CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Identification based on 16S rRNA gene sequence analysis

5.1.1 Polymerase chain reaction (PCR) amplification of 16S rRNA gene

Basically, PCR is a scientific technique in molecular biology field which involves amplification of a single or a few copies of DNA producing thousands to millions copies of the same DNA sequence. According to Roux (1995), variables such as concentrations of Mg2+, buffer pH and cycling conditions in term of annealing temperature are interdependent among them so that every single thing should be handled carefully and conscientiously. For examples, dNTPs directly chelate a proportional number of Mg^{2+} ions, thus, an increase in the dNTPs concentration will decreases the free Mg^{2+} concentration available to influence polymerase function.

The PCR process is depending on thermal cycling which heating and cooling cycles repeated for the DNA melting and enzymatic replication reactions of the DNA. 27f and 1525r primers, the forward and reverse bacterial universal primers, respectively, are the major components for selective and repeating amplification which contain sequences complementary to the target 16S rRNA gene region along with a DNA polymerase (Kolbert & Persing, 1999). As PCR progresses, replication continuously occurred using the DNA as a template and amplified exponentially.

Troubleshooting in PCR sometimes occurred due to some causes which have to be optimized. Roux (1995) suggested some optimization strategies when handling with troubleshooting in PCR especially if weak or no product detected, or if multiple products or a high molecular weight smear is observed. More DNA templates should be used and checked for any inhibitor in template preparation by spiking original PCR mix

with dilutions of known positive template or vary concentrations of other buffer components such as pH, *Taq* polymerase, dNTPs and primers. If smear band obtained, the temperature cycles have to be modified especially the minimum and maximum annealing temperature. The last thing is about primer set which may not suitable for the reaction and should be replaced with the new design one. However, since the forward and reverse primer used in this study were of universal bacterial primers, the main problem for any troubleshooting may due to technical error in preparing master mix of the PCR reaction mixture.

In this study, all tested strains were amplified successfully (Figure 4.13). However, PCR products have to be purified to remove excess primers and nucleotides (Clarridge, 2004) before proceed to sequencing process. Purification of PCR products for sequencing purpose can be done using purification kits. There are a lot of purification kit products available commercially, which can save time and easier to handle. In this study, QIAquick PCR Purification Kit was used to purify the RCP product. The principle implemented in this kit is a silica membrane assembly for DNA binding in provided high-salt buffer and elution with low-salt buffer or water. This procedure cleans up DNA samples by removing primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide and other impurities. Problem caused by loose resins and slurries are also solved by silica-membrane technology. Besides, optimization of specialized binding buffers was done for selective adsorption promotion of DNA molecules (Qiagen, 2011).

5.1.2 Analysis of 16S rRNA gene sequences

The introduction of rRNA-based methods analysis has overcome the limitations of culture-based protocols. Among bacteria, archaebacteria and eukaryotic organisms, the rRNA gene sequences comparison is a powerful method for deducing phylogenetic and

evolutionary relationships (Weisburg *et al*., 1991). 16S rRNA gene which is also designated 16S rDNA (Clarridge, 2004) is a stable part of the genetic code (Woese, 1987) that could be compared to determine phylogenetic relationships of bacteria, and, indeed, all life-forms (Kolbert & Persing, 1999). In bacteria taxonomy, it is also become the most commonly used method for bacterial taxonomy (Tortoli, 2003). Sequencing of material amplified by polymerase chain reaction (PCR) is one of the methods which these sequences been derived previously (Edwards *et al*., 1989).

Once DNA sequences in this study obtained, they were analyzed by examining the sequences from beginning to end. Sequences are considered good if tall distinct peaks with little overlap obtained. Basically, the good sequences started around base 20 and above, whereas poor sequences represented by low or multiple peaks which very typical of beginning and end of a reaction.

Sequences are easier to be conducted by viewing the sequence chromatogram. ChromasLite program was used in this study to view the sequences in order to detect any ambiguity at various nucleotide positions along the DNA sequence. According to Dellis (2009), *"this is due to the bases called by the sequencing machine cannot always precisely determined what nucleotide is represented by either a broad peak or a set of overlaying peaks. In such cases, a letter other than A, C, G, or T is recorded, most commonly "N"*. Any sequence obtained should be proofread in order to ensure all sites are correctly called so the quality of the data can be determined. This is to be done prior to uploading it to GenBank database, NCBI. Both forward and reverse sequencing reactions were carefully checked in this study to correct any possible "miscalls".

The forward and reverse primers used in this study produces about 1500bp length of amplified nucleotide sequences. Basically, the forward sequence is good at the beginning and some kind of messy at the end, but vice versa for that of reverse sequence. Thus, the main task before analyze the sequence in any analyzer program is

to build an almost full sequence of the 16S rRNA gene of the tested strains. That is the importance of MEGA 4 and ChromasLite because both of this software facilitate the combination of the forward and reverse sequences to make an almost full 16S rRNA gene sequence.

5.1.3 Comparison of results from different databases

The next step of analyzing the DNA sequence after getting an almost full sequence is uploading it to BLAST website. As an algorithm, BLAST compares nucleotides of DNA sequences. The BLAST search is used to compare a query sequence with databases of sequences and identify a library sequences that resemble the query sequence. Different types of BLASTs are available, but in this study, nucleotides of DNA sequences were used. However, the results obtained which shows the hits found, may not be the best possible results. This is due to the fact that BLAST is based on a heuristic algorithm so it is not providing all the hits within the database. On the other hand, BLAST also misses hard to find matches and cannot guarantee the optimal alignments of the query and database sequences (Altschul *et al.*, 1990).

According to Cole *et al*. (2005), Sequence Match in Ribosomal Database Project is more accurate than BLAST at finding closely related rRNA gene sequences. They also explained that "*the top 20 s_ab scores will contain the closest sequence by pairwise identity about 95% of the time*." Besides, the emerging trend toward high-throughput rRNA sequence analysis was supported by completely revising the RDP analysis especially in microbial ecology and related disciplines. Cole *et al.* (2005) also explained that three data filters were involved in the DNA analyses process which were*: "(i) include only environmental clone or only isolate sequences; (ii) include only sequences >1200 bases in length (near full-length) or only shorter sequences; and (iii) include only sequences from type strains or only non-type strain sequences."* The

importance of these filters is of major consideration as rRNA-based phylogeny and taxonomy are connected by type strains. In the other word, the original Sequence Match method is completely re-implemented by the Sequence Match in RDP (Cole *et al.*, 2005). The basic strategy used by Sequence Match to find similar sequences to that of user's query sequences is by word matching which is not requiring prior alignment. Thus, the result (related sequences) provided by Sequence Match act as a good starting point to examine the relatedness by other methods.

From RDP, the almost full 16S rRNA gene sequence of tested strains gave quite a number of lists of the highest percentage similarity of *Streptomyces* spp. In this study, another web-based tool, EzTaxon was also used to analyze the sequences in order to double confirm the results obtained in RDP. So, the RDP results were again filtered with the results analyzed using EzTaxon.

The identification concept used by EzTaxon is based on values of pairwise nucleotides similarity and phylogenetic inference methods. Chun *et al.* (2007) explained that it can identify automated and reliable actinomycetes isolates successfully. This is due to the fact that the overall sequence similarity calculation could not be done by BLAST search compared to EzTaxon since it is only based on local alignment. Furthermore, accurate results can be obtained at reasonable computing cost as two methods utilized sequentially by EzTaxon. Thus, there is no doubt that the closest phylogenetic neighbor identification of the query sequence is guaranteed by such combinatory strategy. Stackbrandt & Goebel (1994) in Chun *et al.* (2007) suggested that if the level of similarity in the 16S rRNA gene sequence reach at 97%, it should become the delineation boundary for prokaryotic species as accepted among microbiologists. Employment of the alignment algorithms and consideration of gaps which make the similarity values differ are the reasons why EzTaxon should be used to analyse sequences. Chun *et al.* (2007) explained that "*EzTaxon was successfully developed to*

include the collection of all reference type sequences, and to provide various functions, including a similarity search engine, calculation of pairwise similarity, multiple sequence alignment and phylogenetic treeing algorithms at the server side. This would allow users in places where only poor computing facilities are available to carry out most of the necessary bioinformatic analyses of 16S rRNA gene sequences."

Based on EzTaxon analyses which shown by Table 4.7, strains X34, X42 and X77 match the highest similarity of *S. rochei* (100.0%), *S. albidoflavus* (99.7%) and *S. cavourensis* (99.8%), respectively. According to Table 4.7, strain X34 matches 100% for all three species; *S. rochei, S. plicatus* and *S. enissocaesilis.* However, based on combination of morphological and physiological characterisation comparison, strain X34 was corresponding to *S. rochei.* The same comparison was done to strains X42 and X77. Table N-1 in Appendix N shows the comparison of carbon sources utilization between tested strains and their similar strains that have the same/almost same similarity percentage. Carbon source utilization is a basic characteristic which almost all researchers will do to characterize *Streptomyces* spp. (Shirling & Gottlieb, 1966).

5.1.4 List of prokaryotic names with standard of nomenclature (LPSN)

The designated strains obtained after analyzing the 16S rRNA gene sequences have to be checked for their species name validity. This can be done by checking in an online database in List of Prokaryote with Standing in Nomenclature (LPSN) website at http://www.bacterio.cict.fr/. Accurate name and related information provided in this free online website according to the International Code of Nomenclature of Bacteria. Furthermore, the information available is based on International Journal of Systematic and Evolutionary Microbiology and up to date (Euzeby, 1997).

The three designated strains were validly described species based on LPSN. *Streptomyces rochei, Streptomyces albidoflavus* and *Streptomyces cavourensis* were the species determined for strains X34, X42 and X77, respectively in this study.

5.2 Primary screening for antifungal activity against *Candida albicans* and *Schizosaccharomyces pombe*

Streptomyces spp. are well known of possessing ability to suppress the growth of a wide variety of fungal pathogens (Getha & Vikineswary, 2002). Earlier, the antifungal activities of marine *Streptomyces* have also been reported from India and abroad (Kathiresan *et al*., 2005). In current study, less than 15% of *Streptomyces* spp. strains tested were strongly inhibiting the growth of *Candida albicans* and *Schizosaccharomyces pombe.*

In a study of 85 actinomycete strains tested, Tan (2007) reported 25.9% strains have antagonistic properties against *C. albicans*. Among the active strains, 60% produced less than 10mm zone of inhibition. This report of Tan (2007) exhibited a higher percentage compared to current study which only 6.8% out of 44 strains tested were active against *C. albicans.* However, the inhibition zones produced in Tan (2007) were much lower compared to 20mm of all active strains in this study. This result suggested that the substances produced by active strains in this study showed greater antifungal potency against *C. albicans*. Khatiresan *et al*. (2005) stated that inhibition zone of more than 10mm is produced by 'effective' organism and 'non-effective' for those with less than 10mm inhibition zone.

The percentage difference in number of strains that active against *C. albicans* might be contributed by the two times higher number of strains tested (85) in Tan (2007) compared to this study (44). Besides, the strain of *C. albicans* used in both studies may vary, resulting different patterns of inhibition observed.

16S rRNA gene sequencing analysis for strain X34 showed that this strain is 100% similar to *Streptomyces rochei*. In this current study, strain X34 designated *S. rochei* did not inhibit *C. albicans* growth. This is parallel with the report of Augustine *et al.* (2005a) which demonstrated the same activity pattern of *S. rochei* against *C. albicans*. However, this result is contradicted to the report of Kavitha & Vijayalakshmi (2007) which *S. rochei* inhibited the pathogenic fungi growth by 20mm zone of inhibition. This contradiction may due to the source of both strains were different. Current *S. rochei* strain was isolated from marine; the reported one was from soil. The substance or bioactive metabolites produced may differ due to their origin habitat environment.

5.3 Cultural characterisation of tested *Streptomyces* spp.

The characteristic features of the strains with antifungal potentials are given in Table 4.2. Most of the strains have white colour mycelium and their reverse side colour of the cultures was yellow as none of them produced soluble pigments. Vanajakumar *et al.* (1991) reported that white colour series of actinomycetes were the dominant forms, followed by the grey, yellow and red colour series. These different colour series were also recorded in soil, marine and river sediments (Tan, 2007).

According to Locci (1989), the more striking the substrate mycelium and soluble pigment colours appears (blue, dark green, red and violet), the value are higher. Kutzner (1981) in Locci (1989) reported, among the pigments produced by *Streptomyces* are anthracyclinglycoside, diazaindophenol, naphthoquinone, phenoxazinone and prodigiosin.

5.4 Morphological studies of tested *Streptomyces* spp.

In taxonomical study of actinomycetes, morphology aspect; hyphae arrangement and the shape of the spores are important in distinguishing *Streptomyces* spp. from other sporing actinomycetes. Besides, it also played a major role in characterizing Streptomycete species (Locci, 1989).

In this research, the micromorphological aspect was investigated by scanning electron microscope to study the spores shape and surface. In most ISP media the studied strains had resulting sporulation in their growth (Table 4.2). This was parallel to Kutzner (1981) in Locci (1989) that agar media used in ISP (Shirling & Gottlieb, 1966) with high carbon-nitrogen ratio promoting spore formation. But according to Locci (1989), *Streptomyces* spp. should be grown on poor media as they may not produce spores on rich media. The main point is different strain of *Streptomyces* spp. growth may differ in terms of sporulation in different growth media used.

Based on Figure 4.4 and Figure 4.5, all studied strains were divided into two groups of spore chain arrangement; straight to flexuous (*Rectiflexibles*) and hooks, loops or spirals with one to two turns (*Rectinaculiaperti*). This is according to Pridham (1958) in Locci (1989) whom had proposed three simpler and more practicable categories of spore chain arrangement; a) *Rectiflexibles*, b) *Rectinaculiaperti* and c) *Spirales*. However, Locci (1989) explained that since only spore surface ornamentation is a stable character, it has been adopted as a taxonomic character.

Table 4.3 shows spore ornamentation and spore chain arrangement for all *Streptomyces* strains studied. The spore morphology was smooth, except for strain X35 which were recorded as rugose (wrinkle). Rugose spore type was proposed by Dietz and Mathews (1971) in (Locci, 1989), besides other spore types; smooth, spiny, hairy and warty.

Based on molecular results in this study, strains X34, X42 and X77 are designated to be *Streptomyces rochei*, *S. albidoflavus* and *S. cavourensis*, respectively. The spore shapes of these three strains are compared to that of published and approved list.

As shown in Figure 5.1, both *Streptomyces rochei*; (a) referred to Berger *et al*. (1953) compared to (b) and (c) in this study (strain X34) are similar in terms of spore surface ornamentation. Both spores are smooth and the shape also cylindrical to oblong and of unequal length. Locci (1989) reported that it is difficult to distinguish between smooth, warty and rugose type of spore surface, but by using scanning electron microscope this problems are solved.

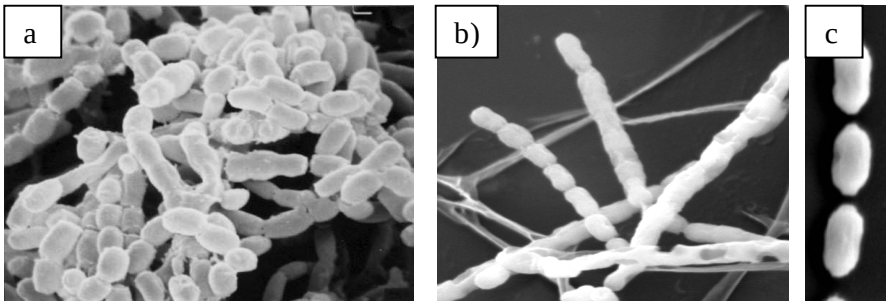

Figure 5.1: Comparison of scanning electron micrograph for *Streptomyces rochei* spores; a) spore of *S.rochei* from approved list reference (Berger *et al*. 1953); b) spore chains of strain X34 and c) spores of strain X34, similar to *S. rochei* via 16S rRNA gene analysis.

Scanning electron micrographs in Figure 5.2 shows the spores of *Streptomyces albidoflavus* which referred to Bergey's Manual of Determinative Bacteriology (sixth edition) and strain X42, that of from current study. Their morphology is comparable which both (a) and (b) spores are shaped cylindrical to oblong and of unequal length. The hyphae arrangements are also straight to flexuous as reported (Table 4.3).

As for strain X77 which designated as *Streptomyces cavourensis*, Figure 5.3 illustrates the similarity of hyphae arrangement and spores of *S. cavourensis* (a) and strain X77 (b). Both have straight to flexuous hyphae with smooth spores (Table 4.3).

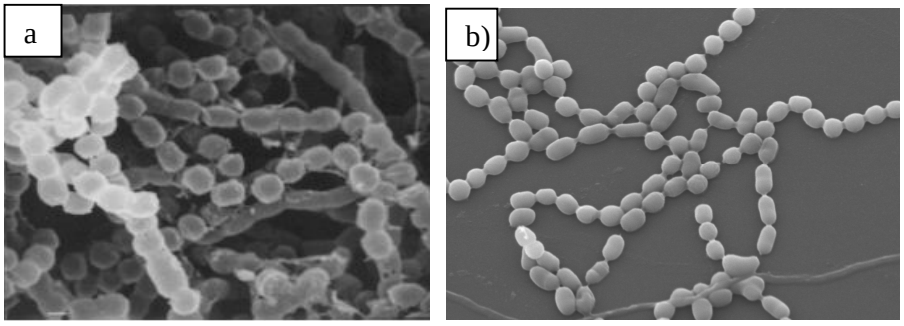

Figure 5.2: Scanning electron micrograph for comparison of *Streptomyces albidoflavus* spores; a) spore of *S. albidoflavus* from approved list reference (Bergey's Manual of Determinative Bacteriology (sixth edition)); b) spores of strain X42, similar to *S. albidoflavus* via 16S rRNA gene analysis.

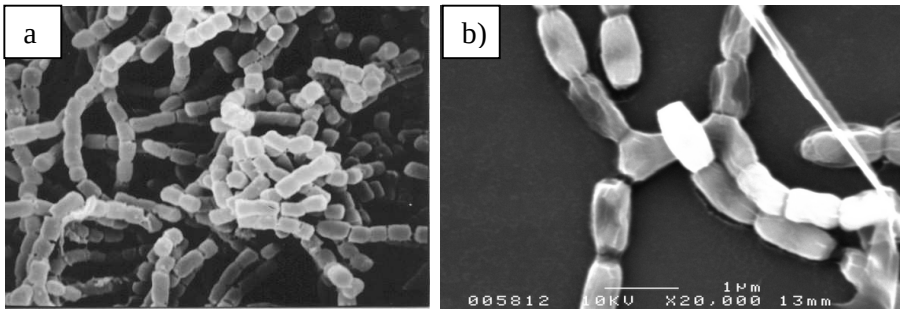

Figure 5.3: Scanning electron micrograph for comparison of *Streptomyces cavourensis* hyphae and spores; a) *S. cavourensis* (Skarbek & Brady, 1978a); b) spores and hyphae of strain X77, similar to *S. cavourensis* via 16S rRNA gene analysis.

5.5 Physiological characterisation of tested *Streptomyces* spp.

5.5.1 Temperature range and optimum temperature for growth

All tested strains grow at temperature between 15˚ to 35˚C, with only one third (strain X34 and X35) are able to survive up to 45˚C. This is parallel to Kutzner (1981) statement in Locci (1989) that most *Streptomyces* spp. grows at range 10˚ to 37˚C, and are thus regarded as mesophiles.

Three designated strains (X34, X42 and X77) as *S. rochei, S. albidoflavus* and *S. cavourensis* were consistent to the published characterization. Reddy *et al.* (2011) reported a marine *S. rochei* has an optimum growth at 30˚C and could grow up to 42˚C, reliable to strain X34 of current study. But in terms of antibiotic production, *S. rochei* has to be incubated at 37˚C (Augustine *et al*., 2005a). However, optimum growth

temperature range and that of optimum growth for antibiotic production are two different aspects to be investigated depending on the objective of study.

Strains X42 and X77 which can grow well at 15˚ to 35˚C are parallel to that of *S. albidoflavus* and *S. cavourensis* based on Bergey's Manual of Determinative Bacteriology (sixth edition) report which both strains have an optimum growth at 30˚C (Table 5.1). This may proved that strains X42 and X77 in current study are similar to *S. albidoflavus* and *S. cavourensis*, respectively.

Table 5.1: Comparison of optimum growth temperature between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	Optimum growth temperature		Reference
		Current study	Published	
X34	*S. rochei*	25-35˚C	30˚C	Reddy *et al*. (2011)
X42	*S. albidoflavus*	25-35˚C	30˚C	Berger *et al.* (1953)
X77	*S. cavourensis*	25-35˚C	30˚C	Skarbek & Brady (1978a)

5.5.2 Formation of melanoid pigment

Actinomycete synthesizes and excretes pigments, melanin or melanoid, which are considered to be useful in characterizing for taxonomical studies (Arai & Mikami, 1972). According to Dastagar *et al*. (2006), in most *Streptomycetes* strains, it is difficult to clarify its pigmentation especially if other fundamental features like colour of the surface aerial mycelium, sporophore morphology and spore surface are combined.

In this study, all tested strains were negative in producing melanoid pigment (Table 4.2). This report is reliable to a taxonomy study of *Streptomyces* strain 3B carried by Nikolova *et al*. (2005) also obtained the same result; no melanoid pigment were produced in any of the investigated for melanin media. When complex media are used, it might be difficult to differentiate the diffusible pigments produced whether it is melanoid (dark brown) or may be just a brown substance.

Focusing to the three strains based on molecular result: strains X34, X42 and X77 which similar to *S. rochei*, *S. albidoflavus* and *S. cavourensis*, respectively; all these approved strains are not producing melanoid pigments or rarely produced (Locci, 1989). This comparison demonstrated that the similarity of these strains in current study to that of approved strains is significant (Table 5.2).

Table 5.2: Comparison of melanoid pigment formation between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	Formation of melanoid pigment		Reference
		Current study	Published	
X34	*S. rochei*	Negative	Negative	Locci (1989)
X42	*S. albidoflavus*	Negative	Negative	Locci (1989)
X77	*S. cavourensis*	Negative	Negative	Locci (1989)

5.5.3 Liquefaction of gelatin

All tested strains were positive in gelatinase enzyme production. Reddy *et al*. (2011), Waksman & Henrici (1948) and Skarbek & Brady (1978a) demonstrated that *S. rochei*, *S. albidoflavus* and *S. cavourensis*, respectively are also positive in gelatin liquefaction test. This reliable result consolidate that strains X34, X42 and X77 are similar to those of approved stated strains (Table 5.3).

Table 5.3: Comparison of gelatin liquefaction between strains X34, X42 and X77 in current study and their designated species.

Strain	Designated species	Liquefaction of gelatin		Reference
		Current study	Published	
X34	*S. rochei*	Positive	Positive	Reddy *et al.* (2011)
X42	*S. albidoflavus*	Positive	Positive	Waksman & Henrici. (1948)
X77	*S. cavourensis*	Positive	Positive	Skarbek & Brady (1978a)

5.5.4 Hydrolysis of starch

Two third of tested strains were positive in starch hydrolysis test. Another one third recorded as negative were strains X34 and X35. The results of this test for those two designated strains (X34 and X42) were consistent to that of approved in Bergey's Manual of Determinative Bacteriology (sixth edition). Strain X42 was able to produce enzymes to hydrolyze starch, as well as *S. albidoflavus* (Atta *et al*., 2011). On the other hand, strain X34 in this study was not producing exoenzyme that could hydrolyze starch, reliable to that of *S. rochei* characterized by Reddy *et al.* (2011) which also lead to negative result (Table 5.4).

The concept behind this test is actually based on *Streptomyces* spp. capability of utilizing starch as their carbon source. Starch appears as a branched polymer as it is a kind of polysaccharide. Due to starch structure which consist a long, series of glucose molecules that hooked together, it would be too big to enter *Streptomyces* spp. cell. Thus, only *Streptomyces* spp. with secreted exoenzyme could enter the cell by hydrolyzing or breaking down the starch (Kaiser, 1999). Because of this unique reaction in nature, this test had become one of the criteria to be physiologically characterized to identify *Streptomyces* spp. strain.

5.5.5 Hydrogen sulfide (H_2S) production

Production of hydrogen sulfide is also a criterion to identify *Streptomyces* spp. and is being implemented by most of researchers in identification method of actinomycetes (Jain & Jain, 2007; Shirling & Gottlieb, 1966; Iwami *et al.*, 1986).

Only one strain out of six among tested strains was positive to produce hydrogen sulfide (H_2S) which was strain X77, designated as *S. cavourensis*. However, according to Skarbek & Brady (1978a), *S. cavourensis* is not producing H_2S, contradicted to current study. This contradiction may be due to different strain used or different

condition applied when the test progressed. As for another two strains X34 and X42 which designated to *S. rochei* and *S. albidoflavus*, respectively, both strains in current study and previous report demonstrated reliable result which was negative in H_2S production (Berger *et al.*, 1953; Reddy *et al.*, 2011; Atta *et al.*, 2011). Again, this comparison demonstrated that the similarity is significant between studied strains and the designated strains as well (Table 5.5).

Table 5.4: Comparison of starch hydrolysis between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	Hydrolysis of starch		Reference
		Current study	Published	
X34	*S. rochei*	Negative	Negative	Reddy *et al.* (2011)
X42	*S. albidoflavus*	Positive	Positive	Atta *et al.* (2011)
X77	*S. cavourensis*	Positive	-	-

Table 5.5: Comparison of hydrogen sulfide production between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	H_2S production		Reference
		Current study	Published	
X34	*S. rochei*	Negative	Negative	Berger *et al.*, (1953)
X42	*S. albidoflavus*	Negative	Negative	Reddy *et al.* (2011) Atta *et al.* (2011)
X77	*S. cavourensis*	Positive	Negative	Berger *et al.* (1953)

The concept behind the criterion of H_2S production is depending on the *Streptomyces* spp. ability to break down sulfur consisting amino acids or reduce inorganic sulfur-containing compounds in order to produce H_2S. This lead the reduced sulfur incorporates into other cellular amino acids or coenzymes. The result of both production of H_2S and sulfur reduce will then lead to combination with the iron salt, in this case peptone-iron agar supplemented with yeast extract, forming visible black ferric sulfide (FeS) in the media, indicating the test is positive (Kaiser,1999).

5.5.6 Sodium chloride (NaCl) tolerance

Half of the tested *Streptomyces* spp. strains were tolerated up to 10% of NaCl, 33.3% were capable to survive up to 8%, whereas 16.6% strain only tolerate up to 6% of NaCl. Entire strains could grow in media without any supplemented NaCl but could not tolerate more than 10% NaCl. All strains were categorized in intermediate group of tolerance since they were in range of 6% to 10% tolerance of NaCl (Tresner *et al*., 1968). MacLeod (1965) in Tan (2007) found that true marine bacteria could only grow at minimum 3% concentration of NaCl. Thus, the result of current study suggested that all the studied *Streptomyces* spp. strains may not originated from marine environment.

Tresner *et al*. (1968) reported that only 1.8% of 313 *Streptomyces* species could not tolerate 4% NaCl; 26.9% could grow at a maximum of 4%; 49.7% could tolerate at maximum 7%; 18.8% could grow at maximum of 10% and only 2.8% could tolerate 13% NaCl. Current study illustrated a non-reliable result compared to that of Tresner *et al.* (1968) report. Less than 20% strain tolerate up to 10% of NaCl in Tresner *et al*. (1968) study compared to 50% in current study. This might be due to the different number of strains involved and the source of *Streptomyces* spp. studied were isolated from different environment.

Generally, it was found that actinomycetes can tolerate to maximum 8% of NaCl (Waksman, 1959). However, in particular to marine isolates, Okazaki & Okami (1972, 1975) in Waksman (1959) explained that marine isolates were commonly have greater capability in salt-tolerance compared to that of terrestrial strains; which sand-dune isolates were more tolerance than that of sea-water isolates. The studied strains in this study were isolated from marine organism. Although early report of Grein & Meyers (1958) in Tan (2007) are not totally agree about the presence of indigenous marine *Streptomyces* spp., there were some reports that supporting the indigenous marine population existence in the marine environment (Tan, 2007). Furthermore, Moran *et al.*

(1995) proved these reports by an important discovery when applied a *Streptomyces*-specific 16S rRNA probe in a hybridization study which demonstrated that *Streptomyces* populations are origin part of marine community, not from terrestrial spores.

Strains X34, X42 and X77 in this study were designated as *S. rochei, S. albidoflavus* and *S. cavourensis,* respectively, based on 16S rRNA gene sequencing. Previous report demonstrated unreliable NaCl tolerance between studied strains and the designated species (Table 5.6). Bergey's Manual of Determinative Bacteriology (sixth edition) revealed that *S. rochei, S. albidoflavus* and *S. cavourensis* were tolerated up to 5% for both *S. rochei* and *S. albidoflavus*, and 7.5% for *S. cavourensis*. Recent study exhibited that *S. albidoflavus* grow well up to 7% of NaCl (Atta *et al.*, 2011) whereas *S. rochei* could survive up to 6 - 7% of NaCl (Reddy *et al.*, 2011; Kavitha & Vijayalakshmi, 2007). However, current study obtained contra percentage of NaCl tolerance in these strains; strain X34 (*S. rochei*) – 8%, X42 (*S. albidoflavus*) – 10% and X77 (*S. cavourensis*) – 6%. The percentage differences between these studied strains and that of previous reports suggested that *Streptomyces* spp. involved are of different strains or the consideration and way of observation in recording the result may also differ between all studies. Furthermore, Tresner *et al*. (1968) discussed that species differentiation in the intermediate tolerance range like in current study is somewhat more difficult because overlapping in ranges which may occur with the 'high' and 'low' tolerance species.

5.5.7 Carbon source utilization

In this test, glucose was used as positive control as Huck *et al*. (1991) in Ismet *et al*. (2004) explained that glucose is well known as a suitable carbon source for microorganism's growth. The results obtained were compared to that of positive control

which demonstrated that maltose, D-mannitol, L-arabinose and L-rhamnose could also be a sole carbon source for entire strains tested.

Table 5.6: Comparison of NaCl tolerance between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	NaCl tolerance		Reference
		Current study	Published	
X34	*S. rochei*	8%	6-7%	Reddy *et al*. (2011)
X42	*S. albidoflavus*	10%	7%	Berger *et al.* (1953)
X77	*S. cavourensis*	6%	7.5%	Skarbek & Brady (1978a)

Seven out of twelve carbon sources used in this study were utilized by almost all strains tested, differing only by two carbon sources which were utilized/not utilized by two strains. This result suggested that most of the strains could grow well even if glucose is changed to other carbon source like D-mannitol, L-arabinose, L-rhamnose, D-xylose and D-fructose.

Huck *et al*. (1991) in the report of Ismet *et al*. (2004) demonstrated that in certain actinomycetes growth and bioactive production, glucose disturb the biosynthesis pathway of many antibiotics. However, so far there is still no report those other carbon sources stated above interfere with it.

The result obtained in current study could be applied in growth process of the strains. As the growth phase started, glucose may enhance growth or stimulate energy for these tested strains. Because of glucose level may reduce in growth process, it could be substituted by other carbon source which slowly utilize by them (Ismet *et al.*, 2004). Again, Huck *et al*. (1991) in Ismet *et al*. (2004) observed that during initial phases of growth, glucose was metabolized. Starch could be utilized as carbon source when glucose reduced in middle phases of growth.

Table 5.7 shows the comparison between strains X34, X42 and X77 to their designated species, in terms of carbon source utilization aspect. Results of Reddy *et al.*

(2011) were compared to current study for strain X34 which designated to *S. rochei*. Three differences in utilization of inositol and sucrose between *S. rochei* and strain X34 were discovered. As for strain X42 designated to *S. albidoflavus*; there was a doubt in D-xylose, inositol, L-rhamnose, maltose and sucrose utilization reliability in both current study and those of Atta *et al*. (2011) report. Apart from that, strain X77 which designated to *S. cavourensis* (Skarbek & Brady, 1978b) differs in utilization of inositol, L-arabinose and L-rhamnose as well. However, the differences in carbon source utilization between all these three strains and their designated species only differ in terms of not utilizing (-) or poorly utilizing (±) the mentioned carbon sources (Table 5.7). These contradictions might be due to technical aspect in conducting test or observing the results. Gottlieb (1960) stressed the importance of technique in carbon source utilization method when he conducted an evaluation of *Streptomyces* spp. strain particularly for utilization of carbon compounds in cooperation with a few groups of researchers in different laboratories. Besides, he came with an idea that some cultures may behave less consistently than others and this should introduce a note of caution.

5.5.8 Nitrate reduction test

More than 80% of the tested strains were able to reduce nitrate in this study, and less than 20% recorded as negative. Focusing on the designated strains X34, X42 and X77 as *S. rochei, S. albidoflavus* and *S. cavourensis*, respectively, Table 5.8 shows the comparison of nitrate reduction between three strains in this study and their designated species referring to Reddy *et al*. (2011) and Atta *et al*. (2011). Unfortunately, there is no report on the nitrate reduction test of *S. cavourensis* recorded yet.

Basically, nitrate reduction test is a criterion to characterize bacteria whether they are able to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or not, by anaerobic respiration (Skerman, 1967). The principle of this criterion is based on the basal medium used in

the test, which is nitrate broth. *Streptomyces* spp. tested will use their enzyme nitrate reductase to reduce nitrate (NO_3^-) to nitrite (NO_2^-). Since nitrate broth contains nitrate source like nutrients and potassium nitrate, it also determines whether *Streptomyces* spp. in the study perform denitrification on nitrate and nitrite or not, in order to produce molecular nitrogen. If the tested strains reduce nitrate to nitrite, addition of sulfanilic acid in inoculated nitrate broth after incubation period will react with the nitrite to form nitrous acid. Next reaction of nitrous acid with addition of α-naphthylamine will produce diazotized sulfanilic acid, and cause the colour of the broth turn to pink or red colour. Thus, the broth colour changes after addition of the nitrate reagents indicating nitrate reduction is positive. However, if nothing colour changes occur after the nitrate reagents addition, there are two possibilities; a) the strain unable to reduce nitrate, or b) the strain produce ammonia or molecular nitrogen by denitrifying the nitrate or nitrite.

Table 5.7: Comparison of carbon source utilization between *Streptomyces* spp. strain in current study and their designated name based on references given.

Strain		**Carbon sources**											
		Fru	Glu	Man	Xyl	Ino	Lac	Ara	Rha	Mal	Raf	Sor	Suc
S. rochei	X34	+	+	+	+	±	+	+	+	+	-	-	±
	Pub1	+	+	+	*+	+	+	*+	+	+	-	-	+
S. albidoflavus	X42	+	+	+	±	±	-	+	±	±	-	+	±
	Pub2	+	+	+	-	-	-	+	-	-	-	ND	-
S. cavourensis	X77	+	+	+	+	±	-	±	±	+	-	-	-
	Pub3	+	+	+	+	-	ND	-	-	ND	-	ND	-

+; good growth, ±; poor growth, -; no growth, ND; not determined
Pub; published strain
Pub1; Reddy *et al*. (2011), *Kavitha & Vijayalakshmi (2007), Pub2; Atta *et al.* (2011), Pub3; Skarbek & Brady (1978b)
Fruc; D-fructose, Glu; D-glucose, Man; D-mannitol, Xyl; D-xylose, Ino; Inositol, Lac; Lactose, Ara; L-arabinose, Rha; L-rhamnose, Mal; Maltose, Raf; Raffinose, Sor; Sorbitol, Suc; Sucrose.

Table 5.8: Comparison of nitrate reduction test between strains X34, X42 and X77 in current study and their designated species.

Strain	Designated species	Nitrate reduction		Reference
		Current study	Previous study	
X34	*S. rochei*	Positive	Positive	Reddy *et al.* (2011)
X42	*S. albidoflavus*	Positive	Positive	Atta *et al*. (2011)
X77	*S. cavourensis*	Negative	-	-

Small amount of zinc powder should be added in the medium in order to confirm which of the possibilities are true. If unreduced nitrate is present in the medium, addition of zinc powder will turns the broth colour to pink or red, indicating the nitrate reduction is negative. This is because the zinc powder reduced the nitrate to nitrite, which forming nitrous acid that reacts with sulfanilic acid. This reaction will produce diazotized sulfanilic acid, and reacts with α-naphthylamine to create red complex. If there is still no colour change, this is due to the absence of nitrate in the medium. Therefore, we can conclude that the second possibility occurred, confirming that the nitrate reduction is positive (Microbugz, 2010).

5.5.9 pH sensitivity

All tested strains were able to grow well on media adjusted to pH 6.5 to 9.5. Since all strains tested were those of antagonistic against both *S. pombe* and *C. albicans*, this result could be applied in antibiotic yielding. This is based on Pandey *et al*. (2005) explanation that the medium pH might be an important factor for antibiotic formation, as media giving high antibiotic yields showed an alkaline pH.

Among the three strains which were designated to *S. rochei* (strain X34), *S. albidoflavus* (strain X42) and *S. cavourensis* (strain X77), only strain X42 had a poor

growth on media adjusted to pH 5.5. This result suggested that strain X42 was a bit sensitive to pH5.5 and any growth or test should not be done at this acidic pH.

Reddy *et al*. (2011) exhibited that *S. rochei* could grow in range of pH6 to pH10.5 and have an optimum growth at pH7. Besides, at this neutral pH, Augustine *et al.* (2005a) and Atta *et al*. (2011) found that both *S. rochei* and *S. albidoflavus*, respectively, achieved their optimum production of antibiotic (Table 5.9). As for *S. cavourensis*, although Skarbek and Brady (1978b) reported flavensomycin production from this species, there was no optimum pH reported.

5.6 Chemical profiling, isolation and identification of active components

5.6.1 Thin layer chromatography (TLC) of tested *Streptomyces* spp. extracts and active fractions of *S. rochei* (strain X34)

Thin layer chromatography (TLC) is a form of chromatography that is most useful for qualitative analysis of crude extracts or isolated compounds (Houghton & Raman, 1998). All the tested *Streptomyces* strains extract in this study exhibited better separation in semi polar solvent system which was hexane: ethyl acetate (3:1). Basically, different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent (Houghton & Raman, 1998). By using three solvent systems in this study which were hexane: ethyl acetate (3:1), toluene, and chloroform, in term of better compounds separation, the suitable solvent can be determined.

As for active fraction F5 of *S. rochei*, the solvent system was adjusted to hexane: ethyl acetate in ratio of 2:1 (Figure 4.16). The proportion difference of solvent system used in crude extract of *Streptomyces* spp. tested and the active fraction may due to the more complex mixture of compounds in the previous extract compared to the compounds contained in the active fraction.

As molybdo (IV) phosphoric acid was used as a compounds detection method, all the spots appeared after heating are probably phenolics, hydrocarbon waxes, alkaloids or steroids, because this reagent is commonly used for staining these compounds (Burstein, 1953).

Table 5.9: Comparison of pH sensitivity between strains X34, X42 and X77 in current study and their designated species.

Strains	Designated species	pH sensitivity		Reference
		Current study	Published	
X34	*S. rochei*	pH 4.5 – 9.5	pH 6 – 10.5	Reddy *et al.* (2011)
X42	*S. albidoflavus*	pH 6.5 – 9.5	pH 7 (optimum)	Atta *et al.* (2011)
X77	*S. cavourensis*	pH 4.5 – 9.5	-	-

5.6.2 Preparative thin layer chromatography (PTLC) of active fraction of *S. rochei* (strain X34)

PTLC was done for *S. rochei* active fraction for compound isolation and purification purpose. It is one of the common and simplest methods to isolate a component or components from a mixture (Houghton & Raman, 1998). About 80mg of active fraction used for PTLC, only less than 50% were obtained after the procedure (Table 4.13). This is parallel to Houghton & Raman (1998) that only small amounts can be obtained from each fractionation procedure.

5.6.3 Antifungal bioassay against *Schizosaccharomyces pombe*

For the screening of chemical profiling and compound isolation part, only crude extract of three potential strains of *Streptomyces* spp. (*S. rochei* (strain X34), *S. albidoflavus* (strain X42) and *S. cavourensis* (strain X77)) were tested against *S. pombe.* The AA disc diffusion method exhibited only *S. rochei* inhibited the growth of *S. pombe* indicated that the crude extract of the strain is possessing antifungal properties. Although all of the three strains did inhibit the tested fungi in primary screening (Figure 4.2), the contradictory result observed in this test may be due to breakdown of unstable

bioactive substances during the extraction process or long storage period of the crude extracts. Furthermore, in the primary screening, agar plug diffusion method was used, where the bioactive substances of fresh culture are diffused directly into the lawned agar (with tested fungi). This may also explained the report of Shomura *et al.* (1979) that the production of bioactive substances on agar but not in submerged culture, where mycelia fragmentation occurred and responsible for non-production of the antibiotics.

Referring to Figure 4.16, one fraction (F5) out of six fractions of *S. rochei* exhibited 60% greater inhibition zone (Table 4.12) against the tested fungi compared to previous test using crude extract before column chromatography was done. The active fraction of F5 may responsible as the major 'active' constituent against *S. pombe.* In certain cases, synergy may occur where the activities of the fractions obtained from a mixture being considerably less than that of the total mixture (Houghton & Raman, 1998).

After PTLC isolation was done to the active fraction F5, all the separated six sub-fractions then tested using PTLC bioassay guided method. This different method of assay was done to limit the usage of the fractions obtained. Marston (2011) stated that this bioassay method requires only small amounts of sample and can be used for the target-directed isolation of the separated constituents. As the assay showed that two sub-fractions demonstrated antifungal activity against *S. pombe* (Figure 4.19), ^{1}H-NMR was done for both of the fractions. Based on the ^{1}H-NMR chromatogram, only sub-fraction F5.1 was carried out to further characterization of its structure. The compound was labeled as compound 1.

5.6.4 Structure identification of active sub-fraction F5.1 of *S. rochei* (strain X34)

NMR spectroscopy is an important technique to obtain physical, chemical, electronic and structural information of molecules. The technique is based on the magnetic

properties of atom nucleus. NMR data provides detailed information of topology, dynamics and three-dimensional structure of molecules in solution and solid state. FTIR was used for identification of functional groups of the compound structure. Basically, FTIR identifies chemical bonding types in a molecule through an infrared absorption spectrum whether it is organic or inorganic. Combination data of FTIR and NMR provides information regarding the molecular structure of analyzed compound.

Yet, there is no report of any *Streptomyces* spp., particularly *Streptomyces rochei* producing compound 1, 2-(3-hydroxybutan-2-yloxy) propanoic acid. Although research of discovering antifungal agent produced by *Streptomyces* spp. was done by many researchers eg. butyrolactols A and B (Kotake *et al.*, 1992); antifungal phospholipids (Cho *et al.*, 1999); antifungal protein (Woo *et al.*, 2002); non-polyene antifungal antibiotic (Augustine *et al.*, 2005b); valinomycin, a peptide antibiotic (Park *et al.*, 2008); antifungal chitinase (Baharlouei *et al.*, 2010), but the antifungal compound identification was only done by several researchers. Atta (2009) reported an antifungal compound belong to 4' phenyl-1-napthyl-phenyl acetamide produced by *Streptomyces olivaceiscleroticus,* AZ-SH514. Besides, production of 4-phenyl-3-butenoic acid by a novel streptomycete named *Streptomyces koyangensis* was also reported by Lee *et al.* (2005). Apart from that, two new compounds were isolated from marine *Streptomyces* sp. FX-58 and were then identified as 5-carboxymethyl-2-propylchromone and 1, 6-dihydroxy-8-propylanthraquinone (Huang *et al.,* 2006).

Focusing on bioactive compounds produced by *Streptomyces rochei* (as the identified compound in this study was isolated from strain X34 which designated to *S. rochei*), this is the first report of compound 2-(3-hydroxybutan-2-yloxy) propanoic acid production from *S. rochei,* which is responsible in antifungal activity against *S. pombe*. Berger *et al.* (1953) listed some secondary metabolites from *S. rochei* with their partial

structure (Appendix P), which are borrelidin, bundling, butyrolactol A, cephalosporin and lankacyclinol.

5.7 Recommendation for future studies

To obtain more crude extracts for purification and verification of unidentified compounds, future work should include large scale of re-fermentation and bioactive extraction of strain X34. This strain should also be tested for other antimicrobial potentials rather than only antifungal property to know the activity spectrum.

Among studies on marine streptomycetes, there were no report on *Streptomyces* spp. inhibited the growth of *Schizosaccharomyces pombe.* As this yeast is an important organism in studying the cellular responses to DNA damage and the process of DNA replication, it could be an indicator for antitumor agent. In relation to this project, the antagonistic result of strain X34 against *S. pombe* may be an indicator that its potent active compounds could act as anticancer or antitumor agent. However, further research should be done on this strain to reach this level as the importance of marine natural product is not only limited to the purpose of research as it may be able to contribute in the biomedical field.

5.8 Conclusions

The first objective of this research was to study the antifungal potential of selected marine *Streptomyces* spp. As predicted, marine streptomycetes do have potential in antimicrobial activity, particularly antifungal property regarding to this study. Six out of forty four marine streptomycetes tested exhibited strong antifungal activity against *Schizosaccharomyces pombe* and *Candida albicans.*

Next aim was to characterize the potential strains using morphological, biochemical and molecular methods. The combination of these three methods was

expected to facilitate the identification of the strains. Three out of six of the potential strains were predicted to have interesting compounds based on pre-screening by high performance liquid chromatography. Therefore, only these three strains which nominated as strains X34, X42 and X77 were further identified by 16S rRNA gene sequencing analysis. The analysis revealed that strains X34, X42 and X77 were similar to *Streptomyces rochei*, *S. albidoflavus* and *S. cavourensis*, respectively.

Finally, to achieve the final objective, the crude extract of these three strains were then tested again against *Schizosaccharomyces pombe*. As only crude extract of strain X34 which was designated to *S. rochei* was positive against *S. pombe*, only this strain was subjected to bioassay guided isolation. In order to identify the active ingredients, chemical profiling, purification and isolation of the bioactive components of strain X34 was done continuously followed by combination of FTIR and NMR analysis. One of the active ingredients was identified structurally as 2-(3-hydroxybutan-2-yloxy) propanoic acid. This is the first report of antifungal activity of this compound isolated from *S. rochei*.