CHAPTER FOUR

RESULTS

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

Six (13.6%) out of forty four putative *Streptomyces* strains tested had good antifungal activity. The antifungal activities of the six strains are presented in Table 4.1. Strains which were active against *C. albicans* gave 21-23mm (in diameter) inhibition zones, whereas strains inhibiting *S. pombe* growth gave 19-21mm (in diameter) inhibition zones. 50% of the strains with antifungal activity against both *S. pombe* and *C. albicans* had the activity comparable to nystatin; which were 23mm and 22mm, respectively. *Candida albicans* was more susceptible than *S. pombe* to bioactive substances produced by strain X13, X32 and X42. It was observed that all strains active against *C. albicans* were also active against *S. pombe*, but not vice versa (Figure 4.1 and 4.2). Substances produced by strains which were only active against *S. pombe* could be more selective compared to substances active against *C. albicans*, thus resulting in the inhibition pattern observed.

4.2 Cultural characterisation of selected *Streptomyces* spp.

Cultural characteristics of the potential strains are presented in Table 4.2. Most strains grew well on ISP 4 and ISP 5 but overall, ISP medium used in this study promoted their spore formation although a few produced sparse growth eg. strain X13 on ISP 2 and ISP 6 media. The aerial and substrate mycelium colour of all strains were white to grey on all media tested (Figure 4.3). There was no soluble pigment observed almost in all strains on all tested media, except strain X13 which had a soluble pigment when

cultured on ISP 3 media. In growth aspect, ISP 4 medium was the best growth medium for all strains followed by ISP 2, ISP 3, ISP 5 and ISP 7 media. Poor growth was observed on ISP 6 medium for all strains.

Table 4.1: Antifungal activity of six potential strains of actinomycete against *C. albicans* and *S. pombe* using agar plug-diffusion method. Six-milimeter agar plugs of seven-day old actinomycete strains were placed on SDA and YPG agar lawned with *C. albicans* and *S. pombe* respectively. Incubation temperature: 37 ± 2ºC (*C. albicans*) and 28 ± 2ºC (*S. pombe*) for 24 – 96 hours. 2.5µg nystatin loaded on sterile six-millimeter Whatman paper disc were used as positive controls. Diameter of inhibition zones were taken as mean of three readings.

Strain	**Diameter of inhibition zone (mm)**	
	C. albicans	***S. pombe***
X13	21 ± 1.5	21 ± 1.0
X32	**23 ± 2.1**	20 ± 2.0
X34	-	**20 ± 0.6**
X35	-	19 ± 1.2
X42	23 ± 3.5	20 ± 2.0
X77	-	19 ± 1.7
Control (Nystatin: 2.5µg/disc)	23 ± 1.0	22 ± 0.6

-: no inhibition

4.3 Morphological studies – scanning electron microscopy (SEM) of selected *Streptomyces* spp.

Observation under scanning electron microscope showed that all the strains were polysporous actinomycetes (eg. *Streptomyces* spp.) producing long spore chains on the aerial hyphae. Strains X13 and X34 (Figure 4.4 (a) and (b)) produced well developed colonies on solid media, consist of extensively branched non-fragmenting substrate mycelium that carries rarely branching flexous to curled, hooks or loops with one turn

(*Rectinaculiaperti*) straight fragment into spores. The spores of strain X13 shaped cylindrical of equal length, whereas the spores of strain X34 appear cylindrical to oblong and of unequal length. The spore surface of both strains are smooth. The aerial mass colour on ISP 4 medium of strain X13 was white to pale yellow, and strain X34 was white to pale grey.

The structure of the spore chains were arranged by straight to flexuous (*Rectiflexbles*) for strain X32, X35, X42 and X77. Strain X32 (Figure 4.5 (a)) have cylindrical equal shaped with smooth spores, whereas strain X35 (Figure 4.5 (b)) consist of round to oval shaped spores with wrinkled surface. Figure 4.5 (c) showing spores of strain X42 which round shaped to oblong and of unequal length with smooth surface. Spores appear oblong or oval shaped with smooth surface for strain X77 (Figure 4.5 (d)).

The spore ornamentation and spore chain arrangement are summarized in Table 4.3. Only strain X35 has rugose ornamented spore, the remaining strains are smooth surface. Basically, the spores are cylindrical to round or oval shape.

4.4 Physiological characterisation of selected *Streptomyces* spp.

4.4.1 Temperature range and optimum temperature for growth

Generally, the optimum temperature for all strains was in range of 25ºC to 35ºC (Table 4.4). All strains could grow in temperature range of 15ºC to 35ºC. At 15ºC, only strains X34 and X35 had poor growth on ISP 4 medium whereas the other strains were fully grown on the media. However, both strains seemed to be well grown at 45ºC. One third of the strains (strains X13 and X77) could not grow at temperature 45ºC; whereas the remaining strains, which were X32 and X42, had poor growth at 45ºC (Figure 4.6).

a) b) c)

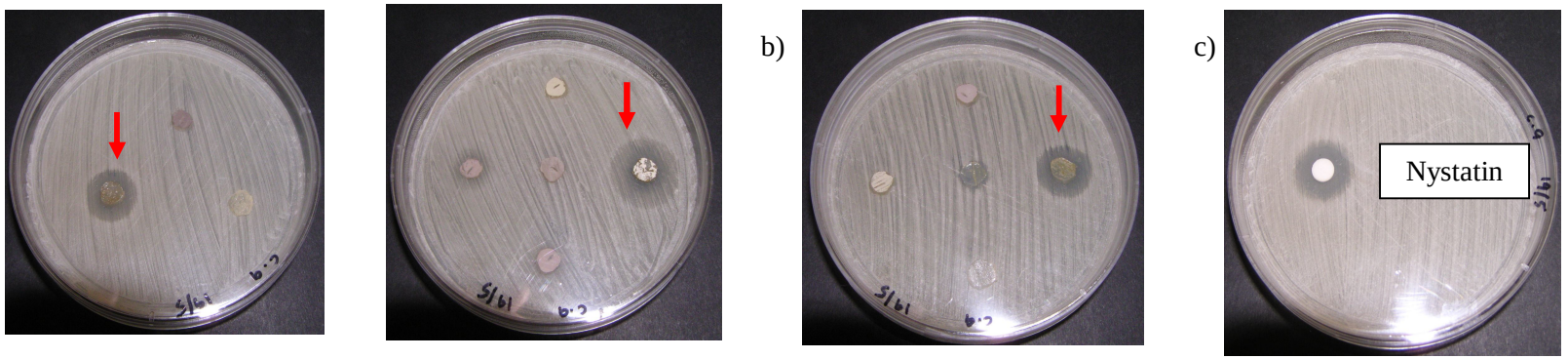
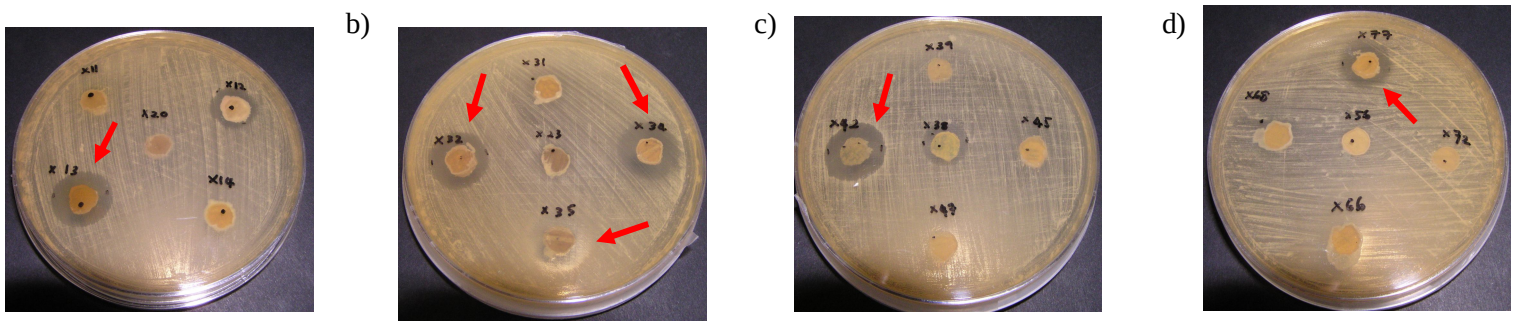


Figure 4.1: Clear zone indicating inhibition of *C. albicans* growth. Test plate consist plugs of actinomycetes strains on SDA media inoculated with *C. albicans* after 72 hours incubated at 37±2°C 28 ±2°C. Positive control is 2.5µg nystatin loaded on 6-mm sterile paper disc incubated in same condition with test plates. a: strain X13, b: strain X32, & c: strain X42 & d: positive control (20µl of 50µg/ml nystatin) on inoculated SDA.

a) b) c) d)



e)

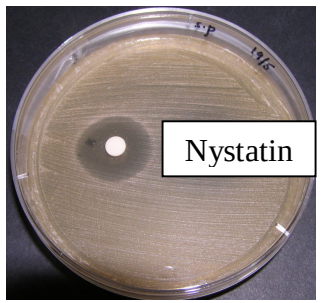


Figure 4.2: Clear zones indicating inhibition of *S. pombe* growth. Test plate consist plugs of actinomycetes strains on YPG media inoculated with *S. pombe* after 72 hours incubated at 28 ±2°C. Positive control is 2.5µg nystatin loaded on 6-mm sterile paper disc incubated in same condition with test plates. a: strain X13, b: strain X32, X34 & X35, c: strain X42 & d: strain X77, & e: positive control (20µl of 50µg/ml nystatin) on inoculated YPG.

Table 4.2: Cultural characteristics of six strains of actinomycetes. Observation was done after 7 days of incubation at 28 ± 2°C. Colour identification was referred to ICI Dulux Colour (2008).

Strain / Cultural characteristics		ISP 2	ISP 3	ISP 4	ISP 5	ISP 6	ISP 7
X13	Growth	+	++	+++	++	+	++
	Aerial mycelium	Orchid White	Nutmeg 461 B.S.3-043	Apple White 13254	Cream Satin 16204	Clear Glossy On Ramin 18017	Cream Satin 16204
	Substrate mycelium	Bamboo	Heritage Red 14360	Ash Grey B.S.9-099	Golden Honey 727	Clear Glossy On Ramin 18017	Honey Pine On Teak Ply 16059
	Soluble pigment	None	Golden Honey 727	None	None	None	None
X32	Growth	++	+++	+++	+++	+	+++
	Aerial mycelium	Barley White 16208	Banana 12138 B.S.10B15	Lily White 16207	Ivory 32 B.S.4-046	Barley White 16208	Barley White 16208
	Substrate mycelium	Bamboo 57	Nutmeg 461 B.S.3-043	Sugar Cane 652 B.S.4-052	Bamboo 57	Nutmeg 461 B.S.3-043	Golden Honey 727
	Soluble pigment	None	None	None	None	None	None
X34	Growth	+++	+++	+++	+++	+	+++
	Aerial mycelium	Tungsten 1042	Dove White Satin 10277	Mercury 10235 B.S.00A05	Banana 12138 B.S.10B15	Clear Glossy On Ramin 18017	Rose White 14115
	Substrate mycelium	Buttermilk 12140 B.S.10C31	Sugar Cane 652 B.S.4-052	Pebbles 16345	Fairlady 16178	Clear Glossy On Ramin 18017	Paper Lace 16143 B.S.10C31
	Soluble pigment	None	None	None	None	None	None

(continuation of Table 4.2)

Strain / Cultural characteristics		ISP 2	ISP 3	ISP 4	ISP 5	ISP 6	ISP 7
X35	Growth	+++	+++	+++	+++	+	+++
	Aerial mycelium	Ash Grey 10526	Lily White Satin 12373	Mercury 10235 B.S.00A05	Dove White 10270	Clear Glossy On Ramin 18017	Dove White 10270
	Substrate mycelium	Sugar Cane 652 B.S.4-052	Bamboo 57	Silver Mist 693 B.S.9-093	Ivory 32 B.S.4-046	Clear Glossy On Ramin 18017	Silver Mist 693 B.S.9-093
	Soluble pigment	None	None	None	None	None	None
X42	Growth	+++	+	+++	++	+	++
	Aerial mycelium	Barley White Satin 16210	Orchid White 11138	Lily White Satin 12373	Lily White 16207	Lily White 16207	Apple White Satin 13256
	Substrate mycelium	Mahogany 12493	Mahogany On Ramin 12092	Sugar Cane 652 B.S.4-052	Nutmeg 461 B.S.3-043	Clear Glossy On Ramin 18017	Ivory 32 B.S.4-046
	Soluble pigment	None	None	None	None	None	None
X77	Growth	+++	++	+++	+++	+	+++
	Aerial mycelium	Lily White 16207	Banana 12138 B.S.10B15	Lily White 16207	Silver Mist 1793 B.S.9-093	Dove White Satin 10277	Pebbles 16345
	Substrate mycelium	Summer Daisy 16454	Bamboo 57	Sugar Cane 652 B.S.4-052	Sugar Cane 652 B.S.4-052	Nutmeg 461 B.S.3-043	Cane 16348
	Soluble pigment	None	None	None	None	None	None

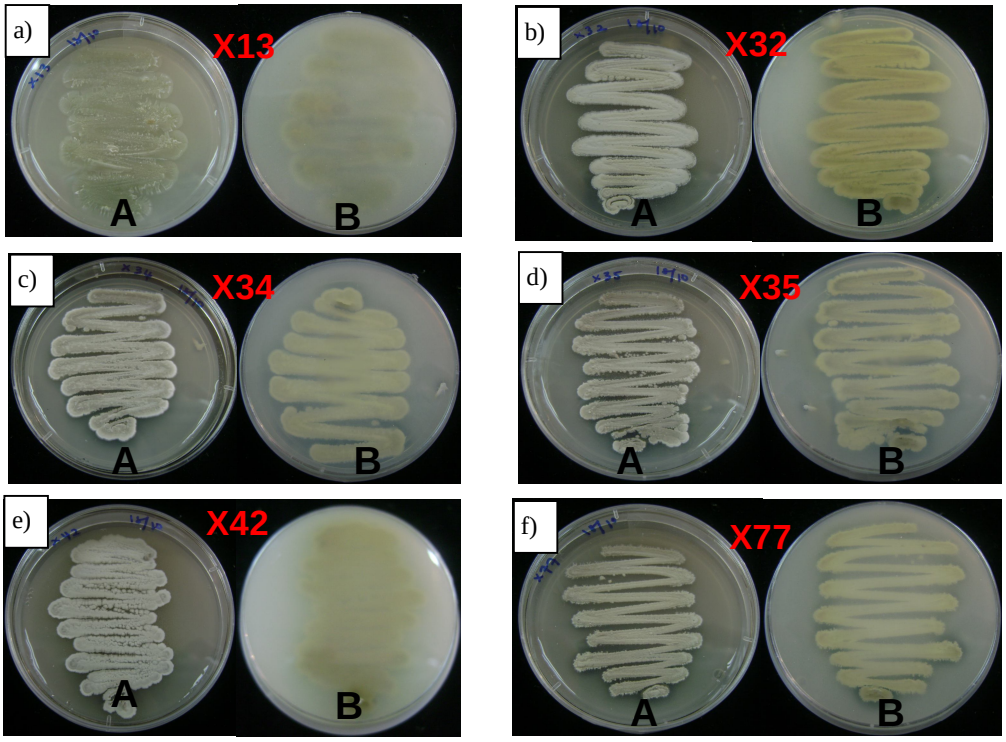


Figure 4.3: Aerial and substrate mycelium of 7-day-old culture of potential strains on ISP4 media. Tested strains were subcultured on ISP 4 media and incubated at 28 ± 2˚C for 7 days. A: aerial mycelium, B: substrate mycelium of a) strain X13, b) strain X32, c) strain X34, d) strain X35, e) strain X42 and f) strain X77.

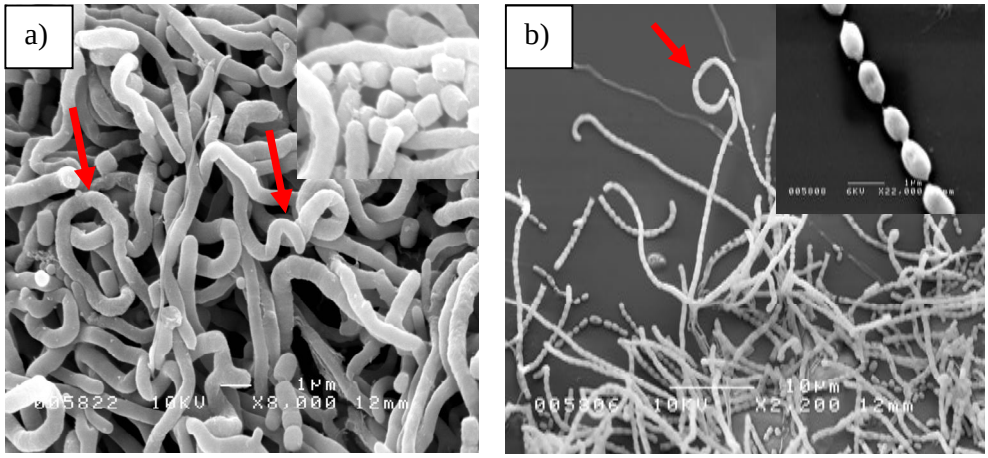


Figure 4.4: Scanning electron micrograph of: a) strain X13 and b) strain X34 spores and hyphae. Strains were grown on ISP 4 media for two weeks at 28 ± 2°C of incubation. Loops spore chains with one turn were observed (*Rectinaculiaperti*).

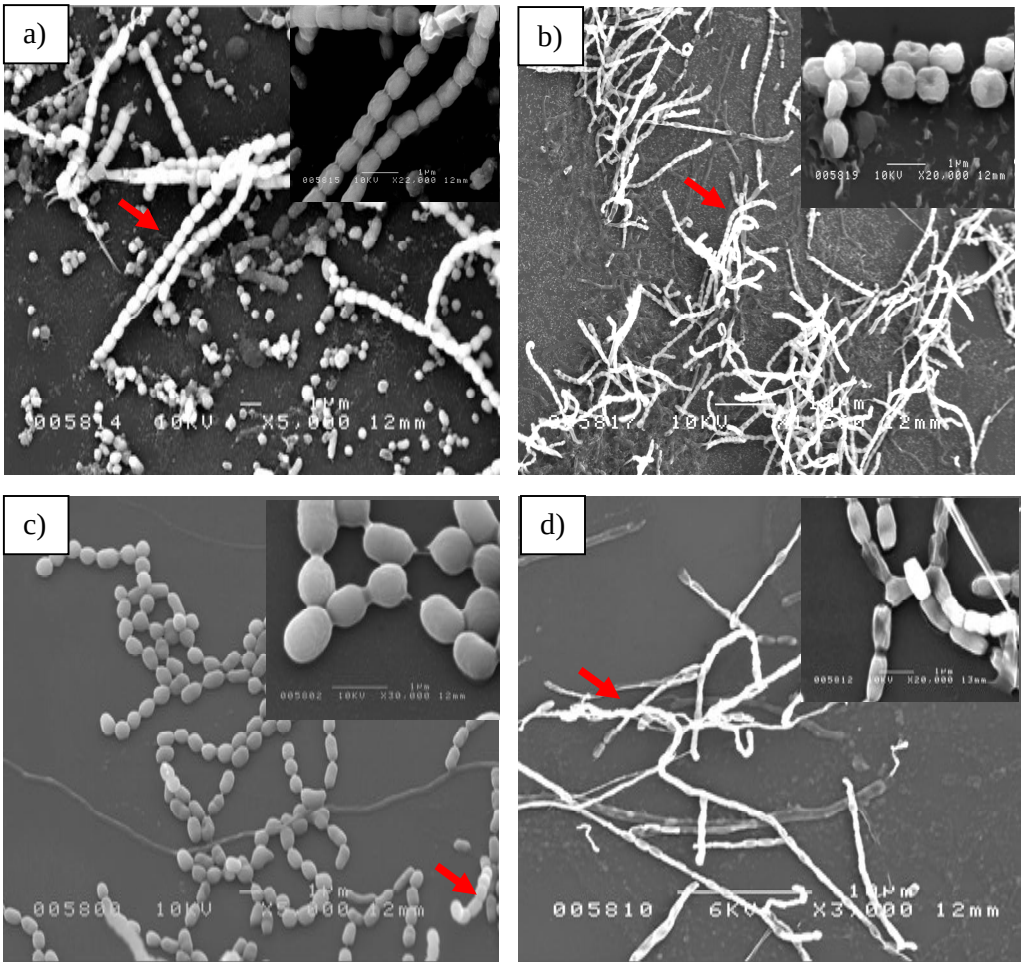


Figure 4.5: Scanning electron micrograph of: a) strain X32, b) strain X35, c) strain X42 and d) strain X77 spores and hyphae. All strains were grown on ISP 4 media for two weeks at 28 ± 2°C of incubation. Straight to flexuous spore chains were observed (*Rectiflexbles*).

4.4.2 Formation of melanoid pigment

Referring to Table 4.2, there was no melanoid pigment was observed in any tested plate when grown on both peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Melanoid pigment is considered positive if there is any diffusible greenish-brown, brown to black pigment in the media observed (Tan, 2007).

4.4.3 Liquefaction of gelatin

Figure 4.7 showed the result of gelatin liquefaction test for all strains. Entire strains were producing gelatinase enzyme, which means they were positive in liquefacting

gelatin. Negative control which only gelatin used was solidified after placed in 4ºC fridge, whereas all tested strains were vice not clear. This result is consistent after 7, 14 and 21 days of incubation.

Table 4.3: Spore ornamentation and spore chain arrangement of studied *Streptomcyes* spp. strains.

Strain	Spore		Spore chain arrangement
	Shape	Ornamentation	
X13	Cylindrical of equal length	Smooth	*Reflexibles*
X32	Cylindrical equal shaped	Smooth	*Rectinaculiaperti*
X34	Cylindrical to oblong and of unequal length	Smooth	*Reflexibles*
X35	Round to oval	Rugose (wrinkle)	*Rectinaculiaperti*
X42	Round to oblong and of unequal length	Smooth	*Rectinaculiaperti*
X77	Oblong or oval	Smooth	*Rectinaculiaperti*

Table 4.4: Growth of all potential strains at 15ºC, 25ºC, 35ºC and 45ºC after 7 days of incubation on ISP 4 media.

Strain	Growth temperature (±2°C)			
	15°C	25°C	35°C	45°C
X13	+++	+++	+++	-
X32	+++	+++	+++	+
X34	+	+++	+++	+++
X35	+	+++	+++	+++
X42	+++	+++	+++	+
X77	+++	+++	+++	-

+++; good growth, +; poor growth, -; no growth

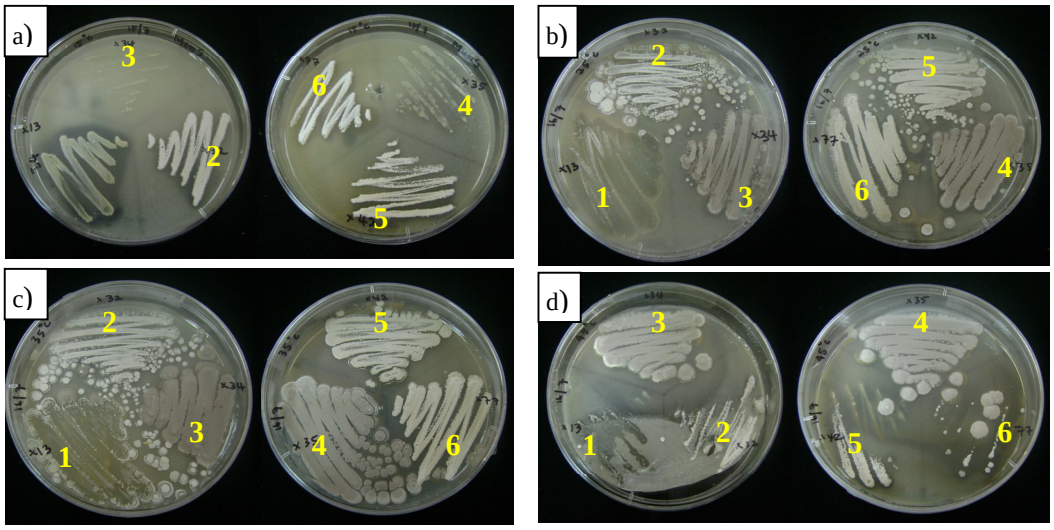


Figure 4.6: Growth of potential strains after 7 days of incubation on ISP 4 media at: a)15 ± 2ºC, b) 25 ± 2ºC, c) 35 ± 2ºC, d) 45 ± 2ºC. Only strains X34 and X35 grow well at 45 ± 2ºC. 1; strain X13, 2; strain X32, 3; strain X34, 4; strain X35, 5; strain X42 and 6; strain X77.

4.4.4 Hydrolysis of starch

According to Figure 4.8, two third of tested strains showed light yellow to brown colour zone around streaked area on ISP 4 media plate upon application of iodine. It was actually clear zone surrounding the streaked area because the starch was no longer there to react with the iodine. Negative control with only ISP 4 media turned to blue-black colour after loaded with iodine, like another one third of the tested strains (Figure 4.8). This is because iodine-starch complex was still inside the media, which was not, hydrolyzed by the strains (X34 and X35) due to lack of exoenzyme to break down the starch (Tan, 2007). The summary of starch hydrolysis test is shown in Table 4.5.

4.4.5 Hydrogen sulfide (H_2S) production

After 18 hours of incubation, only strain X77 (14.3%) was observed positive in producing hydrogen sulfide (Table 4.5). This was determined by formation of brown-black colour on inoculated media indicating that this strain produces the enzyme that

reduce sulfur to hydrogen sulfide (Figure 4.9). The remaining strains did not show the discoloration, meaning that no hydrogen sulfide production occurred.

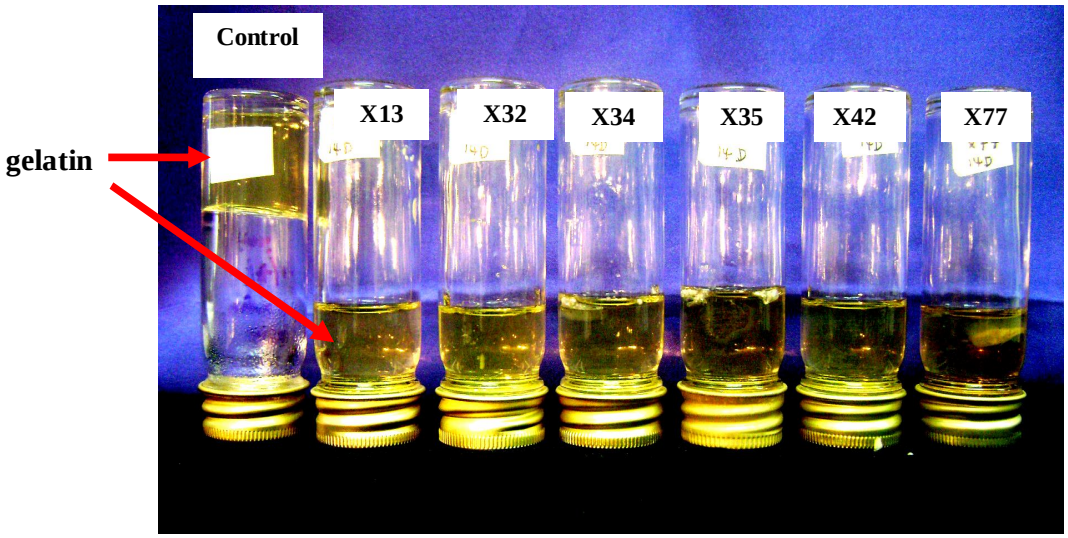


Figure 4.7: Gelatin and inoculated gelatin (with tested *Streptomyces* spp. strains) incubated for 14 days at 28 ± 2ºC. Gelatin without inoculation was used as negative control. After placed in fridge for one hour, all the test bottles with gelatin inside were placed upside down.

4.4.6 Sodium chloride (NaCl) tolerance

Three strains (50%) out of six tested strains grew at 2% up to 10% NaCl (Figure 4.10). These strains were X13, X32 and X42. The remaining strains grew up to 6% NaCl for strain X77 and up to 8% NaCl for strain X34 and X35. The sodium chloride tolerance was observed based on the growth of the tested strains on ISP 4 media added with 2-10% concentration of NaCl. Entire strains could grow on media without addition of NaCl, thus they might be adapted to terrestrial condition since they were marine isolate, which suppose to have adequate NaCl to survive. However, no strains could tolerate up to 12% NaCl (Table 4.5).

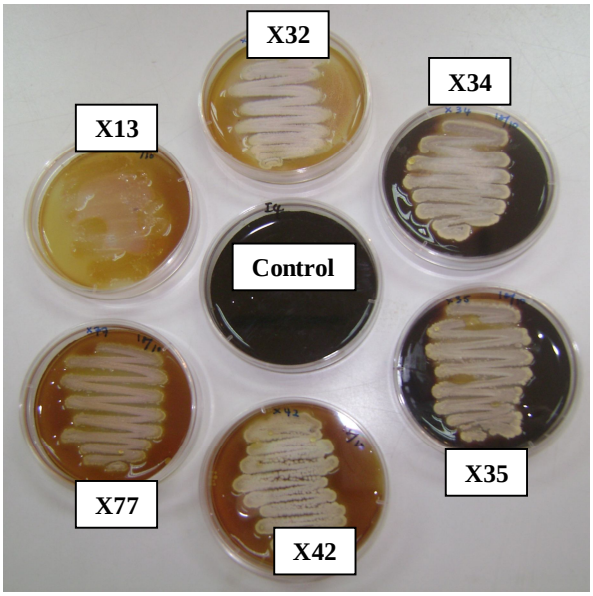


Figure 4.8: Plates of tested strains and negative control (ISP 4 media only) after dropped with iodine. Inoculated ISP 4 media were incubated for 7 days at 28 ± 2˚C. 3ml of iodine dropped on the inoculated media prior to be observed after a few seconds.

Table 4.5: Starch hydrolysis test, NaCl tolerance, H_2S production test, pH sensitivity and nitrate reduction result of all tested *Streptomyces* spp. strains.

Strain	Starch hydrolysis	% NaCl tolerance	H_2S production	pH sensitivity	Nitrate reduction
X13	Positive	0 – 10%	Negative	6.5 – 9.5	Positive
X32	Positive	0 – 10%	Negative	6.5 – 9.5	Positive
X34	Negative	0 – 8%	Negative	5.5 – 9.5	Positive
X35	Negative	0 – 8%	Negative	5.5 – 9.5	Positive
X42	Positive	0 – 10%	Negative	6.5 – 9.5	Positive
X77	Positive	0 – 6%	Positive	5.5 – 9.5	Negative

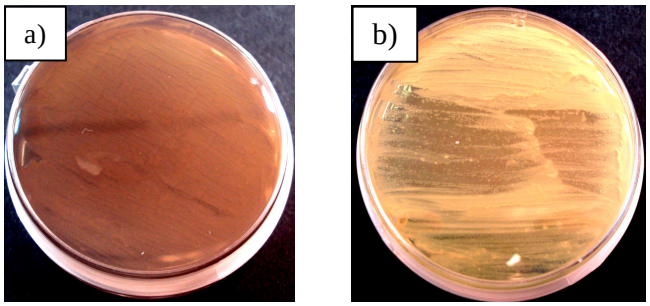


Figure 4.9: Hydrogen sulfide production test of: a) strain X77 (positive) and b) X13 (negative). Tested strains were lawned on Difco peptone-iron agar supplemented with 0.1% of Difco yeast extract. Inoculated media was incubated at 28±2˚C for 18 hours.

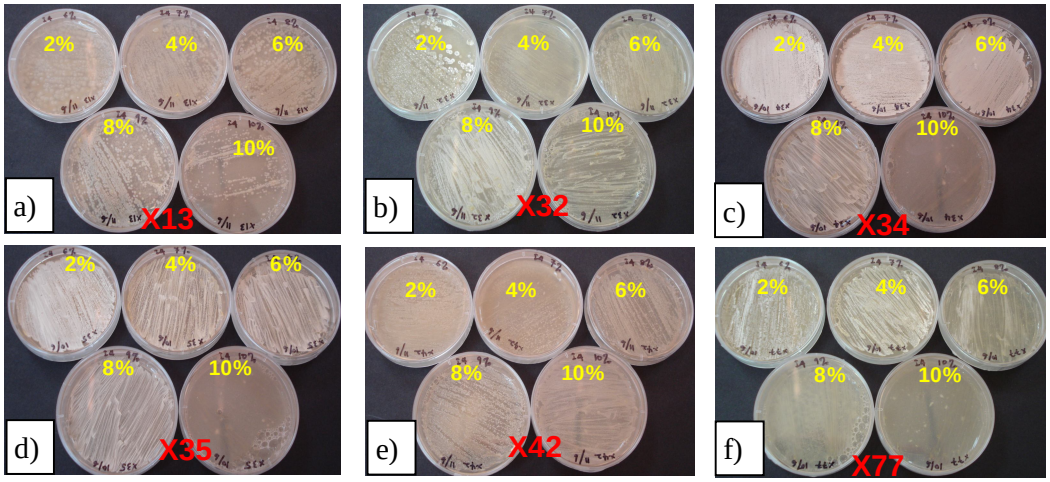


Figure 4.10: NaCl tolerance for all tested strains which were grown on ISP 4 agar incorporated with different concentrations of NaCl. a) strain X13, b) strain X32, c) strain X34, d) strain X35, e) strain X42 and f) strain X77. ISP 4 plates without NaCl were used as control. Plates were incubated at 28 ± 2ºC for one to two weeks before tolerance was determined.

4.4.7 Carbon sources utilization

In this study, each strain exhibited almost uniform utilization pattern, except one or two carbon sources were utilized/not utilized (Table 4.6). Poor or almost no growth were observed on basal medium ISP 9 due to no carbon sources supplemented but only mineral salts (Appendix A). Almost all strains grew well on ISP 9 media incorporated with D-glucose, D-mannitol, L-arabinose, L-rhamnose and maltose. Growth of five strains on ISP 9 incorporated with D-fructose were comparable to the positive control (glucose), suggesting that this carbon source including D-mannitol, L-arabinose, L-rhamnose and maltose were utilized as their sole carbon sources (Tan, 2007). Two third (66.67%) of the tested strains grew on ISP 9 supplemented with inositol; the same percentage of the tested strains also observed which did not utilize lactose as carbon sources. Besides, only strain X42 grew on ISP 9 incorporated with sorbitol. All strains did not grow on media added with raffinose as carbon source.

Table 4.6: Growth of potential strains on basal medium (ISP 9) with carbon sources. Spore suspension of 7-day-old cultures tested strains were loaded onto walls in test plates (added with carbon sources) and incubated at 28 ± 2ºC for ten days.

Strain	Carbon sources											
	Fru	Glu	Man	Xyl	Inos	Lact	Ara	Rha	Malt	Raff	Sorb	Suc
X13	-	+	+	+	-	-	+	±	+	-	-	-
X32	+	+	+	+	-	-	+	+	+	-	-	+
X34	+	+	+	±	±	+	+	+	+	-	-	±
X35	+	+	+	-	+	+	+	+	+	-	-	-
X42	+	+	+	±	±	-	+	±	±	-	+	±
X77	+	+	+	+	±	-	±	±	+	-	-	-

+; good growth, ±; poor growth, -; no growth.
Fruc; D-fructose, Glu; D-glucose, Man; D-mannitol, Xyl; D-xylose, Inos; Inositol, Lact; Lactose, Ara; L-arabinose, Rha; L-rhamnose, Malt; Maltose, Raff; Raffinose, Sorb; Sorbitol, Suc; Sucrose.

4.4.8 Nitrate reduction test

Five out of six strains (83%) showed positive results in nitrate reduction test which means these strains (strain X13, X32, X34, X35 and X42) are able to reduce nitrate (Table 4.5). After addition with sulfanilic acid and dimethyl-α-naphthylamine, pink to red color appeared indicated the presence of nitrite (nitrate → nirite). In this study, only strain X77 (16.67%) cannot reduce nitrate which was confirmed by the discoloration to pink colour after addition of zinc dust. Strain X77 also showed the same result as negative control (Figure 4.11).

4.4.9 pH sensitivity

A somewhat distinct pH sensitivity pattern was observed for each of the tested strain. Entire strains had good growth with sporulation on all ISP 4 media between pH 6.5 to 9.5, but not growing at all on media with pH 4.5 (Table 4.5). However, the result exhibited half of the test strains grew poorly on media with pH level 5.5, whereas the

remaining strains had quite good growth with sporulation on the media with the same pH level. Strains which were not growing well on media with this pH level were strains X13, X32 and X42 (Figure 4.12).

a)

b)

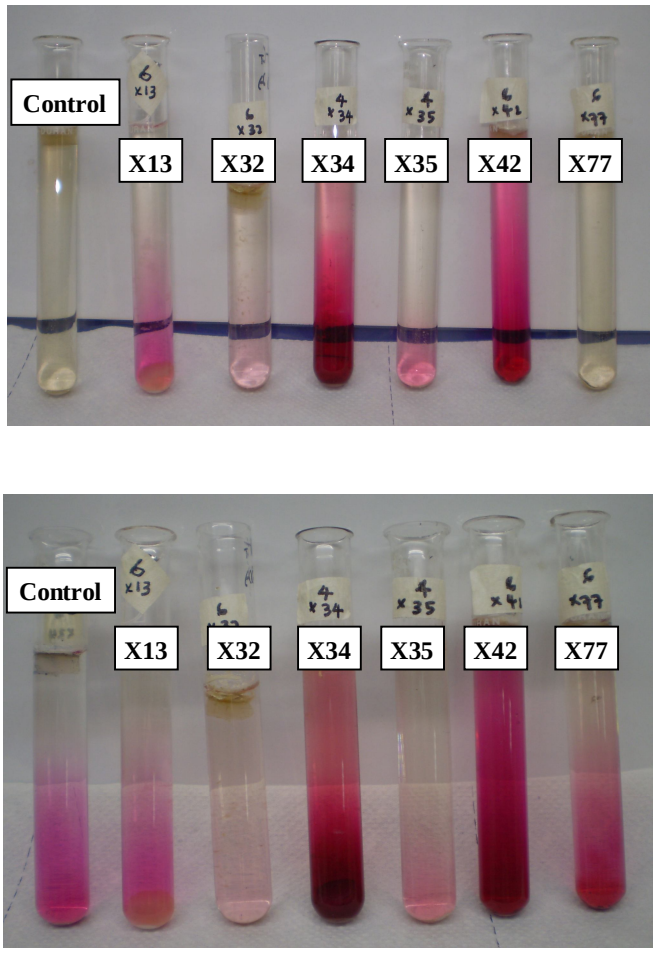


Figure 4.11: Nitrate reduction test of potential strains. A 6mm-plug of 7-day-old culture was put into synthetic nitrate broth and incubated at 28 ± 2ºC for ten days. After incubation, the inoculated solution was added with sulfanilic acid and dimethyl-α-naphthylamine before added with zinc dust. Solution without culture plug was used as negative control. a) Tested strains and control after added with sulfanilic acid and dimethyl-α-naphthylamine. b) Tested strains and control after added with zinc dust.

4.5 Identification based on 16S rRNA gene sequence analysis

According to HPLC chromatograms of six potential strains of tested *Streptomyces* spp. (Appendix K), only three strains out of six were selected to be identified based on 16S rRNA gene sequence analysis and chemically profiled as they were expected to have interesting compounds (4.6.1). The strains were X34, X42 and X77.

4.5.1 PCR amplification of 16S rRNA genes of potential strains

Samples of successfully amplified 16S rRNA gene (approx. 1.5kb) using PCR are shown in Figure 4.13. A single band of each tested strain was clearly seen in the correct size of 1500bp as compared to the marker. These PCR product could be further purified before sent for sequencing. Product purification was needed to remove excess primers and nucleotides (Clarridge, 2004).

4.5.2 Analysis of 16S rRNA gene sequences

Forward and reverse sequences (Appendix C) obtained from 1st Base Laboratory were analyzed using MEGA 4 and ChromasLite softwares to get almost full sequences of strains X34, X42 and X77 which were 1109, 1342 and 1278 long of nucleotides, respectively.

The almost full sequences (Appendix D) were checked using BLAST. There are about hundreds of *Streptomyces* spp. name that appeared in the search result still show the probable similarity ID here (top 20 list in Appendix E). However, BLAST search is only used to compare the query sequence and the databases available are not very accurate in terms of the hits found. In other word, BLAST result is not providing all the hits within the databases obtained (Altschul, 1990).

By using EzTaxon, there were almost 30 lists of *Streptomyces* spp. obtained. The top three among the lists are listed in Table 4.7. These lists of the most probable *Streptomyces* spp. are having the highest pairwise similarity compared to the strains to be identified in this study.

When comparing the Fasta format of the full sequences of the *Streptomyces* spp. strains in RDP Sequence Match, 20 lists of the highest ab_score obtained (Appendix F). According to Ribosomal Database Project (1992);

A sequence match score (s_ab) are the number of unique 7-base oligomers shared between our own sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences.

The top four list of *Streptomyces* spp. in RDP search for each strain to be identified are as shown in Table 4.8. These lists are not positioned in rank of similarity, but score of s_ab. As noted, there are identical *Streptomyces* strain for strains X34, X42 and X77 in both EzTaxon and RDP search which are *S. rochei*, *S. albidoflavus* and *S. cavourensis*, respectively. List of *Streptomyces* spp. in Table 4.8 are chosen based on full name given with species name.

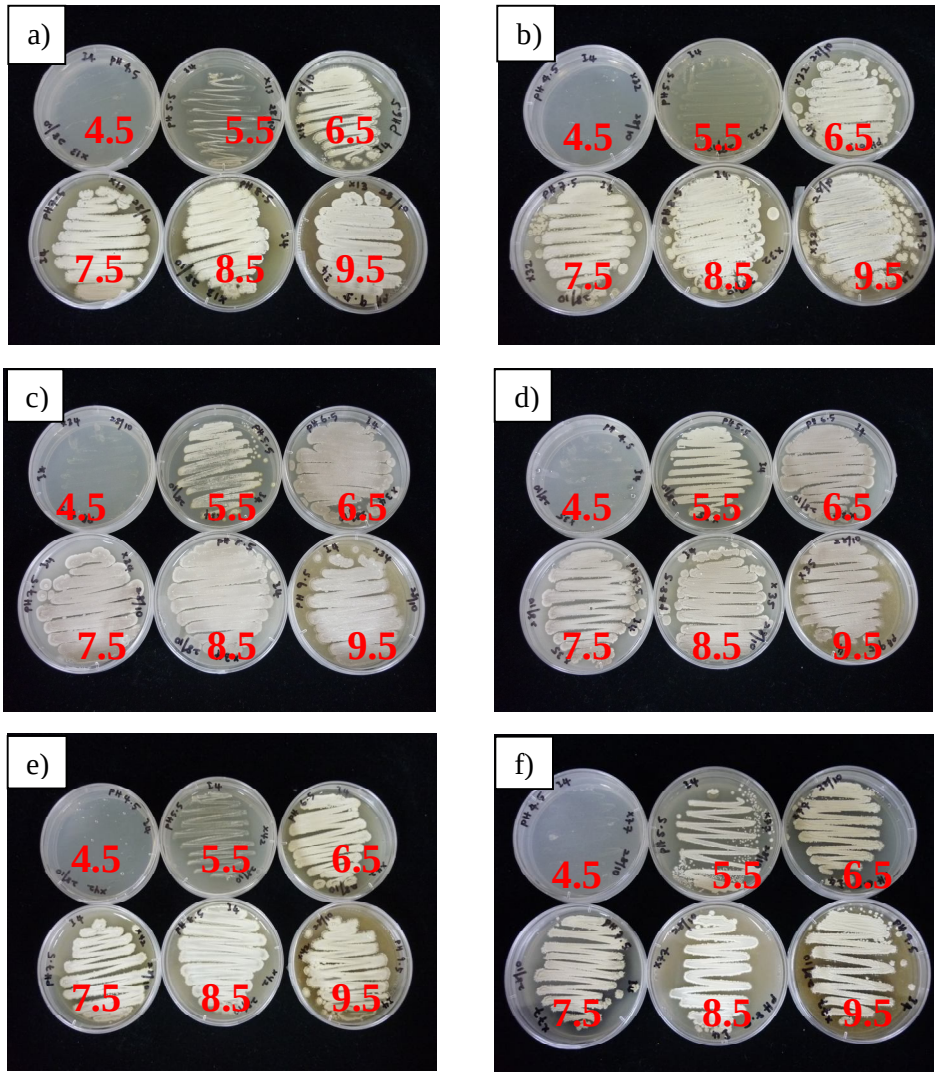


Figure 4.12: pH sensitivity of tested strains. All strains were lawned on ISP 4 media adjusted to pH 4.5 – 9.5 prior to inoculation at 28±2˚C for seven days. a) strain X13, b) strain X32, c) strain X34, d) strain X35, e) strain X42, and f) strain X77.

All *Sreptomyces* spp. listed in Table 4.8 have the same s_ab score, which is 1.000. Since the pairwise similarity are not calculated, the first 20 list of s_ab score are considered having the most similar sequence; which is about 95% by pairwise identity (Cole *et al.*, 2005).

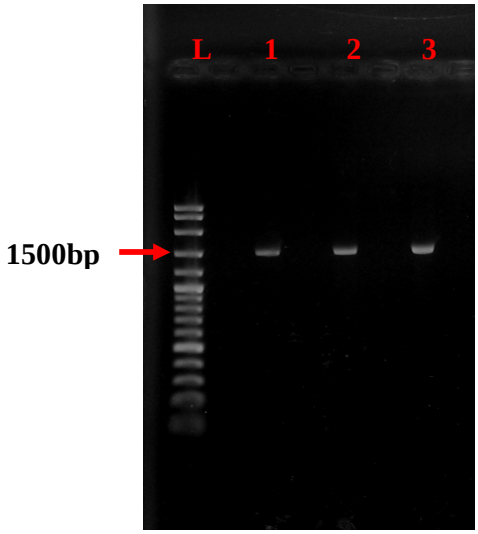


Figure 4.13: Successfully amplified 16S rRNA genes of three potential strains by PCR. L: 100bp ladder, lane 1: Strain X34, lane 2: Strain X42, lane 3: Strain X77.

Table 4.7: List of top three ranks of *Streptomyces* spp. strains by EzTaxon search.

Strain	Rank	Name	Strain	Accession No.	Similarity (%)
X34	1	*Streptomyces rochei*	NBRC 12908 (T)	AB184237	100.000
	2	*Streptomyces plicatus*	NBRC 13071 (T)	AB184291	100.000
	3	*Streptomyces enisssocaesilis*	NRRL B-16365 (T)	DQ026641	100.000
X42	1	*Streptomyces albidoflavus*	DSM40455 (T)	Z76676	99.702
	2	*Streptomyces somaliensis*	NBRC 12916 (T)	AB184243	99.627
	3	*Streptomyces hydrogenans*	NBRC 13475 (T)	AB184868	99.627
X77	1	*Streptomyces cavourensis*	NBRC 13026 (T)	AB184264	99.765
	2	*Streptomyces celluloflavus*	NBRC 13780	AB184476	99.765
	3	*Streptomyces albolongus*	NBRC 13465 (T)	AB184425	99.765

Based on comparison in EzTaxon and RDP search, list in Table 4.9 are the most probably species of the *Streptomyces* strains studied. In order to ensure the validity of the species name, these lists were referred to LPSN which are available online at www.bacterio.cict.fr/allnamessz.html. The results are the same as shown in Table 4.9; all the species are included in valid Approved List, with their references as well.

Thus, for the next part of this thesis, the studied strains of *Streptomyces* are considered as *S. rochei* for strain X34, *S. albidoflavus* for strain X42 and *S. cavourensis* for strain X77.

Table 4.8: Probable *Streptomyces* spp. list with highest s_ab score (1.000) of strains X34, X42 and X77 full 16S rRNA sequences in RDP search.

Compared Strains	**Probable Sequence Full Name**	**Accession No.**
X34	*Streptomyces maritimus* BD26	AF 233338
	S. rochei NBRC 12908 (T)	AB184237
	S. plicatus NBRC13071	AB184291
	S. vinaceusdrappus NBRC13099	AB184311
X42	*S. griseus* IFO13550	AB045867
	S. sampsonii ATCC25495	D63871
	S. champavatii NRRL B-5682	DQ026642
	S. albidoflavus JAM-AC0101	AB362247
X77	*S. globisporus* subsp. *globisporus* NBRC12208	AB184066
	S. cavourensis subsp. *cavourensis* NBRC13026	AB184264
	S. bacillaris NBRC13487	AB184439
	S. cavourensis NRRL2740	DQ445791

Table 4.9: Validity status of the most probable species for *Streptomyces* spp. strains studied according to List of Prokaryotic Names with Standing in Nomenclature (LPSN). Details of information of these strains are available in Appendix G.

***Streptomyces* spp. strain**	**Most probable species**	**Status**	**Reference**
X34	*S. rochei* NBRC 12908(T)	Approved List 1980	Bergey *et al*. 1953
X42	*S. albidoflavus* DSM40455(T)		(Rossi Doria 1891) Waksman & Henrici 1948
X77	*S. cavourensis* NRRL2740		Skarbek & Brady 1978

4.6 Chemical profiling, isolation and identification of active components

4.6.1 High performance liquid chromatography (HPLC) for interesting compounds screening

HPLC chromatograms of six potential strains extracts are shown in Appendix K. Three out of six strains were selected based on the presence of peaks that suggested interesting compounds were present in the extracts. The selected extracts were of strains X34, X42 and X77. These strains were identified based on 16S rRNA gene sequence analysis before further profiling to identify their active compounds by bioassay guided isolation.

4.6.2 Thin layer chromatography (TLC) of selected *Streptomyces* spp.

Among the three solvent systems used to develop the TLC plates, hexane: ethyl acetate in ratio 3:1 produced the best compound separation of the three *Streptomyces* strains tested. Figure 4.14 shows visible spots of compounds after spraying with molybdo (VI) phosphoric acid and heated over hot plate at 100˚C. Blue spots as shown in Figure 4.14 would indicate the presence of hydrocarbons, terpenes and sterols.

4.6.3 Antibiotic assay (AA) disc diffusion method of tested *Streptomyces* spp. crude extracts

For the antifungal test against *S. pombe,* only *S. rochei* (strain X34) exhibited positive result with inhibition zone of 1.5cm (Figure 4.15 and Table 4.10). The other two strains which were *S. albidoflavus* (X42) and *S. cavourensis* (X77) did not inhibit the growth of *S. pombe* at all. Therefore, for the isolation and identification of active compounds, only *S. rochei* crude extract was profiled.

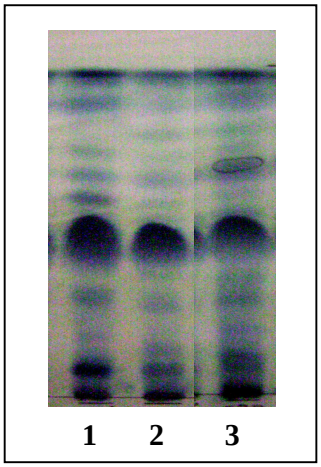


Figure 4.14: TLC plate of 1; *S. rochei*, 2; *S. albidoflavus* and 3; *S. cavourensis*. This plate was developed in hexane: ethyl acetate (3:1) solvent system. 1; *S. rochei* (strain x34), 2; *S. albidoflavus* (strain X42), 3; *S. cavourensis* (strain X77).

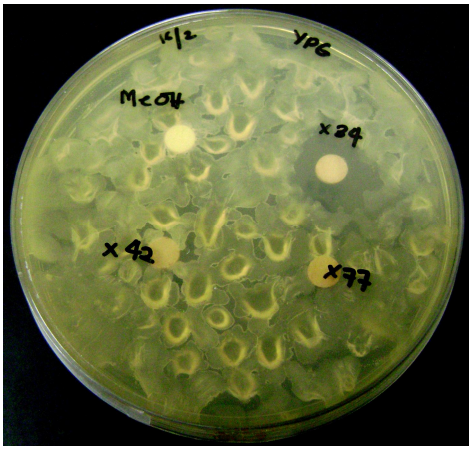


Figure 4.15: Agar diffusion test using crude extract of *S. rochei* (strain X34), *S. albidoflavus* (strain X42) and *S. cavourensis* (strain X77) against *S. pombe*. 25µl of crude extract (10mg/1ml methanol) were loaded onto the paper disc and put on YPG agar seeded with tested fungi before incubated at 30˚C for three days.

4.6.4 Column chromatography of *S. rochei* (strain X34) crude extract

Column chromatography was done in order to separate the compounds of the crude extract of *S. rochei* according to their polarity. Table 4.11 shows the yield of every fraction obtained after evaporation of the eluants. The highest weight of yield obtained was F6, followed by F5, F4, F1, F3 and F2. All these fractions were subjected to TLC, ^{1}H-NMR and antifungal bioassay to screen their bioactivity against *S. pombe.* Only fraction with antifungal property will be further carried out for the next step of experiments.

Table 4.10: Antifungal activity of *S. rochei, S. albidoflavus* and *S. cavourensis* against *S. pombe.* Results were recorded after three days of incubation at 30˚C.

Crude extract of *Streptomyces* Strains	**Diameter of inhibition zone (cm)**
S. rochei (strain X34)	1.5
S. albidoflavus (strain X42)	-
S. cavourensis (strain X77)	-

Table 4.11: Fractions of extracts obtained from 500mg of *S. rochei* (strain X34) extract after column chromatography.

Fraction	**Eluant**	**Ratio**	**Eluant Volume**	**Yield (After evaporation) (mg)**
F1	Hexane: ethyl acetate	9:1	250ml each fraction	54.9
F2		8:2		23.7
F3		7:3		24.6
F4		6:4		58.9
F5		1:1		87.3
F6	$ChCl_3$: MeOH: dH_2O	65:25:4		107.1

$ChCl_3$: chloroform, MeOH: methanol, dH_2O: distilled water

4.6.5 Thin layer chromatography (TLC) of *S. rochei* (strain X34) fractions

All the *S. rochei* fractions collected after column chromatography were concentrated and spotted on TLC plate to see their chemical nature. Figure 4.16 shows the separation of the compounds of each fraction. At this level, hexane: ethyl acetate in ratio 2:1 was the best solvent system as the standard solvent system [hexane: ethyl acetate (3:1)] could not separate the compounds.

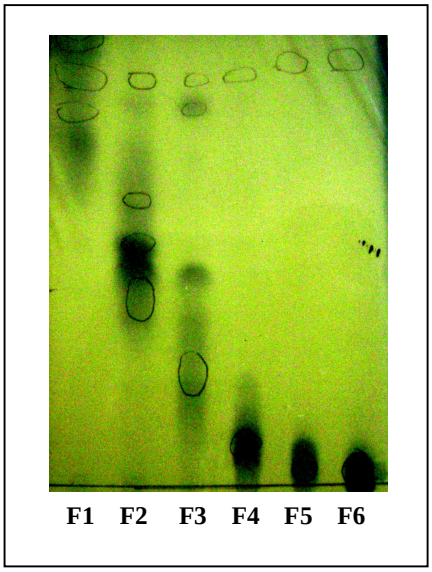


Figure 4.16: TLC plate of 6 fractions (F1-F6) of *S. rochei* (strain X34) after column chromatography. The plate was developed in hexane: ethyl acetate (2:1).

4.6.6 ^{1}H – NMR of six fractions of *S. rochei* crude extract (strain X34)

For further structure information of the compounds, all the fractions were subjected to ^{1}H-NMR. All chromatograms of the proton NMR are shown in Appendix I.

4.6.7 AA disc diffusion assay of *S. rochei* (strain X34) fractions

To detect which fraction has antifungal activity against *S. pombe,* all *S. rochei* fractions (F1-F6) were tested again using AA disc diffusion technique. Only fraction F5 exhibited positive result in this test. In terms of diameter of inhibition zone, a better result was observed (Figure 4.17) compared to previous assay using crude extract of *S.*

rochei (Figure 4.15). The active fraction F5 inhibited *S. pombe* growth by diameter of 2.5cm (Table 4.12), compared to 1.5cm in previous test (Table 4.10). The inhibition zone was also more clearly indicating the fraction may have fungicidal activity against *S. pombe* (Figure 4.17), as after three weeks, there was no growth of *S. pombe* around the inhibition zone observed.

Based on this assay result, only the active fraction (F5) will be carried out for further bioassay guided experiments for isolation of active compound/s.

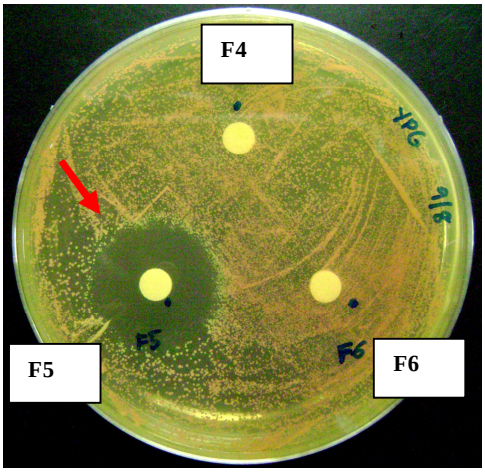


Figure 4.17: Plate of agar diffusion test of fractions F4, F5, and F6 (fractions of *S. rochei* (strain X34)) against *S. pombe*. 25µl of crude extract (10mg/0.5ml methanol) were loaded onto the paper disc and put on YPG agar seeded with *S. pombe* before incubated at 30˚C for three days.

Table 4.12: Antifungal activity of *S. rochei* fractions (F1-F6) against *S. pombe* using AA disc diffusion method. Results were recorded after three days of incubation at 30˚C.

***S. rochei* fractions**	**Diameter of inhibition zone (cm)**
F1	-
F2	-
F3	-
F4	-
F5	2.5
F6	-

4.7 Bioassay guided isolation of active fraction (F5) of *S. rochei* (strain X34)

4.7.1 High performance liquid chromatography (HPLC) of active fraction F5

Four conditions of HPLC were used to profile the active fraction F5 in this study (Table 3.4). Chromatograms of the HPLC result of the active fraction, F5 are shown in Appendix J.

4.7.2 Preparative thin layer chromatography (PTLC) of active fraction (F5) of *S. rochei* (strain X34)

Preparative thin layer chromatography (PTLC) was done for active fraction of F5 to isolate and purify separated compounds. About 80mg of the fraction F5 was separated to another six sub-fractions (F5.1-F5.6) after the process. Table 4.13 shows the yield of sub-fractions obtained after evaporation. All the sub-fractions were subjected to thin layer chromatography (TLC) and ^{1}H-NMR for chemical profiling, besides tested for antifungal bioassay again to confirm the bioactivity of the separated compounds.

Table 4.13: Yield of sub-fractions obtained from fraction 5 (F5) of *S. rochei* (strain X34) after preparative thin layer chromatography (PTLC).

Sub-fractions	Yield obtained (mg)
F5.1	9.7
F5.2	2.8
F5.3	5.3
F5.4	4.3
F5.5	6.9
F5.6	1.8
Total	30.8

4.7.3 Thin layer chromatography (TLC) of separated fraction F5 (sub-fractions F5.1-F5.6) of *S. rochei* (strain X34)

There were a few spots (circled) observed under UV light besides visible blue to greenish spots appeared after molybdo (VI) phosphoric acid was sprayed and heated (Figure 4.18). The TLC plate was developed in hexane: ethyl acetate (2:1) solvent system. All these sub-fractions were tested again for antifungal activity against *S. pombe* to confirm which compounds are responsible for the antifungal property.

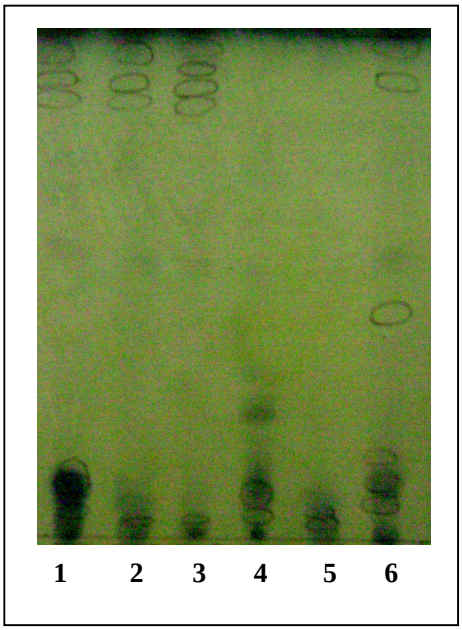


Figure 4.18: TLC plate of six sub-fractions of fraction 5 (F5.1-F5.6) of *S. rochei* (strain X34) after preparative thin layer chromatography (PTLC). The plate was developed in hexane: ethyl acetate (2:1). 1; sub-fraction F5.1, 2; sub-fraction F5.2, 3; sub-fraction F5.3, 4; sub-fraction F5.4, 5; sub-fraction F5.5, 6; sub-fraction F5.6.

4.7.4 ^{1}H – NMR of six sub-fractions of active fraction (F5) of *S. rochei* (strain X34)

^{1}H-NMR was done for all sub-fractions of F5 (F5.1-F5.6) obtained after PTLC. The chromatograms of the sub-fractions are shown in Appendix L. The sub-fraction F5.1 indicated some interesting compounds that may contribute to the antifungal property against *S. pombe.*

4.7.5 Preparative thin layer chromatography (PTLC) bioassay guided

Due to limited *S. rochei* sub-fractions of the PTLC yield obtained, the next antifungal bioassay was done by PTLC bioassay guided technique to minimize the usage of the fractions. Sub-fractions F5.1 and F5.4 (yellow circled dashes) exhibited zone of antifungal activity against *S. pombe* (Figure 4.19 (a)), which were compared to the spots of compounds (red circled dashes) in control plate (Figure 4.19 (b)). Both the active sub-fractions F5.1 and F5.4 were subjected to NMR for more information of the compounds structure.

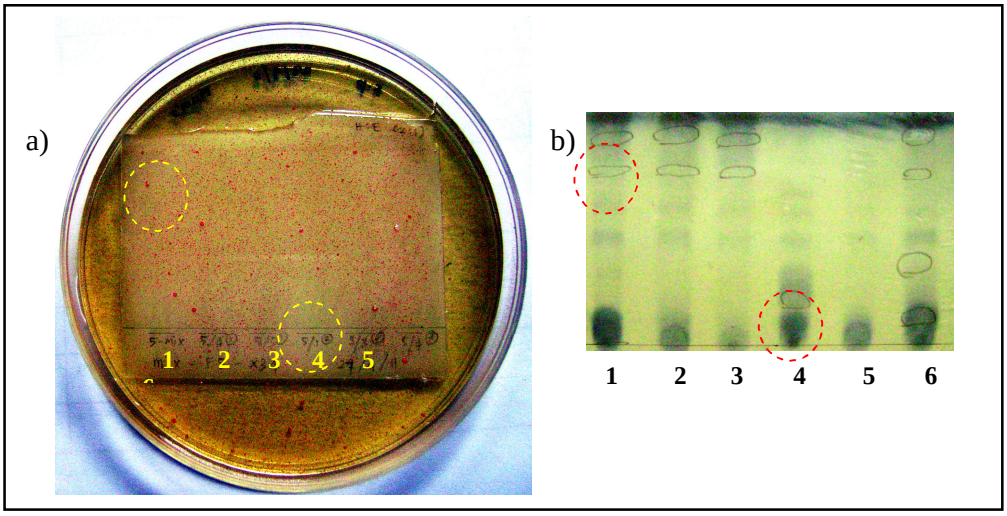


Figure 4.19: a) PTLC bioassay guided plate of fraction 5 (F5.1-F5.6) after PTLC. The plate was developed in solvent system of hexane: ethyl acetate (2:1) before tested in YPG agar seeded with *S. pombe*, and incubated for three days at 30˚C. b) Plate of reference for PTLC bioassay. This plate was developed in the same solvent system with the tested one. 1: Sub-fraction F5.1, 2: Sub-fraction F5.2, 3: Sub-fraction F5.3, 4: Sub-fraction F5.4, 5: Sub-fraction F5.5 and 6: Sub-fraction F5.6. Circle dashes in control plate (b) are the spots of compounds which may responsible of the inhibition zone in plate (a).

4.7.6 Structure identification of active sub-fraction

At this level, only sub-fraction F5.1 was subjected to all NMR procedures as the ^{1}H-NMR and ^{13}C-NMR of sub-fraction F5.4 were not good (Appendix M). NMR chromatograms of active sub-fraction F5.1 are shown in Appendix O. Fourier

transformer infrared resonance (FTIR) result of active sub-fraction F5.1 is shown in Figure 4.20.

Compound 1 was isolated as oil with an [a]D value of +23.7. A total of seven carbon atoms were observed upon ^{13}C-NMR (Figure O-2 in Appendix O). Based on DEPT experiment, it was clear that three were methyl carbons (d: 14.4, 18.4 and 20.5; C_6D_6), three oxymethine carbons (d: 67.3, 69.6, and 75.9) and one carbonyl carbon (d:175.7). All the methyl's were secondary methyl's and were seen as doublets. The remaining three protons were present as oxymethines attached to the above-mentioned oxycarbons. Partial structures and the connectivity are shown in Figure 4.21 and Figure 4.22.

Based on the partial structures and HMBC connections, relative structure of Compound 1 was deduced as 2-(3-hydroxybutan-2-yloxy) propanoic acid (Figure 4.23). This analysis indicates a suggested empirical formula of $C_7H_{14}O_4$. Table 5.10 gives the chemical shifts of the carbons and protons of Compound 1. Presence of hydroxyl functionality was further confirmed by FTIR analysis as shown in Figure 4.20, with the presence of absorbance at 3390.77 cm^{-1} and presence of carbonyl functionality at 1731.50 cm^{-1}.

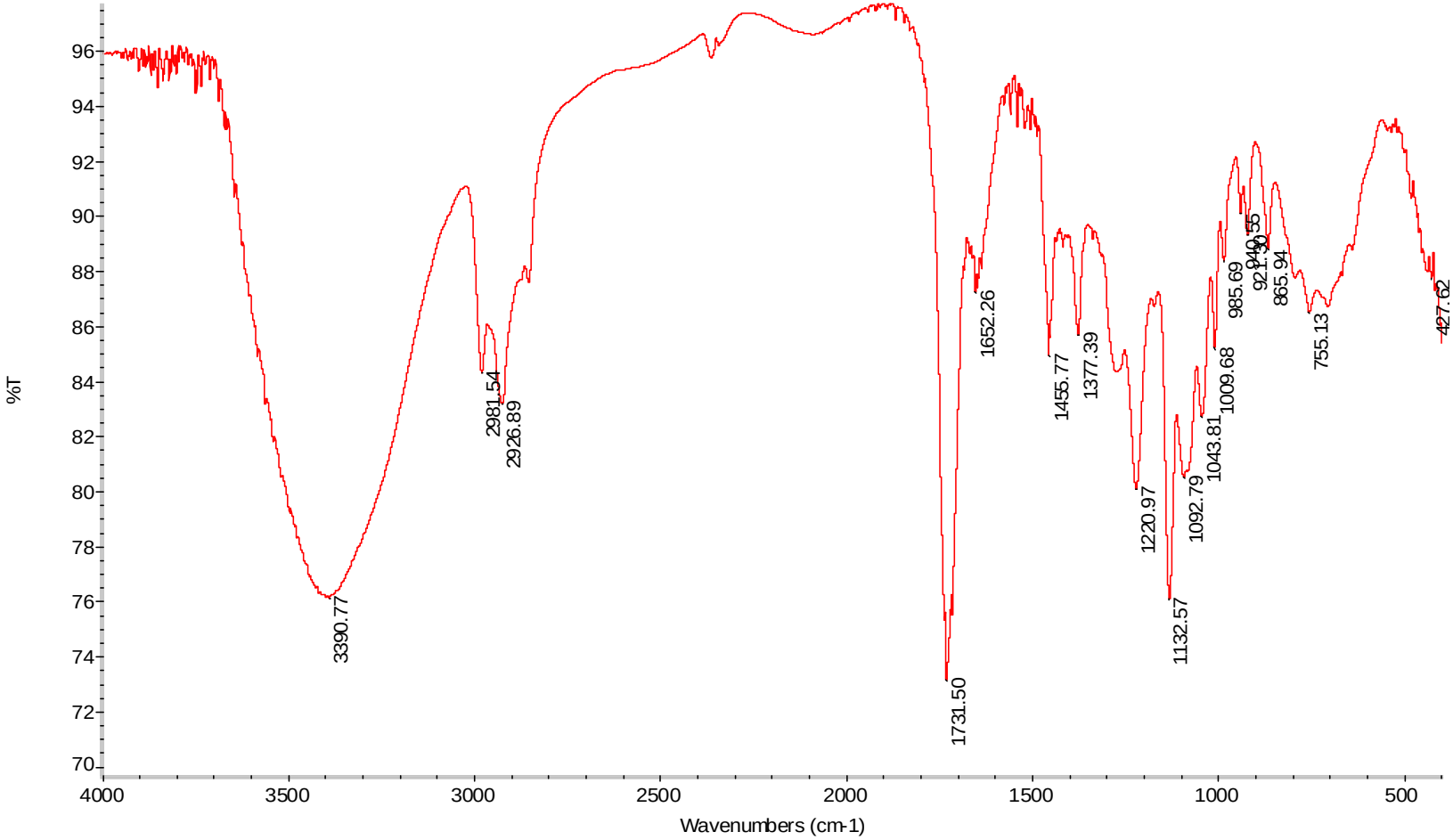


Figure 4.20: FTIR spectrum of active sub-fraction F5.1

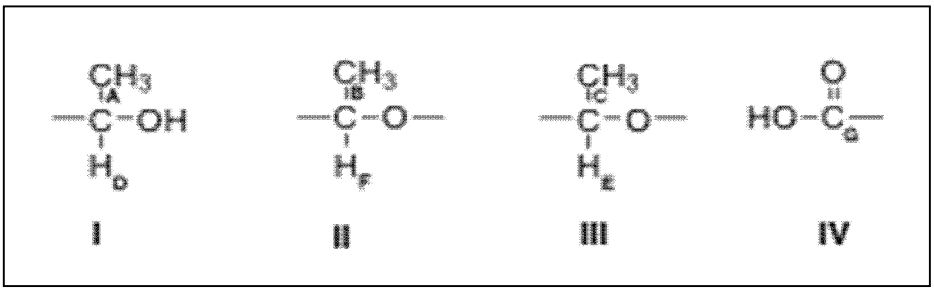


Figure 4.21: Partial structures of Compound 1

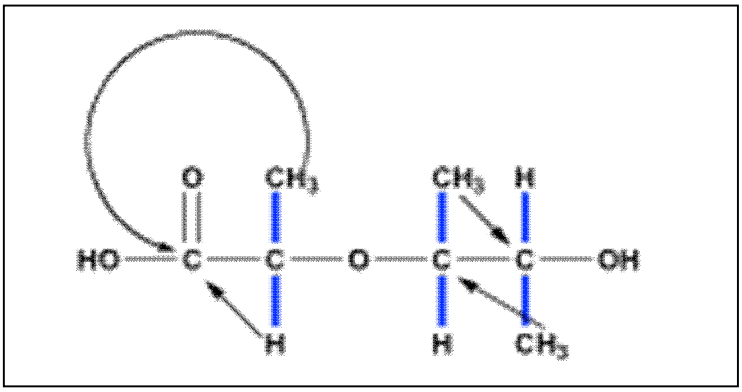


Figure 4.22: Connectivity of partial structures of Compound 1

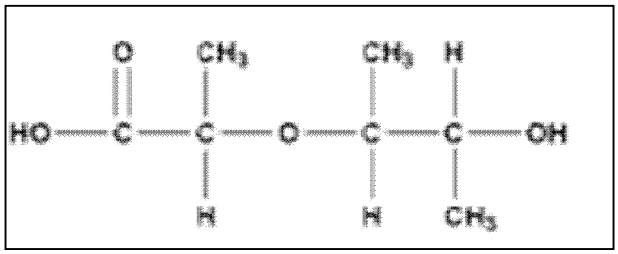


Figure 4.23: Structure of Compound 1, 2-(3-hydroxybutan-2-yloxy) propanoic acid, $C_7H_{14}O_4$.

Table 4.14: Chemical shifts of C and H in Compound 1 taken in deuterated benzene (C_6D_6).

No.	^{13}C d	^{1}H d	Multiplicity (Hz)
1.	20.8	1.25	d, 6.87 Hz
2.	67.3	4.08	m
3.	175.7	-	
4.	69.6	3.46	m
5.	75.9	4.76	m
6.	18.4	0.84	d, 6.87 Hz
7.	14.4	0.96	d, 6.19 Hz