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Full Length Research Paper

Biodeterioration of binding media in tempera paintings by *Streptomyces* isolated from some ancient Egyptian paintings

Akmal A. Sakr[1,3], M. F. Ghaly[1], M. F. Ali[2] and M. E. F. Abdel-Haliem[1]*

[1]Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt.
[2]Conservation Department, Faculty of Archaeology, Cairo University, Cairo, Egypt.
[3]Conservation Department, Civilization Museum, Cairo, Egypt.

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Eight out of 46 *Streptomyces* isolates representing highest deterioration symptoms of disfiguration and scaling were isolated from mural paintings within tombs at Tell Basta and Tanis, Lower Egypt. These isolates were identified using traditional and 16S rDNA sequencing methods as *Streptomyces albidofuscus*, *Streptomyces ambofaciens*, *Streptomyces canarius*, *Streptomyces chibaensis*, *Streptomyces coelicolor*, *Streptomyces corchorusii*, *Streptomyces nigrifaciens* and *Streptomyces parvullus*. These identified *Streptomyces* isolates decomposed animal glue into amino acids with a high percentage of L-glutamic acid and ammonia as end product, depolymerized arabic gum into free mono-sugars, decomposed egg yolk into amino acids with low percentage of L-glutamic acid and ammonia and decomposed bees wax enzymatically into short chains of stearic acid. Finally, these isolates caused flaking of paintings layers due to penetration of filamentous mycelium within painting layers with the assistance of enzymatic hydrolysis.

Key words: Animal glue, arabic gum, bees wax, biodeterioration, egg yolk, *Streptomyces*, Tell Basta, Tanis, Egyptian tombs.

INTRODUCTION

Paintings in ancient Egyptian tombs were carried out using tempera technique, by mixing earthy pigments such as hematite, limonite, azurite blue, lamp black and green malachite or synhesis pigments such as Egyptian blue with different binding media such as arabic gum, animal glue, egg yolk and bees wax (Newman and Serpico, 2000).

The ability of *Streptomyces* to deteriorate mural paintings was attriubted to utilizing a wide range of carbon sources, possessing different adaptation methods against adverse environmental conditions such as biocides and heavy metals (McCarthy and Williams, 1992) and producing a wide range of specific enzymes that could decompose binding media, so every medium has its own deterioration mechanism and products (Salvadori et al., 2003).

Animal glue is composed mainly of amino acids and is exposed to biodeterioration by collagenase enzyme produced by *Streptomyces* that causes cross linking of amid bonds in collagen of animal glue into amino acids (Chadefaux et al., 2009), so these paintings lose its cohesiveness detected from plaster layers and stone support (Goshev et al., 2005). Biodeterioration of animal glue in mural paintings was seen, whereas *Streptomyces* colonized fresco paintings in Cathedral of Nativity of the Virgin in the Pafnutii-Borovski monastery, Russia, decomposed animal glue used as binding medium and restoration material (Karpovich-Tate and Rebrikova, 1991), paintings of Boyana church, Bulgaria, also *Streptomyces griseus*, *Streptomyces albus*, *Streptomyces flavus* and *Streptomyces fradiae* caused flaking and powdering of these paintings (Hadjivulcheva and Gesheva,1982) and gesso layers on Virgin Mary wooden statue in Church of

*Corresponding author. E-mail: farrag1999@yahoo.com. Tel: +2001114598085. Fax: +2552308213.

Gora, Croatia, and *Streptomyces* sp. vigorously attacked animal glue in these layers (Briški et al., 2001).

Furthermore, arabic gum was the most common binding medium in ancient Egyptian paintings. This gum is a complex one and composed mainly of arabinogalactan oligosaccharides, and polysaccherides (Onyari et al., 2008). These polysaccherides are subjected to enzymatic depolymerization by *Streptomyces* into monmeric units of free mono sugars that modified red color estimated spectrophotometrically at 420 nm (Colombini et al., 2002).

The third binding medium was egg yolk that was used in many ancient Egypt paintings, or as a varnish layer to protect the paintings from adverse environmental conditions (Derric et al., 1999). Egg yolk contains 20 to 40% lipid, two thirds of these lipids from triglycerides that composed mainly of phosphatidylcholines lecithin (Odlyha et al., 2000), whereas lethcianease enzyme could decompose phospholipids into ammonia as end product (Arthur and Henerici, 1970).

Bees wax was used as protective layer and latter was used as binding medium. Bees wax subjected to biodeterioration by *Streptomyces* that could decompose hydrocarbons by producing biosurfactants that emulsify hydrophobic compounds and cause ring fission and carbon assimilation (Roper, 2004) or by producing esterase enzyme, since *Geodermatophilus* isolated from limestone blocks from the pyramid of the King Chevern and tombs of workers at Deir el Madeina, Upper Egypt, produced esterase enzyme (Gtari et al., 2012).

Finally, binding media were subjected to biomechanical deterioration by *Streptomyces* that could penetrate its hyphae within painting layers and cause dislodging of various layers, so these painting layers were detached from the plaster layers and stone supports (Altenburger et al., 1996).

Due to the lack of literature about the role of *Streptomyces* in biodeterioration of binding media in ancient Egyptian paintings and deterioration mechanisms and products, and since it was suggested that different binding media are susceptible to deterioration by *Streptomyces*, this study was conducted.

Streptomyces isolated from mural paintings were identified morphologically, biochemically and using 16S rDNA sequence method. Biodeterioration of binding media used in ancient Egyptian mural paintings such as animal glue, arabic gum, egg yolk and bees wax by *Streptomyces* were completely analyzed.

MATERIALS AND METHODS

Isolation locations

Forty six (46) *Streptomyces* isolates were collected from deterioration symptoms of scaling and disfiguration from mural paintings in tombs of Ankh m b3st, Ist, Ankh h.f and Ihi at Tell Basta and tomb of The King Oserkon II at Tanis (Figure 1), Lower Egypt, using sterile cotton swab method. These bacterial isolates were isolated and purified onto starch-nitrate-agar (SNA) plates (agar 20; starch 20; KH_2PO_4 1; $MgSO_4$ 0.5; NaCl 0.5; KNO_3 2 and $CaCO_3$ 3 g / l distilled water (Kuster and Williams, 1964) supplemented with antifungal (Dermatine 10 to 50 µg / l) to inhibit the growth of competitive fungi. The plates were incubated for 45 days at 30°C.

Identification of bacterial isolates

The most biodeterioration active bacterial isolates (eight isolates) were identified morphologically and biochemically according to the identification keys presented by Kämpfer (2006) and confirmed using 16S rDNA sequence method.

16S rDNA sequencing

Total DNA was extracted from eight isolated colonies of actinobacteria according to the method presented by Sambrook and Russel (2001). The gene coding for 16S rRNA was amplified from each isolate by polymerase chain reaction (PCR) with universal primers (forward primer [F27] 5'-AGAGTTTGATCCTGGCTCAG-3' (Chénbey et al., 2000) and reverse primer [R1492] 5'-GGTTACCTTGTTACGACTT-3') (Turner et al., 1999). These primers bind to universally conserved regions and permit the amplification of an approximately 1500 bp fragment. The PCR amplification was carried out in a Gene-Amp PCR system 9600 thermocycler (Perkin Elmer).

The amplification conditions were as follows: 94°C for 10 min and 35 cycles of denaturation at 95°C for 30 s, annealing-extension at 56°C for 1 min, 72°C for 1 min and an extension at 72°C for 10 min. Presence and yield of specific PCR products (16S rRNA gene) were monitored by running 1% agarose gels. Then, PCR product was cleaned up by using GeneJET™ PCR purification kit (Fermentas).

Amplified DNA fragments were partially sequenced at GATC Biotech AG (Konstanz, Germany) using ABI 3730xl DNA sequencer using forward primer (F27). The 16S rDNA sequences which have been determined in the present study were deposited at NCBI web server (www.ncbi.nlm.nih.gov; Table 1). Sequence analysis and comparison with published sequences was made using the Basic Local Alignment SearchTool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1997).

Deterioration of animal glue

Streptomyces isolates were cultured on starch-nitrate-agar free from carbon source whereas 2% animal glue was used as sole carbon source in 250 ml flasks (Petrova et al., 2006). Each flask contained 100 ml of broth meduim (pH was adjused to 7), inoculated with 10% spore suspension (1×10^6 spores / ml) and incubated at 30°C for two weeks. After the end of incubation period, the biomass was filtered off and the filterate was cleared by centrifugation at 3000 rpm for 15 min.

One milliliter of the filterate was analysed by high performance liquid chromatography (HPLC) amino acid analyzer LC300 Eppendorf Germany (National Research Centre, Dokky, Giza) for quantitive anaylsis of amino acids in the deteriorated samples.

Biodeterioration of arbic gum

Biodeterioration of arabic gum by *Streotpmyces* isolates was determined by estimating the functional groups in both deteriorated and control arbic samples by infra-red (IR) spectroscopy (JASCO FT-IR 61000, National Research Centre, Dokky, Giza).

Biodeterioration of egg-yolk

Biodeterioration of egg-yolk was investigated whereas *Streptomyces*

Figure 1. Location of sampling: **(a)** Azurite blue in tomb of Oserkon II with white crust, Tanis. **(b)** Red ochre, tomb Ankh m b3st. **(c)** Azurite blue, tomb of Ihi, Tell Basta. **(d)** Red and yellow paintings, tomb Ankh h3 f, Tell Basta. **(e)** Black color from tomb of Ankh h3 f, Tell Basta. **(f)** White crust of soduim chloride, tomb of Ist, Tell Basta.

isolates were cultured on liquid starch-nitrate broth (SNB) medium free carbon and nitrogen source, whereas 2% egg yolk was used as sole carbon and nitrogen source and incubated at 30°C for two weeks. Released ammonia was detected in the obtained filterate using Fourier transform infrared (FT-IR) spectroscopy (JASCO FT-IR 61000, National Research Centre, Dokky, Giza).

Table 1. Phylogenetic affiliation of inoculated strains (Homology of 16S r DNA and similarity in comparison with NCBI Data).

Location	Homology approximately	Similarity enter genes 16S r DNA (%)	Authors' accession number	G + C content (%)
Azurite blue, tomb of Ihi, Tell Basta.	*S. albidofuscus*	99	Later name is *S. pyridomyceticus* BankIt1507621 JQ625331	58
Yellow color of Southern wall of Ankh h3ftomb.	*S. ambofaciens*	99	BankIt1507642 JQ625332	60.6
Blue color, ceiling burial tomb of Oserkon II, Tanis.	*S. canaries*	99	BankIt1507650 JQ625337	58.9
Black color, tomb Ankh h.f, Tell Basta.	*S. chibaensis*	100	BankIt1507649 JQ625336	58.9
Red color, tomb Ankh m b3st, Tell Basta.	*S. coelicolor*	99	BankIt1507648 JQ625335	59.2
Limestone, tomb of Oserkon II, Tanis.	*S. corchorusii*	98	BankIt1507647 JQ625334	58.9
North wall, tomb of Ist, Tell Basta.	*S. nigrifaciens*	98	Later name is *S. flavovirens* BankIt1507149 JQ625330	~56
Yellow color of Southern wall of Ankh h3ftomb.	*S. parvullus*	99	BankIt1507645 JQ625333	56.3

Deterioration of bees wax

Degradation of bees wax by *Streptomyces* isolates was detected by inoculating *Streptomyces* isolates onto SNA plates supplemented with 2% bees wax as sole carbon source emulsified with 80 0. 1 ml/L Tween. Incubation period was two weeks at 30°C. Inoculated and non-inoculated samples were analyzed by mass spectra (JOLE, JMS-AX500 Mass spectra, National Research Centre, Dokky, Cairo).

Mechanical deterioration

The mechanical deterioration of binding media by *Streptomyces* was determined by covering glass slides with animal glue and inoculated with *Streptomyces* isolates, incubated at 30°C for two weeks then examined with scanning electron microscope (JOLE, SEM 6300, National Research Centre, Dokky, Cairo). The penetration of *Streptomyces* mycelium within the painting layers was observed according to colonizied method of Milanesi et al. (2006).

RESULTS AND DISCUSSION

Eight selected and identified isolates from different paintings (Figure 1) were attributed to genus *Streptomyces* (*Streptomyces albidofuscus*, *Streptomyces ambofaciens*, *Streptomyces canarius*, *Streptomyces chibaensis*, *Streptomyces coelicolor*, *Streptomyces corchorusii*, *Streptomyces nigrifaciens* and *Streptomyces parvullus*) with similiarity more than 98% and the authors' accession numbers in the International GenBank were illustrated in Table 1, since *Streptomyces* spp. are considered the typical and first colonizers of deteriorated cultural heritage objects (Lanoot, 2005) and involved significantly in biodeterioration of binding media due to their ability to decompose relatively complex organic substances such as cellulose, pectin, chitin and proteins (Borrego et al., 2012).

Our results indicate that ainmal glue enhanced growth and sporulation of *S. ambofaciens* more than other *Streptomyces* isolates. Furthermore, HPLC amino acid analyser data revealed that *S. ambofaciens* and *S. nigrifaciens* decomposed animal glue into amino acids, and ratios of amino acids of argnine and histidine were lower than that obtained for alanine and glycine. Also, ammonia was revealed (Figures 2 and 3), and its ratio reached 1024.76 and 1244.84 µg/ml with *S. ambofaciens* and *S. nigrifaciens,* respectively (Tables 2 and 3). On the other hand, FT-IR patterns of deteriorated animal glue revealed presence of L-glutamic acid at 1454 cm^{-1} and ammonia group at 3426 cm^{-1} (Figures 4 and 5). L-glutamic acid was the most present amino acid in the deteriorated samples, with ratio 824.32 µg / ml and 794.961 µg / ml for *S. ambofaciens* and *S. nigrifaciens,* respectively (Tables 2 and 3).

On the other hand, *Streptomyces* isolates increased pH of medium supplemented with animal glue as carbon source, whereas *S. ambofaciens* reduced pH of medium from 7 to 6.1 after two weeks, then increased final pH into 8.67, while *S. nigrifaciens* increased pH from 7 to 9 after three weeks of incubation (Table 4). This may be explained by the fact that animal glue was decomposed enzymatically by *Streptomyces* through two steps due to collagenolytic activity of isolated *Streptomyces* (Górny, 2004). The first step was decomposition of animal glue into free amino acids and the second step was decomposition of amino acids into ammonia as end product that increased pH of media, making favorable setting for the growth of *Streptomyces* and providing nutrition for other associated mciroorganisms in the ecosystems (Tomek and Pechova, 1992).

Streptomyces involved in the biodeterioration of arabic gum varied in their ability to depolymrise arabic gum,

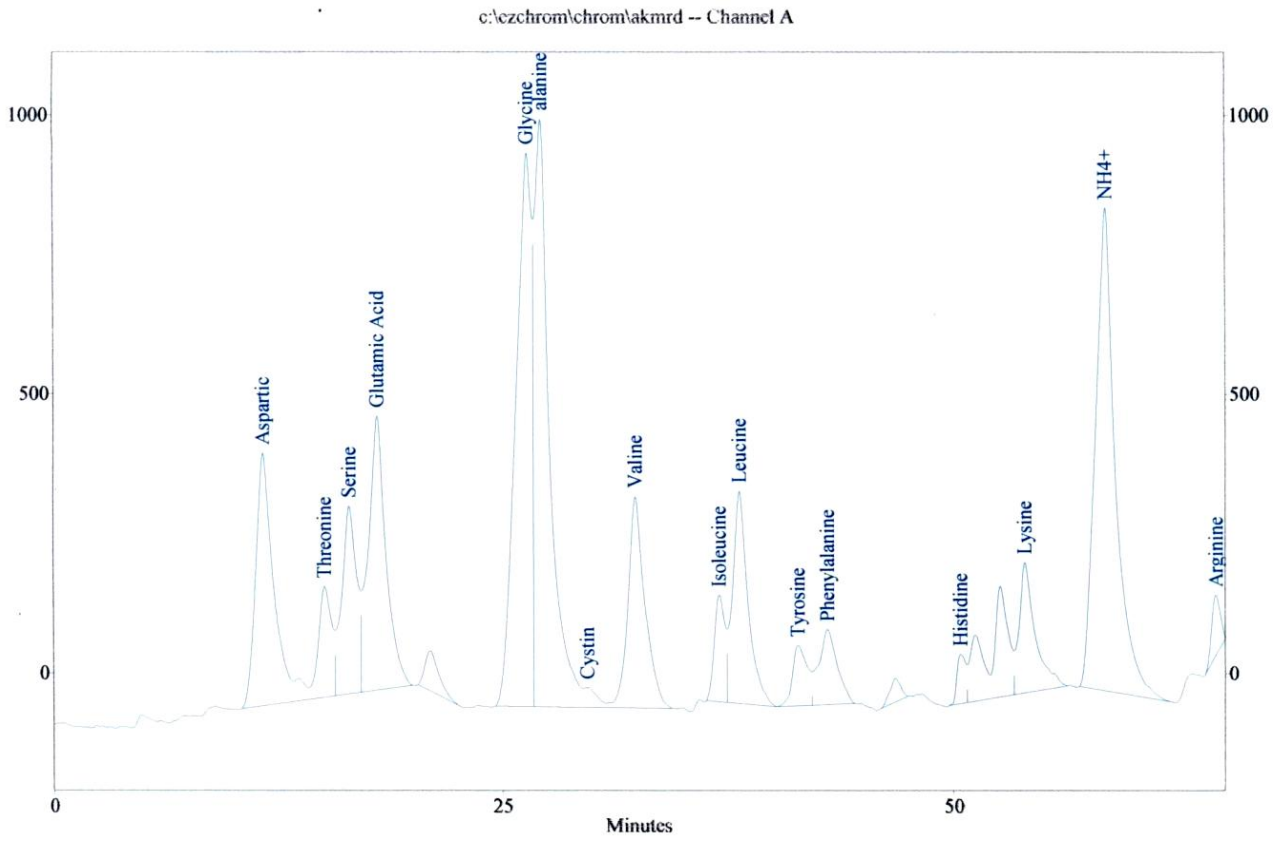

Figure 2. Quantitative analysis of amino acid from decomposition of animal glue by *S. ambofaciens*.

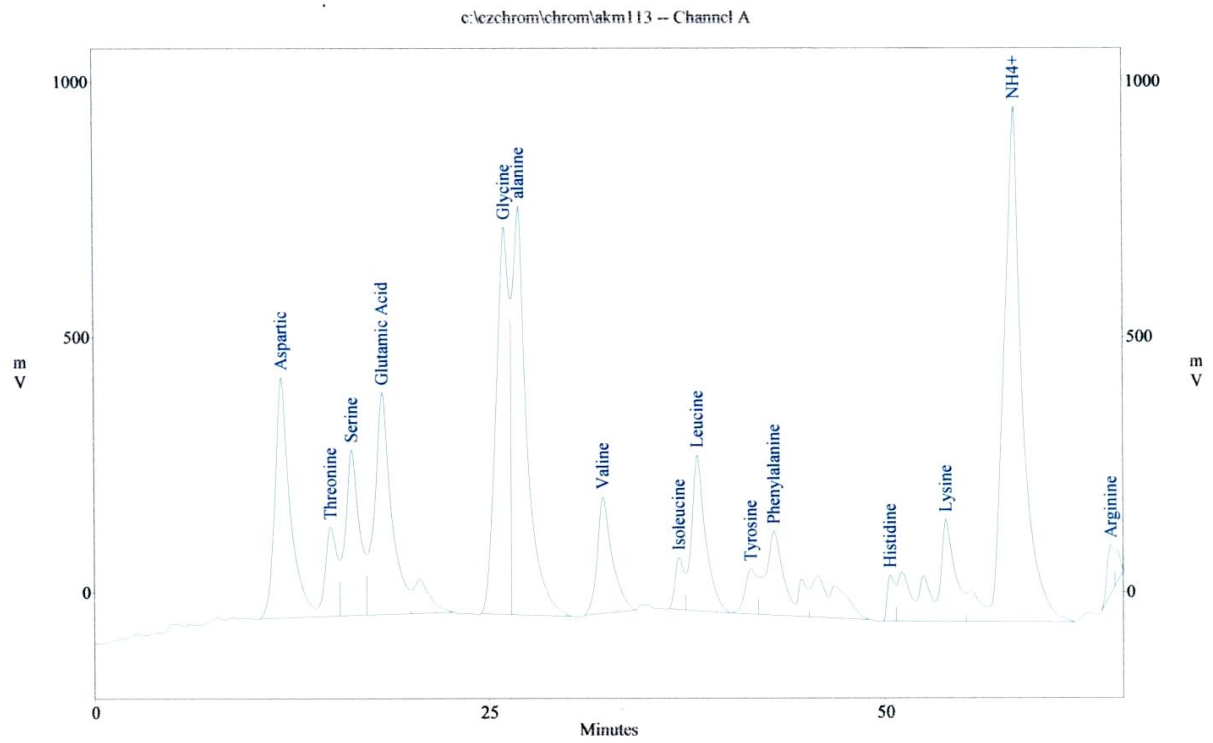

Figure 3. Quantitative analysis of amino acid from decomposition of animal glue by *S. nigrifaciens*.

Table 2. Quantitative analysis of amino acid resulting from decomposition of animal glue by *S. ambofaciens*.

Peak number	Name	Time (min)	Area	Concentration (µg/ml)
1	Aspartic	11. 63	30502852	605.28
3	Threonine	15.07	101135930	170.4
4	Serine	16.45	19306890	268.28
5	Glutamic acid	18.02	33690412	824.32
7	Glycine	26.33	60421520	400.88
8	Alanine	27.13	628234552	795.48
9	Cystin	29. 68	1764352	79.48
10	Valine	32.33	24626244	350
11	Isoleucine	36,97	7747364	168.84
12	Leucince	38.08	22831084	517.92
13	Tyrosine	41.33	6081601	202.8
14	Phenylalnine	42.98	9526860	285.88
16	Histidine	50.38	2989894	112.36
19	Lysine	53.95	13983382	356.28
21	NH4	58.40	6663752	1024.76
22	Arginine	64.47	3569638	155. 56

Table 3. Quantative analysis of amino acids resulting from decomposition of animal glue by *S. nigrifaciens*.

Peak number	Name	Time (min)	Area	Concentration (µg/ml)
1	Aspartic	11.90	313052	621.16
2	Threonine	14.98	9149939	135.8
3	Serine	16.33	20009218	278.04
4	Glutamic acid	18.27	32490380	794.961
6	Glycine	25.98	34583220	289.16
7	Alanine	26.90	51494672	625
8	Valine	32.20	14926400	212.12
9	Isoleucine	36.98	3947304	86.04
10	Leucince	38.15	18896858	428. 68
11	Tyrosine	41. 52	4588704	153.04
12	Phenylalnine	42.98	1240370	372.44
16	Histidine	50.30	2931907	110.2
19	Lysine	53.82	12272261	312.68
21	NH_4	58.13	80950976	1244.84
22	Arginine	64.20	2955407	128.8

whereas *S. ambofaciens*, *S. parvullus*, *S. nigrifaciens* and *S. canarius* had high depolymerisation ability of poly-saccherides, *S. corchorusii* had a good ability, whereas *S. albidofuscus* had a moderate one, but *S. chibaensis* and *S. coelicolor* had a low growth rate (Figure 6). Furthermore, spectrophotometric data indicated that free mono-sugars obtained from depolmerization of arabic gum enzymatically by *Streptomyces* and the produced monosugar are not obtained from decompose phenolic compounds in the arabic gum, whereas FT-IR pattern gave 3897 cm^{-1} intense band characterizing for phenolic groups in both standard and deteriorated arabic gum samples (Figures 7 and 8). These results are similar to that of Onyari et al. (2008). Phenolic compounds in the arabic gum could not be decomposed by *Streptomyces* isolates, whereas FT-IR pattern gave an intense band at 3897 cm^{-1} characterized for phenolic groups (Derric et al., 1999), that appeared in both standard and deteriorated samples, since the presence of phenol groups in both deteriorated and the standard samples was attributed to toxicity of these groups for *Streptomyces* isolates (Derric et al., 1999).

Our finding indicates that growth rate of *Streptomyces* cultured onto charcoal with arabic gum as binding meduim was increased, this may be due to adsorbing phenolic compounds from arabic gum by charcoal and

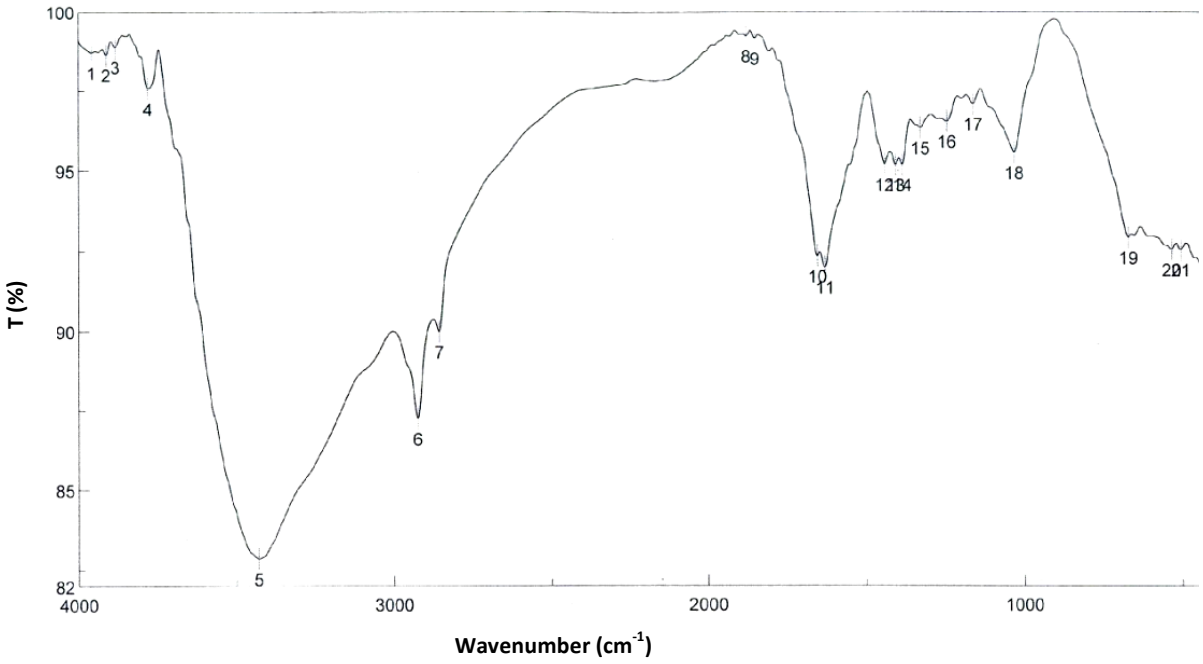

Figure 4. IR spectrum for standard animal glue.

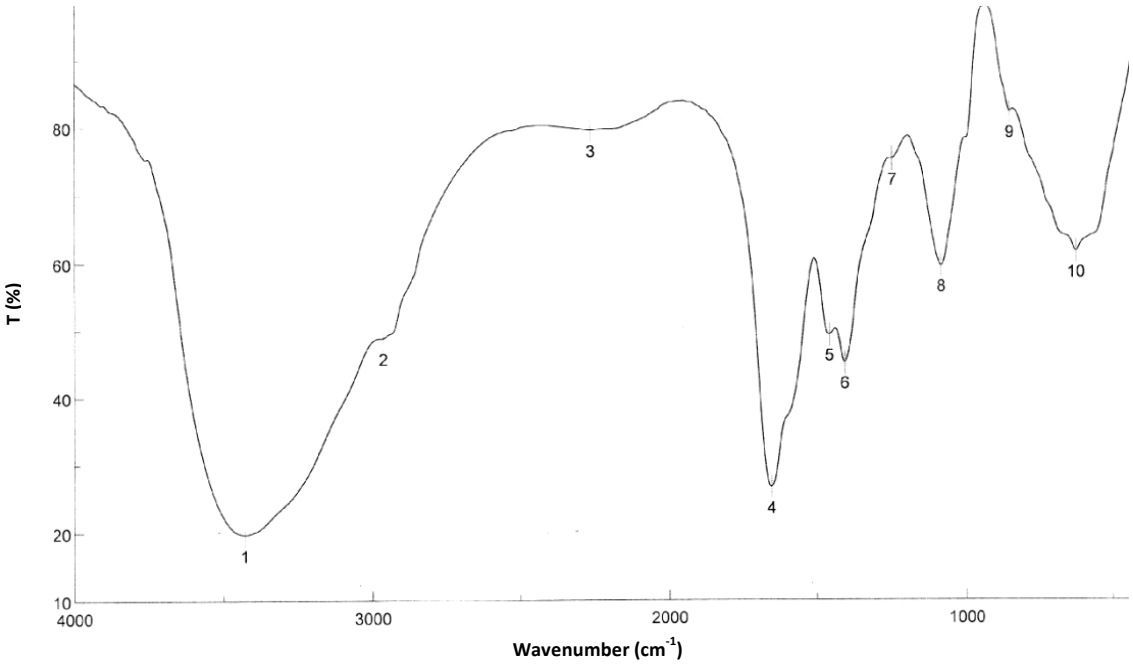

Figure 5. IR spectrum for deteriorated animal glue.

prevented them from access to *Streptomyces* mycelium (Normand, 2006).

Furthermore, FT-IR patterns of deteriorated egg yolk samples gave an intense band at 1500 cm^{-1} of (C-N-H) group orginating from amino acids and the band which appeared at 3426 cm^{-1} was characterized for ammonia group (NH_3), whereas egg yolk was decomposed by leucinase enzyme produced by *Streptomyces* isolates into amino acids and ammonia as end product (Peris-Vicente et al., 2006).

Table 4. Final pH after 2 to 3 weeks of incubating isolates *S. ambofaciens* and *S. albidofuscus* on starch-nitrate broth medium where animal glue was used as carbon and nitrogen source.

Isolate	Initial pH	Final pH after different periods	
		2 weeks	3 weeks
S. albidofuscus	7.0	8.1	8.1
S. nigrifacien	7.0	8.7	9
S. ambofaciens	7.0	6.1	8.67

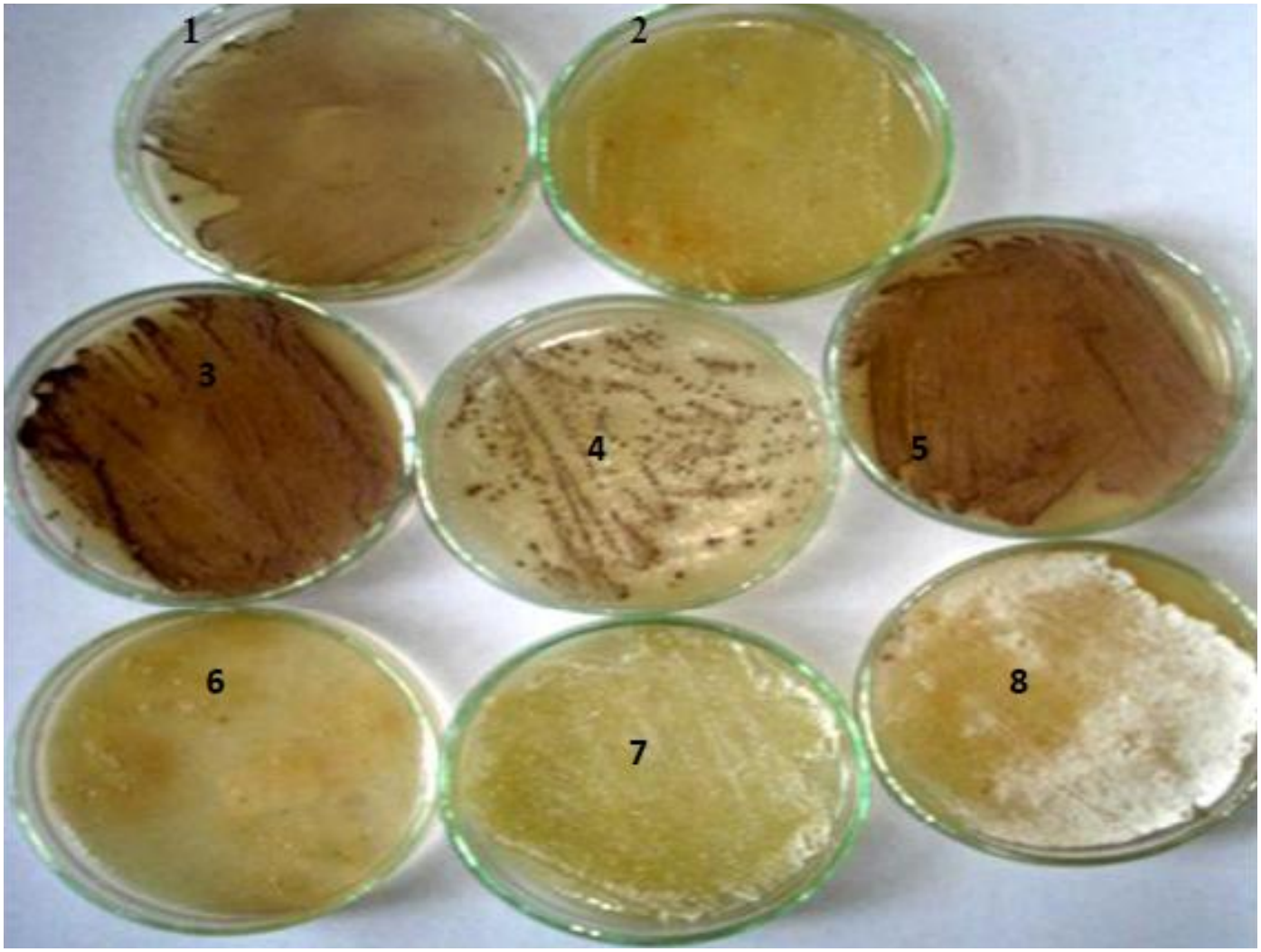

Figure 6. Growth of isolated *Streptomyces* on arabic gum as carbon source in starch-nitrate-agar media. **(1)** *S. corchorusii*. **(2)** *S. coelicolor*. **(3)** *S. parvullus*. **(4)** *S. ambofaciens*. **(5)** *S. nigrifaciens*. **(6)** *S. chibaensis*. **(7)** *S. albidofuscus*. **(8)** *S. canarius.*

Table 5 shows that *Streptomyces* isolates varied in their biodegradability of bees wax, whereas *S. ambofaciens*, *S. nigrifaciens*, *S. coelicolor* had high growth rate, whereas *S. corchorusii*, *S. chibaensis*, *S. albidofuscus* had middle growth rate and *S. canarius* had low rate (Figure 9). These isolates decomposed bees wax, where *S. corchorusii* and *S. ambofaciens* decomposed long chains of bees wax into short chains ($C_{18}H_{36}$) (Figure 10). These results are confirmed by the previous results of Regert et al. (2005). Massspectra data indicated that bees wax was decomposed by *Streptomyces* into short chains of hydrocarbon of stearic acid, since it was found that the number of carbon atoms gave the characteristic number of stearic acid (C_{18}:n) the main component of bees wax (Regert et al.,

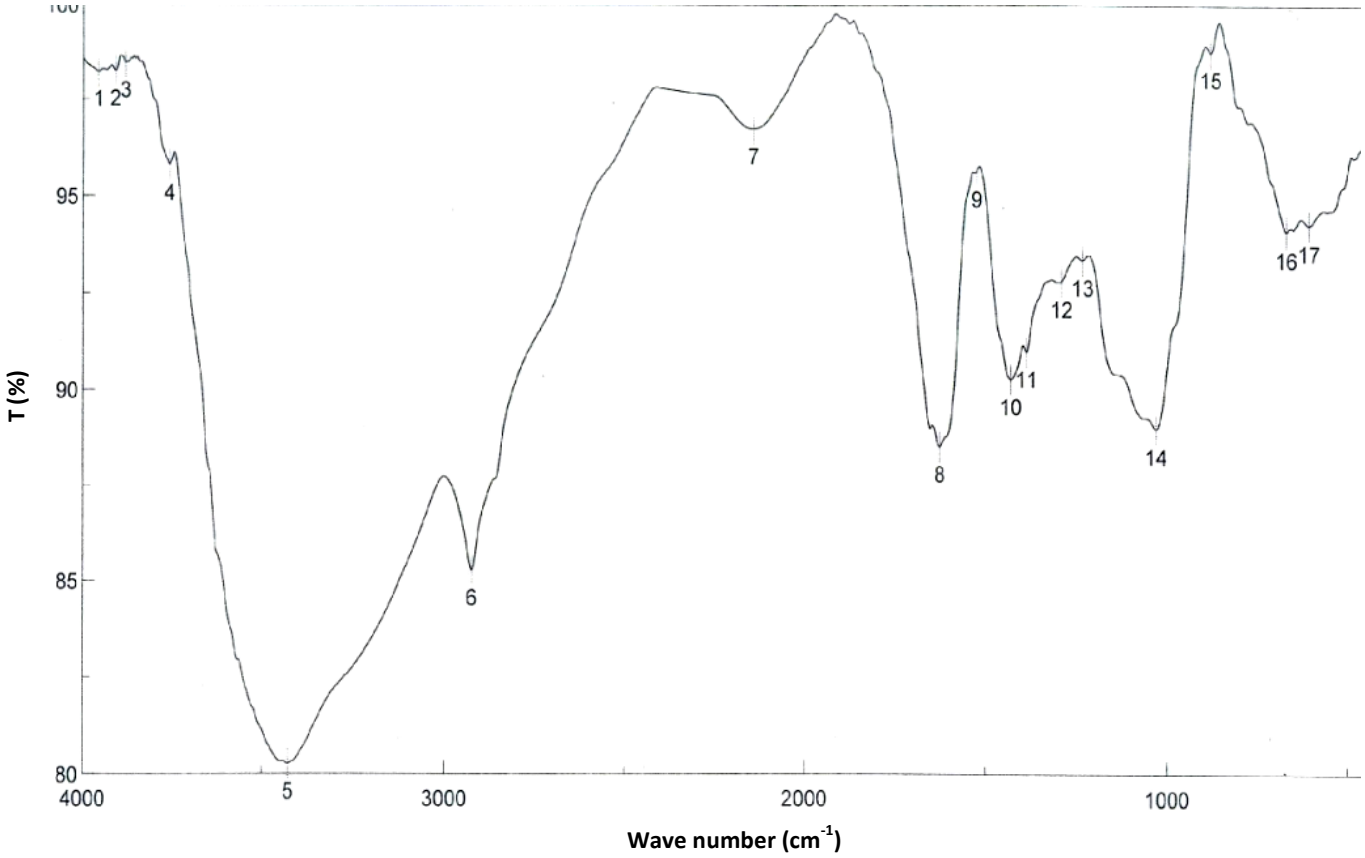

Figure 7. IR spectra of standard arabic gum.

Table 5. Growth of *Streptomyces* strains on bees wax.

***Streptomyces* isolate**	**Growth rate**
S. albidofuscus	++
S. ambofaciens	+++
S. canarius	++
S. chibaensis	++
S. coelicolor	+++
S. corchorusii	++
S. nigrifaciens	+++
S. parvullus	+++

+++, Good growth; ++, moderate growth.

2001). Decompostion of bees wax resulted in enzymatic action of esterase enzyme, whereas *Geodermatophilus* isolated from paintings in workers' tombs, Luxor, Upper Egypt, could decompose bees wax into short chains of stearic acid due to production of esterase enzyme (Essoussi et al., 2010), or this decomposition may have occurred as a result of biosurfactants produced by *Streptomyces* that emulsify hydrophobic compounds and cause ring fission and carbon assimilation (Pizzul et al., 2006). Due to decomposition of bees wax by *Streptomyces*, varnish layers lost its hydrophobicity and plasticity because of decrease of hydrocarbons in the ancient bees wax (Roper, 2004).

Finally, SEM micrographs of inoculated and non inoculated glass slides covered with film of animal glue revealed that *S. ambofaciens* made holes in this film (Figures 11a and b). On the other hand, mycelium of *S. coelicolor* penetrated within painting layers of red hematite and azuirte blue mixed with arabic gum as binding meduim, whereas the spores connected with mycelium were observed (Figure 11c) and caused flaking of these layers deteched from plaster and stone support (Pepe et al., 2009).

Moreover, mycelium of *S. coelicolor* and *S. canarius* penetrated into painting layers of red hematite and azuircte blue, since the biodeteriorative role of *Streptomyces* includes both chemical and mechanical destruction due to swelling and shrinking of mycelium within painting layers (Altenburger et al., 1996).

Conclusions

Streptomyces colonizing mural paintings and deteriorating

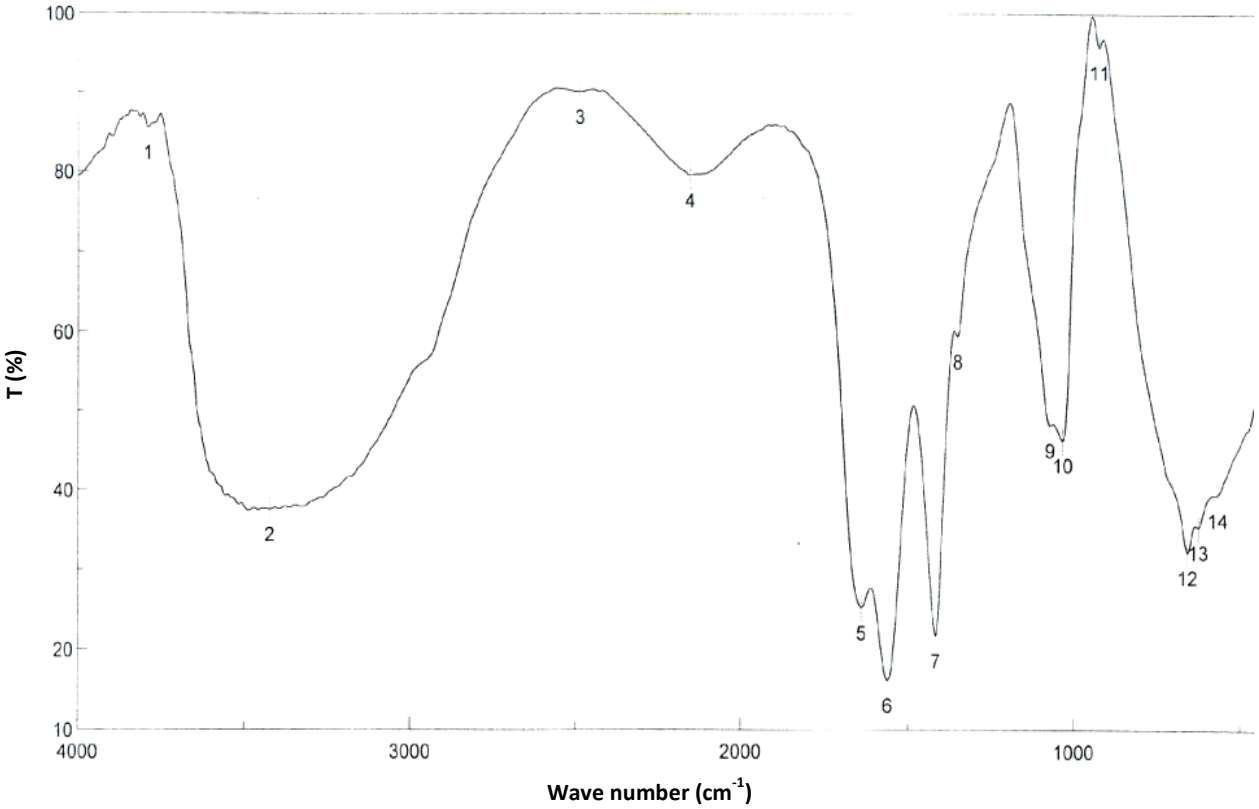

Figure 8. IR spectra of deteriorated arabic gum.

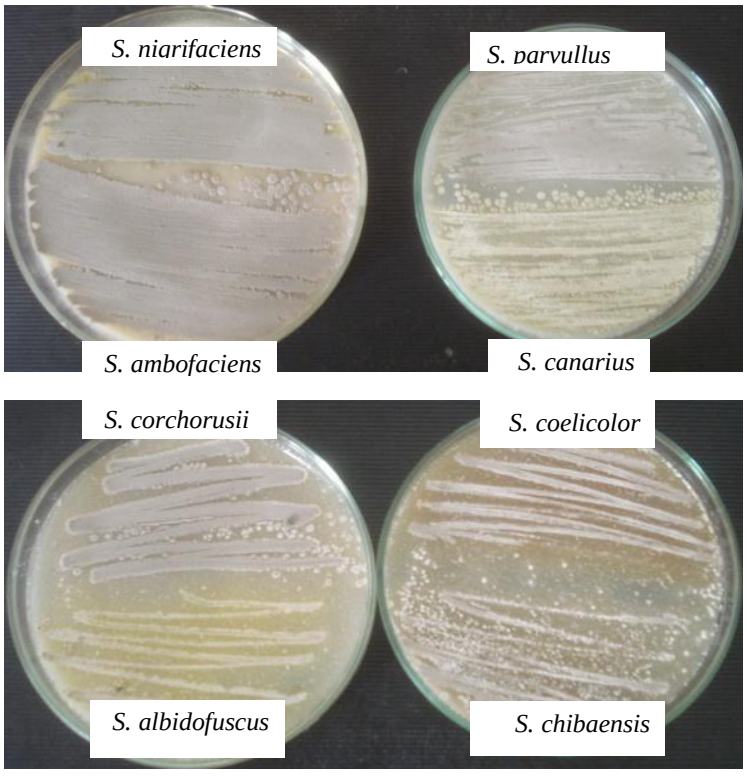

Figure 9. Effect of *Streptomyces* isolates on bees wax.

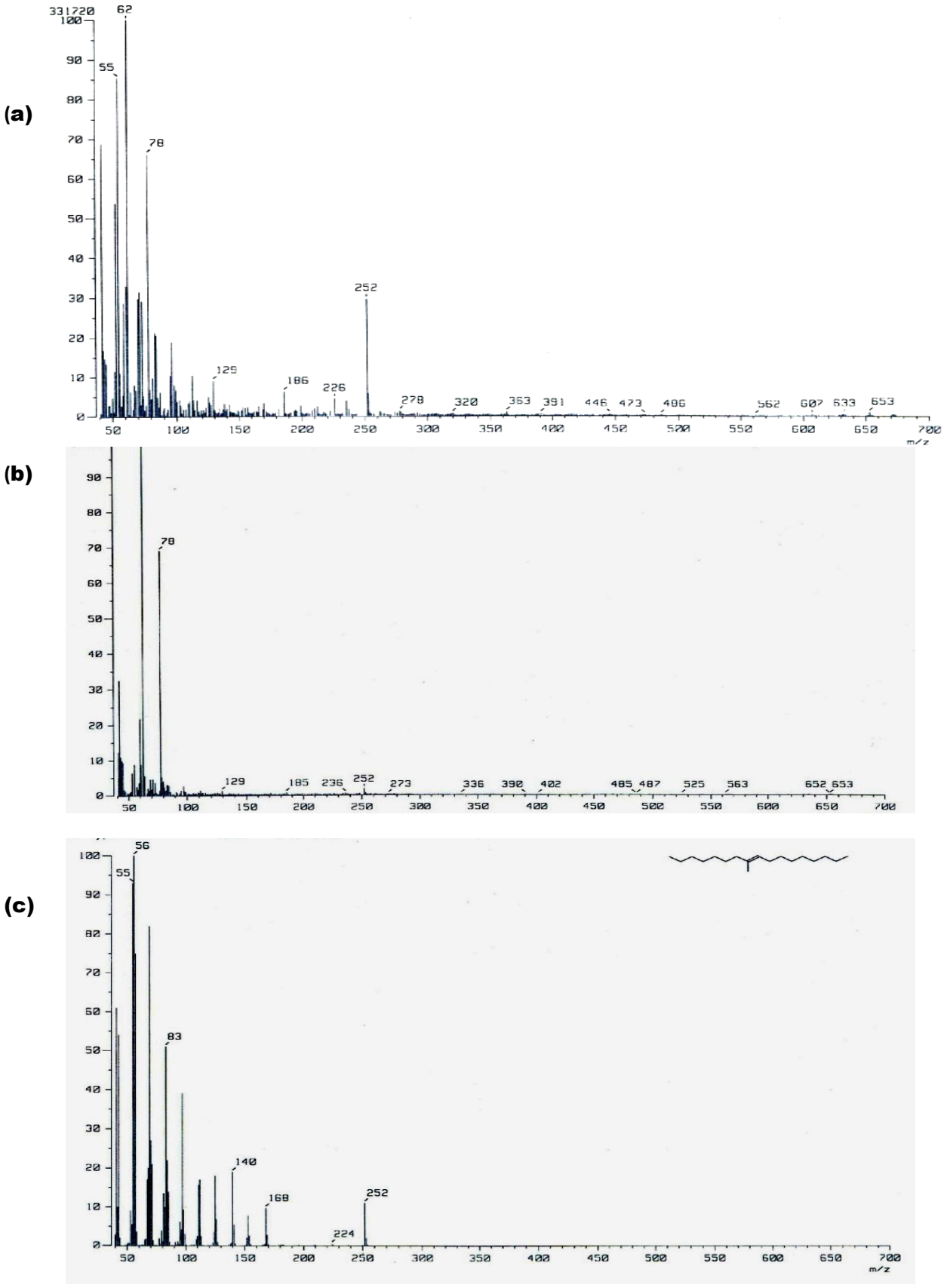

Figure 10. Mass spectra of bees wax inoculated: (a) control, (b) *Streptomyces ambofaciens*, (c) *Streptomyces nigrifaciens*.

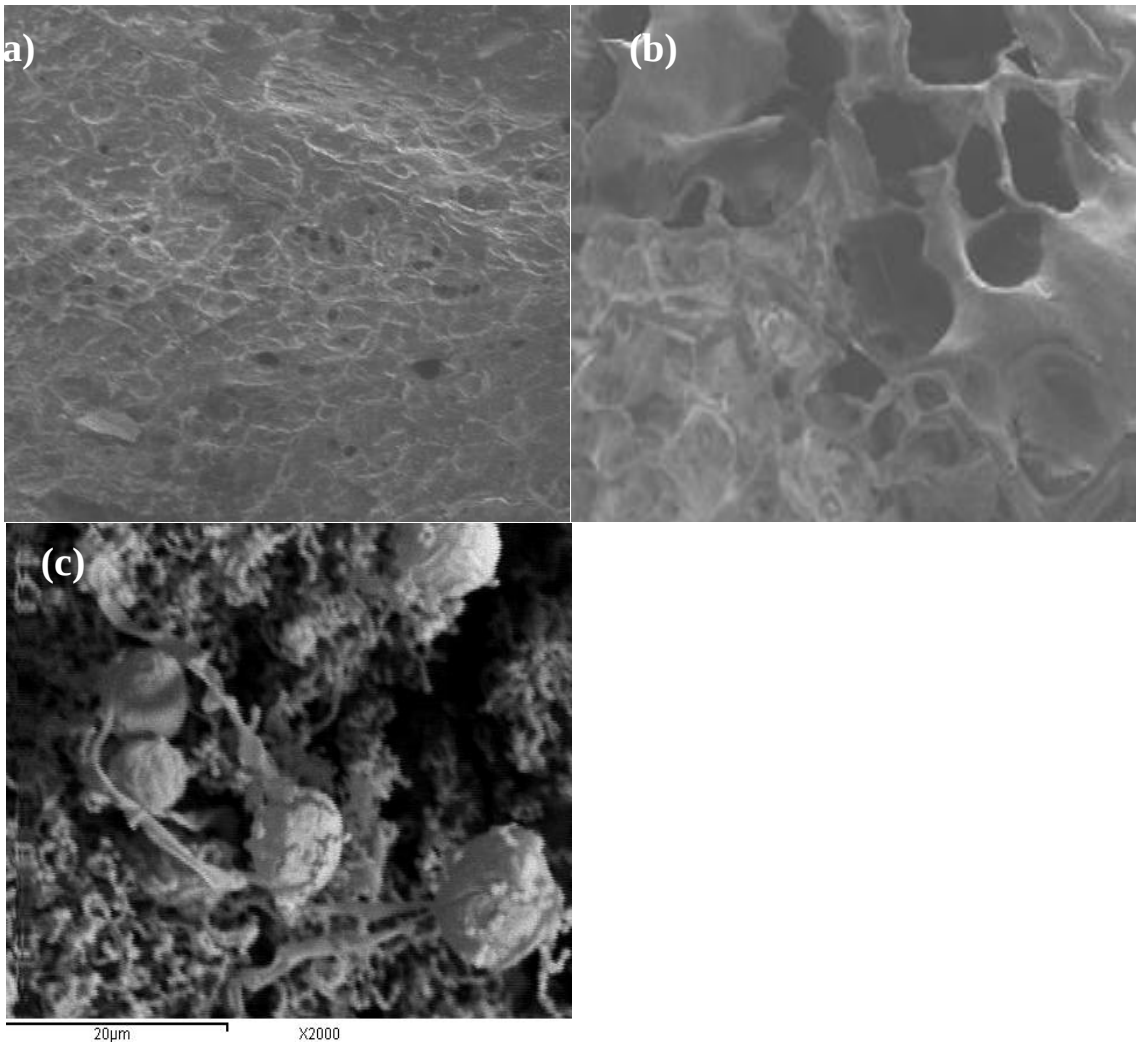

Figure 11. SEM micrograph of animal glue; (a) control, (b) inoculated by *S. ambofaciens*, 1200x, (c) pentration of hyphae of *S. coelicolor* within binding meduim of arabic gum causing flaking of painting layers, 2000x.

binding media were characterized. Scientific analyses bring new information to the light on mechanisms of deterioration of binding media by *Streptomyces* and the end products of their biodeterioration.

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