformis* (Axenellida) were collected from Menjangan Island National Park West Bali (Indonesia) through Scuba diving. Since the stringent surface sterilization procedures have previously proven to be too rigorous for delicate sponge tissues, the sponges were either briefly sterilized for 30s in 70% v/v ethanol followed by three subsequent washing in sterile commercial sea water or merely washed three times in sterile seawater. The sponge tissue was then cut into approximately 1.5mm segment and planted on Sabouroud agar growth medium with sea water, 250ug/mL chloramphenicol, and cultivated at 25°C. Emerging fungal mycelia were isolated and taken into the culture.

Antimicrobial activity of fungi

The concentration of microbes used was 107 Colony Forming Unit. The microbial suspension was inoculated into 10mL nutrient-agar medium (*E. coli, S. aureus*) and Saboroud Dextrose Agar (*C. albicans*), then poured into a petri dish.

Sterilized disc paper, sprayed as much as 10µL of fungi methanol extract dissolved with a concentration of 10 mg/mL. As a positive control, 1mg/mL of tetracycline was used for antibacterial test and used 1mg/mL of griseofulvin for an antifungal test, and methanol as a solvent control. Paper discs were placed on top of pre-prepared solid media. Petri was incubated overnight at 35ºC-37°C. Observed zone barriers formed around the paper disc (Niyomkam, *et al.*, 2010).

Identification of fungi

Identification of fungi associated sponge was carried out by observing fungi macroscopically, microscopically, and molecularly based on the genetic analysis of partial locus Internal Transcribed Spacer (ITS) ribosomal DNA fungi.

DNA isolation was initiated by growing fungal isolates in Potato Dextrose Broth liquid medium (PDB) and incubated for 72h. The biomass of the fungal mycelia is further harvested for the DNA extraction process. DNA fungi extraction was performed using PHYTOpure nucleon reagents (Amersham LIFE Science). PCR Amplification on ITS using primer ITS4. Purification of PCR product was performed by PEG precipitation method and continued with cycle sequencing. The results of the sequencing cycle are reversed with ethanol purification method. Analysis of sequence sequencing of nitrogen-based using an automated DNA sequencer (ABI PRISM 3130 Genetic analyzer) (Applied Biosystems). The sequenced raw data was then streamed and dissembled using the Bioedit program. Sequencing data was already assembled at BLAST with genome data registered at DDBJ/DNA Data Bank of Japan or NCBI/National Centre for Biotechnology Information to determine which taxon/species has the greatest homology/similarity and is molecularly related (Atashpaza, *et al.,* 2010; Santosa, 2011).

RESULTS AND DISCUSSION

S. flabelliformis sponges (Figure 1) are red with a thin shape and soft, porous surface with irregular protrusions on the whole body, inside yellowish-white body. Sponge classification is as follows: Kingdom Animalia, Division Porifera, Class Demospongiae, Subclass Ceractinomorpha, Order Halicondrida, Family Axenellida, Genus Stylissa, Species *S. flabelliformis* (Hooper and Soest, 2002).

The screening was done to obtain the active microbial (fungi) potential. Isolation of fungus from *S. flabelliformis* sponge observed at least 10 fu as (Figure 2). All of the 10 fungi were then being tested against the 3 kinds of microbes such as *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. These microbes used are representative of gram-positive bacteria, gram-negative and yeast.

The antimicrobial activity of fungi against several bacteria showed that most of the collected fungi were possible to inhibit the growth of microorganisms tested. Fungi No. 3, 5, 6, 7, 9 and 10 were inhibiting *S. aureus, C. albicans* and *E. coli*. The fungus has broad-spectrum properties because it can inhibit the growth of almost all tested bacteria and yeast *in vitro*.

Table I also shows that 7 of the 10 fungi can inhibit *Staphylococcus aureus.* It is known that *S. aureus* causing various infections, such as acne, ulcers, pneumonia, endocarditis, and implantation of any part of the body (Jawetz *et al*., 1996). The prevalent treatment to deal with bacterial infections is to use antibiotics such as methicillin, amoxicillin, penicillin, oxacillin, and others. Nonetheless, what seems to be happening is the occurrence of *S. aureus* resistance to these antibiotics. One of the most commonly reported cases of resistance is Methicillin Resistant *S. aureus* (MRSA), a methicillin-resistant *S. aureus* bacterium. The mechanism of methicillin resistance does not depend on the formation of beta-lactamase. However, it is more due to changes in Protein Binding Penicillins (PBPs), a penicillin-receiving protein that occurs as a result of chromosome mutations (Hugo and Russel, 2011). There are 5 fungi that can inhibit the growth of three microbes. In addition, sponge-derived strain collections that comprise isolates that tested negative for antimicrobial activity at first may have done so, because the compound of interest is not produced under standard laboratory conditions (Indraningrat, *et al*, 2016).

Result of molecular identification

Sequencing by ITS rDNA Isolate

Figure 3. Isolated DNA from Sal 1; 2. Isolated DNA from Sal 2; 3. Isolated DNA from Sal 3; 4. Isolated DNA from Sal 4; 5. Isolated DNA from Sal 5; 6. Isolated DNA from Sal 6; 7. Isolated DNA from Sal 7; 8. Isolated DNA from Sal 8; 9. Isolated DNA from Sal 9; 10 Isolated DNA from Sal 10. All DNA samples were loaded at concentration of 1/100 with condition 0.8% agarose gel. Amount of DNA ladder loaded per lane: 0.2ug each. Volume of sample loaded per lane : 1uL each.

Sal 1: Based on BLAST results on the NCBI database against the ribosomal RNA 18S sequence Gene sample number 1702.00467 obtained homology of 100% with *Aspergillus flavus* strain UPMZ02 18S ribosomal RNA gene, partial sequence; Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; And 28S ribosomal RNA gene, partial sequence KY698416.1 *Aspergillus flavus* strain UPMZ02 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S re; KU561919.1 *Aspergillus flavus* strain aC4 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial e; KT828546.1 *Aspergillus flavus* strain KAU internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial e; KR296888.1 *Aspergillus flavus* strain PUXX-FS06 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28e; KU561917.1 *Aspergillus flavus* strain sM4 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial (Figure 4).

Sal 2: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00468 obtained a homology of 99% with *Aspergillus fumigatus* strain CD1621 18S ribosomal RNA gene, partial sequence; Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; And 28S ribosomal

RNA gene, partial sequence HQ285554.1 *Aspergillus fumigatus* strain DS-C3 18S ribosomal RNA gene partial sequence; JX232280.1 Aspergillus fumigatus strain SGE69 18S ribosomal RNA gene partial sequence; JX092088.1 *Aspergillus fumigatus* strain CD1621 18S ribosomal RNA gene partial sequence; KM207771.1 *Aspergillus fumigatus* strain SK1 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KU321562.1 *Aspergillus fumigatus* strain 004 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene (Figure 5).

Sal 3: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00469 obtained homology of 100% with *Trichoderma reesei* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA, partial and complete sequence, strain JCM 2267 KP263685.1 *Trichoderma reesei* strain JCM 2267 internal transcribed spacer 1 partial sequences 5.8S ribosomal RNA gene; KC847188 *Trichoderma reesei* strain IPBCC06 325 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; KC847186 *Trichoderma reesei* strain IPBCC93 260 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; JN704346.1 *Hypocrea jeccorina* voucher HR241489 1 partial 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gen (Figure 6).

Sal 4: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00470 obtained a homology of 100% with *Aspergillus nomius* strain KUB105 18S ribosomal RNA gene, partial sequence; Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; And 28S ribosomal RNA gene, partial sequence KR737578.1 *Aspergillus nomius* strain KUB105 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S re; NR 121218.1 *Aspergillus nomius* NRRL 13137 ITS region from TYPE material; KF312154.1 *Aspergillus nomius* strain JA2 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial e; KF312151.1 *Aspergillus nomius* strain P3 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial se; DQ467992.1 *Aspergillus nomius* strain KS2 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribose (Figure 7).

Sal 5: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00471 obtained a homology of 100% with *Aspergillus* sp. strain TLWK-09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; And large ribosomal subunit of RNA gene, partial sequence KX353855.1 *Aspergillus* sp. strain TLWK-09 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KJ650333.1 *Aspergillus oryzae* strain SCIM1 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KR076749.1 Aspergillus flavus strain meijun2 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; LN482514.1 *Aspergillus flavus* genomic DNA containing ITS1 5.8S rRNA gene and ITS2 strain TUHT118; LN482494.1 *Aspergillus flavus* genomic DNA containing ITS1 5.8S rRNA gene and ITS2 isolate TUHT98 (Figure 8).

Sal 6: Based on BLAST results on the NCBI database against the ribosomal RNA 18S sequence Gene sample number 1702.00472 obtained homology of 100% with *Aspergillus flavus* strain MC-10-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; And 28S ribosomal RNA gene, partial sequence (Figure 7). KU095081.1 *Aspergillus oryzae* strain J12 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KP764897.1 *Aspergillus flavus* isolate AfKSA13-15 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KP764870.1 *Aspergillus flavus* isolate AfKSA13-04 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KP764857.1 *Aspergillus oryzae* isolate AoKSA13-01 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KU527785.1 *Aspergillus flavus* strain MC-10-L internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene (Figure 9).

Sal 7: Based on BLAST results on the NCBI database against the ribosomal RNA 18S sequence Gene sample number 1702.00473 obtained homology of 99% with *Penicillium* sp. strain RMA-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal

transcribed spacer 2, complete sequence; And large ribosomal subunit of RNA gene, partial sequence KP296145.1 *Penicillium griseofulvum* isolate 018 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KC506183.1 Fungal sp. AM2013 strain 16 Mp internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene KF914643.1 *Penicillium* sp. 4M3 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KY883661.1 *Penicillium* sp. strain RMA-2 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KY439022.1 *Penicillium citrinum* strain 1S.12 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene (Figure 10).

Sal 8: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00474 obtained a homology of 100% with *Aspergillus* sp. strain TLWK-09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; And large ribosomal subunit of RNA gene, partial sequence. Figure and fungus identification data for Sal 8 are the same as figure and data in Sal 5 (Figure 8), so Sal 8 is not further elaborated

Sal 9: Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene sample number 1702.00475 obtained a homology of 100% with *Aspergillus fumigatus* small ribosomal subunit RNA gene, partial sequence; Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; And large ribosomal subunit of RNA gene, partial sequence. EF634403.1 *Aspergillus fumigatus* isolate NRRL 35223 18S ribosomal RNA gene partial sequen internal transcribed spacer 1 5.8S ribosomal RNA gene; KX098468.1 *Aspergillus fumigatus* small subunit ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; KY859370.1 *Aspergillus fumigatus* isolate 97 6E4 small subunit ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; KY617052.1 *Aspergillus fumigatus* voucher MHE 24 MC internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA JQ767180.1 *Aspergillus fumigatus* strain ZH1 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S (Figure 11).

Sal 10 : Based on BLAST results on the NCBI database on the ribosomal RNA 18S sequence Gene Sal 10 obtained a homology of 99% with *Trichoderma reesei* strain TV221 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; And 28S ribosomal RNA gene, partial sequence. KP263685.1 *Trichoderma reesei* strain TV221 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KP263682.1 *Trichoderma reesei* strain TV217 internal transcribed spacer 1 partial sequence 5.8S ribosomal RNA gene; KC847188.1 Trichoderma reesei strain IPBCC06 325 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; KC847186.1 *Trichoderma reesei* strain IPBCC93 260 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene; JN704346.1 Hypocrea jecorina voucher HR241489.1 partial 18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gen (Figure 12).

From the results of the data above can be seen that the 10 fungi, 7 fungi belong to the genus *Aspergillus*, 2 fungi belong to the genus *Trichoderma* and 1 fungi belong to the genus *Penicillium*. These three genera are included in the Ascomycota division. Although the genus is the same (*Aspergillus*) not all fungi have antimicrobial effects on the microbes tested. Most fungi of the Ascomycota division having an antimicrobial effect are *Aspergillus*, as expressed in the Indraningrat *et al* paper (Indraningrat *et al*, 2016). Sponge-associated Ascomycota found to produce antimicrobials can be further classified into 12 genera. Of these 12 fungal genera *Aspergillus* (30%) and *Penicillium* (23%) are currently the two most prominent groups of sponge-associated fungi reported as antimicrobial producers.

CONCLUSION

S. flabelliformis sponges have 10 fungal isolates. Fungi Sal 3 (*Trichoderma reesei* strain JCM 2267), Sal 5 (*Aspergillus sp* strain TLWK-09 Sal 6 (*Aspergillus flavus* strain MC-10-L) and Sal 10 (*Trichoderma reesei* strain TV221) have antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *Candida albicans* ATCC 27583. These 10 fungus are *Aspergillus flavus* strain UPMZ02, *Aspergillus fumigatus* strain CD1621, *Trichoderma reesei* strain JCM 2267, *Aspergillus nomius* strain KUB105, *Aspergillus* sp. strain TLWK-09, *Aspergillus flavus*

strain MC-10-L, *Penicillium* sp. strain RMA-2, *Aspergillus* sp. strain TLWK-09, *Aspergillus fumigatus* and *Trichoderma reesei* strain TV221.

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Figure 1. *Stylissa flabelliformis sponge.*

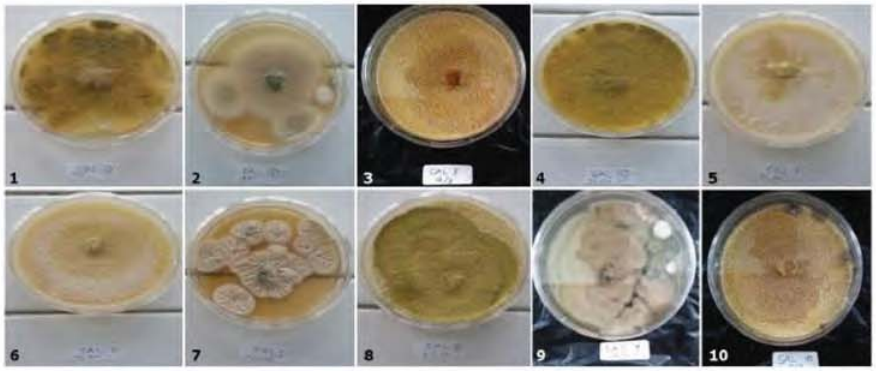

Figure 2. Isolate of fungus from *Stylissa flabelliformis*

Table I. Activity of fungus association sponge against several microbes

No of fungus SAL	Activity of fungus against several microbes			
	S. aureus ATCC 29213	*E. coli* ATCC 25922	*C. albicans* ATCC 10231	*Methicillin Resisten S. aureus* (MRSA)
1	-	-	-	-
2	x	-	-	-
3	x	x	x	-
4	-	-	-	-
5	x	x	x	x
6	x	x	x	x
7	x	x	x	x
8	-	-	x	-
9	x	x	x	x
10	x	x	x	x

x = it has antimicrobial activity

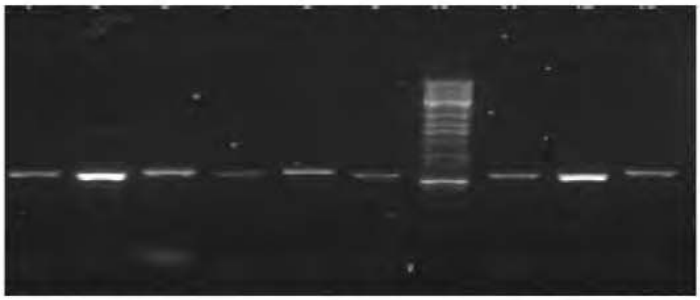

Figure 3. Isolated DNA from Sal 1; 2. Isolated DNA from Sal 2; 3. Isolated DNA from Sal 3; 4. Isolated DNA from Sal 4; 5. Isolated DNA from Sal 5; 6. Isolated DNA from Sal 6; 7. Isolated DNA from Sal 7; 8. Isolated DNA from Sal 8; 9. Isolated DNA from Sal 9; 10. Isolated DNA from Sal 10. All DNA samples were loaded at concentration of 1/100 with condition 0.8% agarose gel. Amount of DNA ladder loaded per lane: 0.2ug each. Volume of sample loaded per lane : 1uL each.

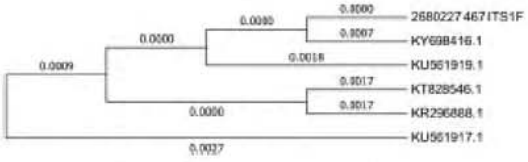

Figure 4. Phylogeny tree of fungi Sal 1

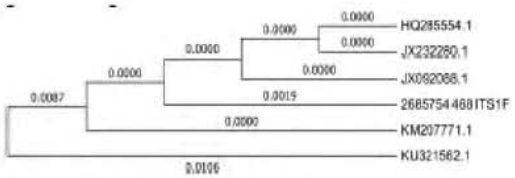

Figure 5. Phylogeny tree of fungi Sal 2

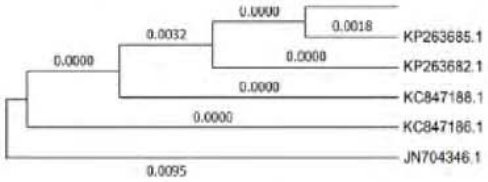

Figure 6. Phylogeny tree of fungi Sal 3

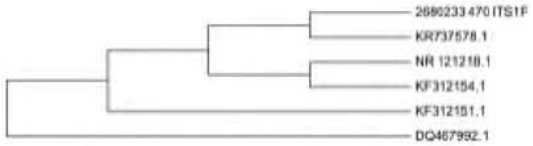

Figure 7. Phylogeny tree of fungi Sal 4

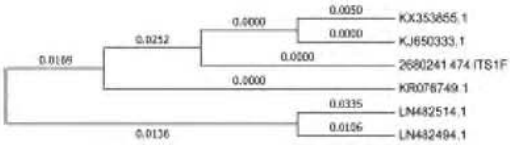

Figure 8. Phylogeny tree of fungi Sal 5

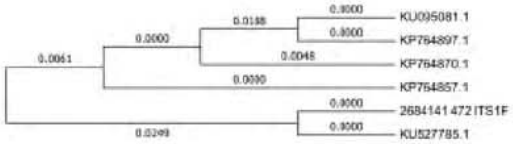

Figure 9. Phylogeny tree of fungi Sal 6

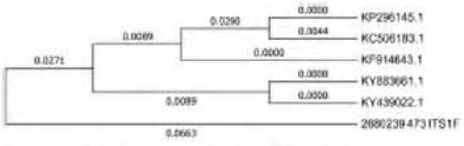

Figure 10. Phylogeny tree of fungi Sal 7

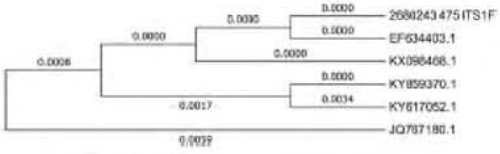

Figure 11. Phylogeny tree of fungi Sal 9

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