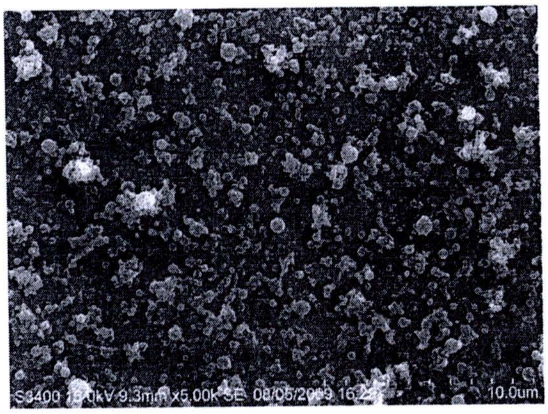
CHAPTER IV

RESULTS AND DISCUSSION

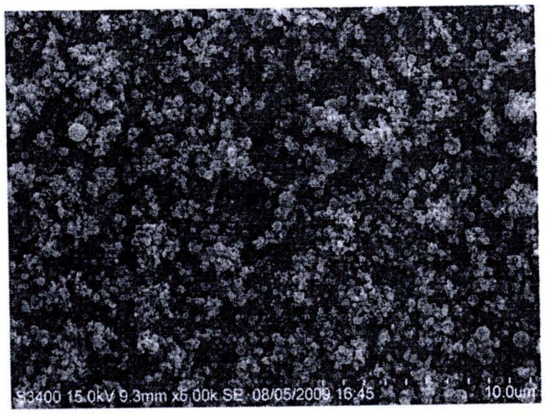
This chapter is divided into 5 parts: 1) electrospinning of pure chitosan, 2) the effects of chitosan hydrolysis and PVA addition on electrospinability, 3) morphology of chitosan nanofibers fabricated by electrospinning technique, 4) bacterial cell attachment on chitosan nanofibers, 5) viability of bacterial on chitosan nanofibers.

4.1 Electrospinning of Pure Chitosan

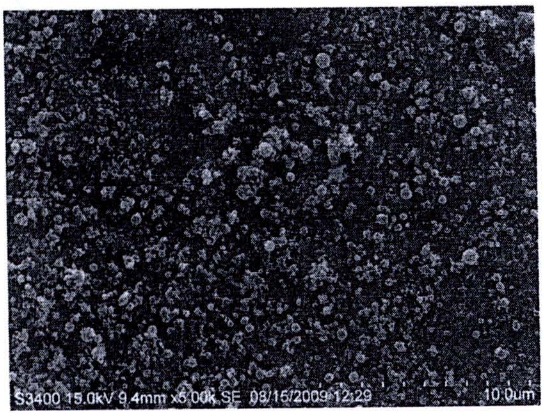
For the electrospinning of pure chitosan, usings of chitosan solution with the concentration of 2%, 1.5% and 1% for the chitosan molecular weight of 100, 400 and 760 kDa, respectively, the results are shown in Figure 4.1. It should be noted that the concentration of chitosan in the solution investigated was the highest concentration that allowed to be electrospun. The solutions with the concentration higher than these values were too viscous to be spinnable. The SEM images in Figure 4.1 reveal that only sprayed droplets were obtained in all conditions. No fiber was found. Generally, the formation of droplets or fibers is controlled by viscosity of the solution [11, 12]. However, for chitosan, which is a cationic polysaccharide with amino groups at the C2 position, the repulsive interaction among the polycations on the chitosan chains has been thought to prevent sufficient chain entanglement requires for the formation of fibers via electrospinning [13]. It was presumed that the jet of chitosan solution was stable for a short period after being ejected from the tip of the needle. After that, the jet broke up, while the solvent evaporation took place, resulting in the formation of particles. All attempts to produce pure chitosan nanofibers from the raw chitosan failed. Therefore, other approachs, i.e., the use of hydrolyzed chitosan or the use of PVA as the spinning aid, were further investigated.



(a)



(b)



(c)

Figure 4.1 SEM micrographs of products from the electrospinning of pure chitosan solution prepared by using chitosan with molecular weight of 100 (a), 400 (b) and 760 kDa (c), respectively.

4.2 The Effect of Chitosan Hydrolysis and PVA Addition on Electrospinability

The processing parameters, as well as solution parameters play important role in the formation of the fibers by electrospinning. In relative order of their impact on the electrospinning process, viscosity and conductivity of the solution are considered to be important factors. Very different results can be obtained using the same kind of the solution and the same electrospinning set up if the viscosity and conductivity of the solution are changed. Therefore, the effects of chitosan hydrolysis and PVA addition, on viscosity and conductivity of the solution were firstly investigated. The discussion of each effect is provided in the following subsections.

4.2.1 Viscosity of hydrolyzed chitosan and chitosan/PVA solutions

In preliminary experiments, solutions of pure chitosan with molecular weight of 100, 400 and 760 kDa was found to have viscosity of 65, 164.27 and 210.73 cP, respectively. As mentioned in the previous section, these solutions could be not electrospun into fibers. Only collections of spherical beads were found on the collector. After being hydrolyzed, chitosan could form a solution with increased viscosity, as shown in Figure 4.2. The viscosity of the solutions of chitosan hydrolyzed for 6 to 48 h were increased to 229.63 and 1951.10 cP, respectively. However, for the chitosan being hydrolyzed for 6 and 12 h, the electric force could not initiate the formation of fibers, in the similar manner as that for pure chitosan. This might also be the result of low viscosity as seen in Figure 4.2.

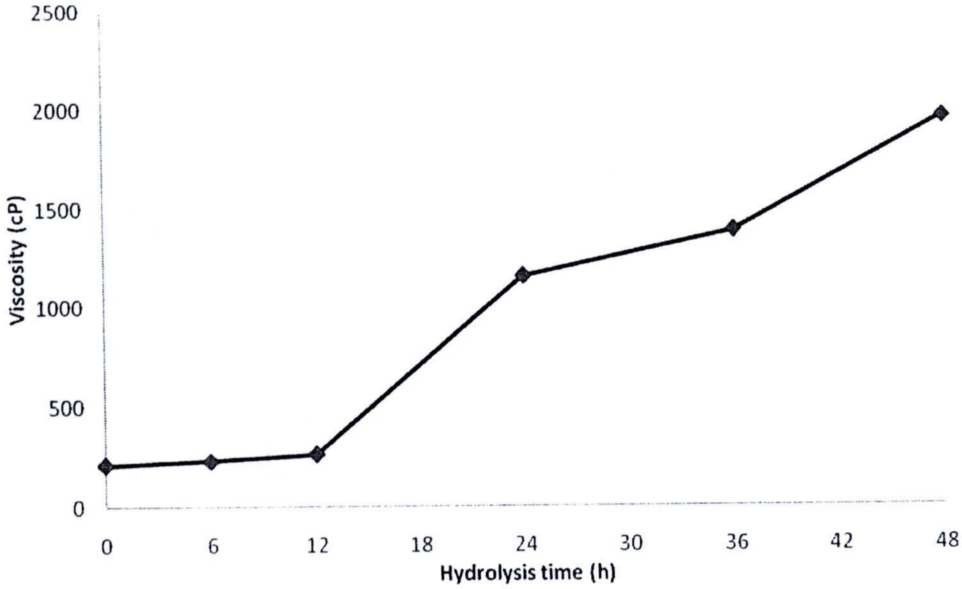


Figure 4.2 Viscosity of the solution of hydrolyzed chitosan in 90% (v/v) acetic acid, as a function of hydrolysis time for chitosan. The molecular weight of chitosan was 760 kDa.

For addition of PVA to chitosan solution, similar trend was observed. The increased amount of PVA resulted in the increase in the viscosity of the solution. The viscosity of chitosan solution was also increased with increasing of chitosan molecular weight. The result was shown in Figure 4.3 as well as in Table 4.1.

Table 4.1 Viscosity of chitosan/PVA solution

PVA Content (%w/v)	100 kDa chitosan		400 kDa chitosan		760 kDa chitosan	
	Chitosan Content (%w/v)	Viscosity (cP)	Chitosan Content (%w/v)	Viscosity (cP)	Chitosan Content (%w/v)	Viscosity (cP)
0.10	0	825.00±1.00	0	825.00±1.00	0	825.00±1.00
0.08	0.004	1079.00±8.93	0.003	1161.00±6.00	0.002	1204.83±2.67
0.06	0.008	664.00±9.54	0.006	847.33±1.53	0.004	1015.67±2.52
0.04	0.012	428.00±4.00	0.009	536.30±2.31	0.006	685.50±8.81
0.02	0.016	290.00±5.57	0.012	314.50±0.96	0.008	463.10±4.43
0	0.020	65.00±2.65	0.015	164.27±2.51	0.010	210.73±1.46

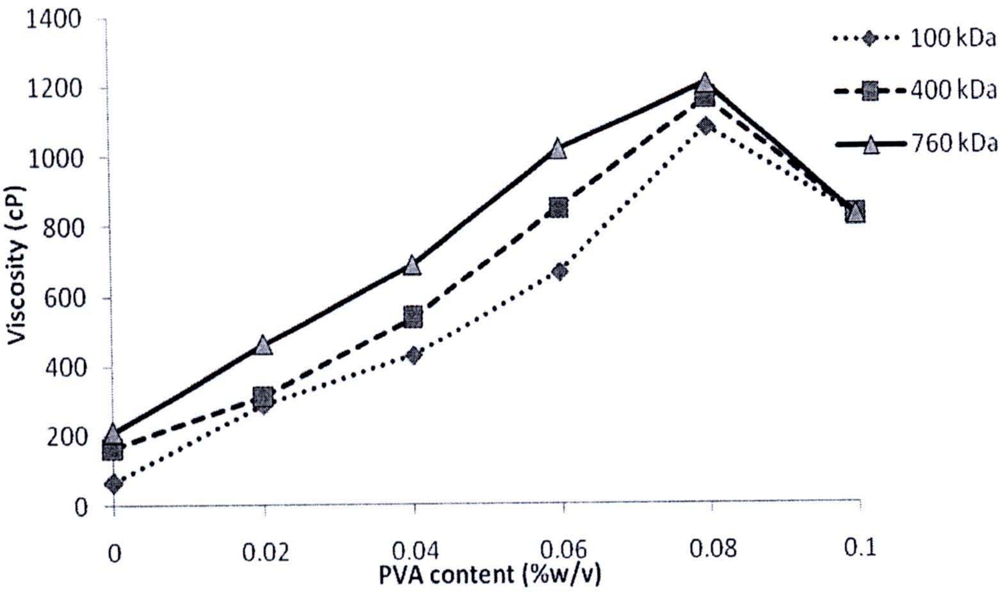


Figure 4.3 Viscosity of the solution of chitosan/PVA at different of chitosan molecular weight.

4.2.2 Conductivity of hydrolyzed chitosan and chitosan/PVA solution

The conductivity of the hydrolyzed chitosan solution was affect by hydrolysis time, while that of the chitosan/PVA solution was affected by both PVA content and the molecular weight of chitosan. The conductivity of the hydrolyzed chitosan solution is shown in Figure 4.4. For short period of hydrolysis, the conductivity of the solution was not significantly changed. However, after 24 h of hydrolysis with NaOH, the conductivity greatly increased. When the hydrolysis time was increased, the resistance of groups imposed by the arrangement of the C2 and C3 substituent in the sugar ring that affected the deacetylation of the polymer chain was increased, in addition to the increase in the positive charge of polymer chain [14]. Consequently, the conductivity of the hydrolyzed chitosan solution was greatly increased.

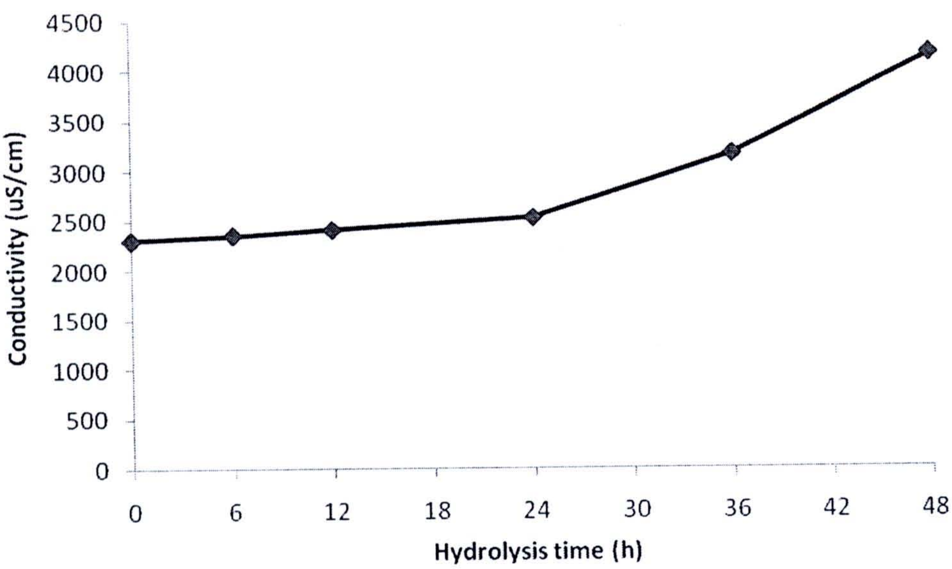


Figure 4.4 The conductivity of hydrolyzed chitosan solution.

For the addition of PVA as the spinning aid, it was found that the conductivity of blended solution was decreased by the content of PVA. This might be due to the strong interaction of hydrogen bond occurred between chitosan and PVA molecule in the blended solution tesulting in decreased amount of free ions in the solution. Thus the conductivity of the solution with the addition of PVA was decreased. On the other hand, increasing the molecular weight of chitosan would result in the decrease in the conductivity. It would be explained by the role of the intermolecular interactions. Increasing in molecular weight, chitosan could have block arrangement of acetylated and deacetylated units and might reduce available sites of amino groups on the chitosan molecule [15], resulting in the decreased in conductivity. The results are shown in Table 4.2 and Figure 4.5.

The addition of cationic would increase the conductivity of polymer solution and resulted in higher number of charges in the solution so that charge repulsion may obstruct the entanglement of polymer chains. Thus the fiber jet of higher conductivity solution could be subjected to higher tensile force in the presence of an electric field than a fiber jet from a solution with low conductivity.

Table 4.2 The conductivity of chitosan/PVA solution.

PVA content	Conductivity (μS/cm)		
(%w/v)	100 kDa chitosan	400 kDa chitosan	760 kDa chitosan
0.10	747	747	747
0.08	1389	1138	1034
0.06	1451	1313	1103
0.04	2020	1824	1391
0.02	3421	2610	2250
0	4850	3280	2300

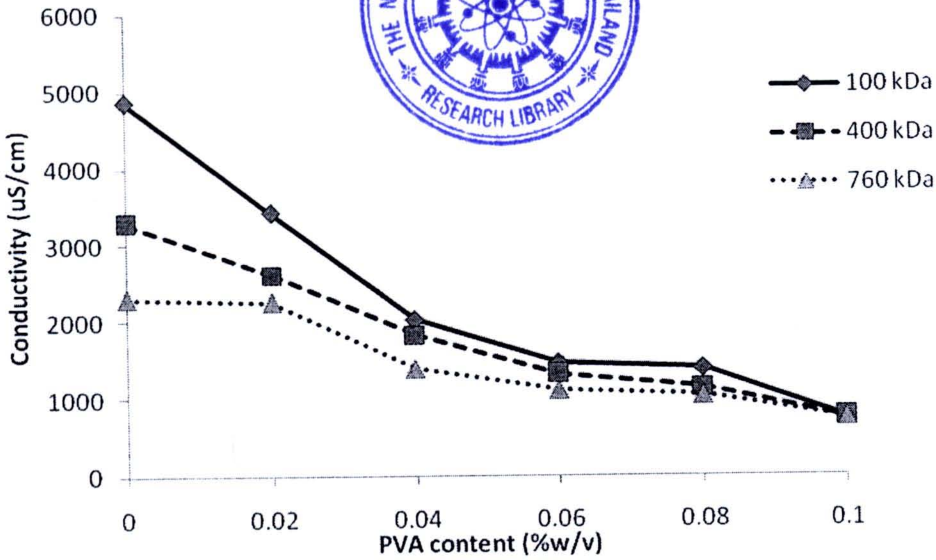


Figure 4.5 The conductivity of chitosan/PVA solution.

4.3 Morphology of Chitosan Nanofibers Fabricated by Electrospinning Technique

4.3.1 Morphology of electrospun hydrolyzed chitosan

Electrospinning of hydrolyzed chitosan solution was conducted using 10 cm tip-to-collector distance and 25 kV of applied electric field. The solutions were prepared using the highest amount of the hydrolyzed chitosan dissolvable into 90 % acetic acid, which depended on the duration of the hydrolysis period that chitosan had experienced. As shown in Figure 4.6, for a hydrolysis period in the range of 0 - 48 h, the maximum soluble amount of hydrolyzed chitosan increased with the hydrolysis time.

Nevertheless, it was found that the solution prepared from chitosan that had been hydrolyzed for 0, 6 and 12 h could not be spun into fibers. Only spherical droplets were found on the collector. By increasing the hydrolysis time up to 24 h, nanofibers with average diameter of 117.4 nm could be generated. The prolonged period of hydrolysis time to 36 and 48 h resulted in the decreased fiber diameter of

39.2 and 25.2 nm, respectively, as shown in Figure 4.7. These behaviors can be explained by the role of NaOH in the further deacetylation of chitosan polymeric chains. When the hydrolysis time was increased, in addition to the increase in the positive charge of the polymers chain, the molecular weight of chitosan was also decreased, which enabled chitosan molecules to align more effectively in the electrical field during the electrospinning process [16, 17]. It was also suggested that the average length of the polymeric chain of chitosan after hydrolysis may be below the required length for entanglement coupling formation. Regarding electrospinning parameters, the chitosan concentration affected spinability of the solution by affecting on the viscosity of the solution, which was directly related to chitosan chain entanglement. On the other hand, the conductivity of the solution influenced the fiber size and fiber morphology. Increasing conductivity was resulted in decreased fiber diameter and nonuniform fiber morphology, which leaded to a dramatic bending instability as well as a broad diameter distribution.

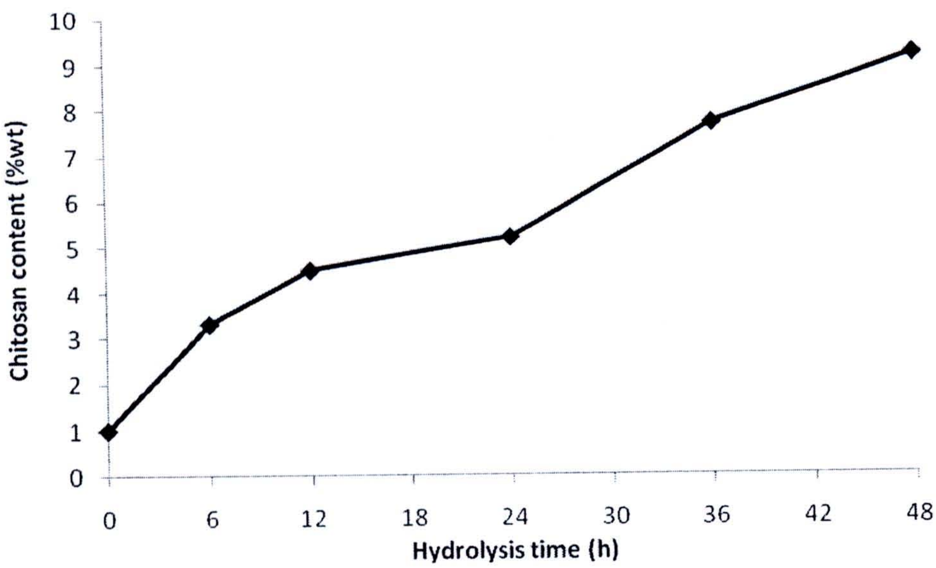


Figure 4.6 Maximum solubility of chitosan hydrolyzed for various period of hydrolysis.

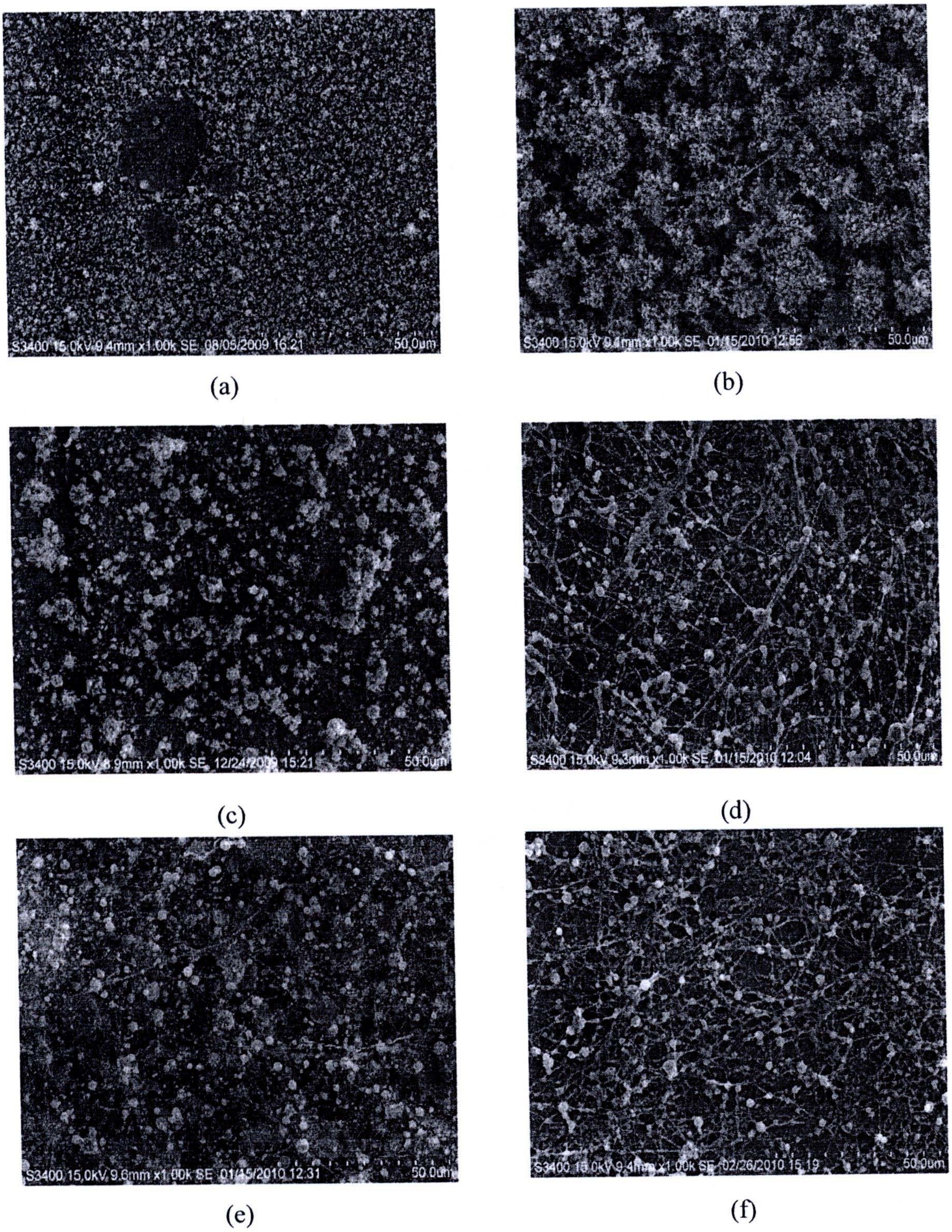


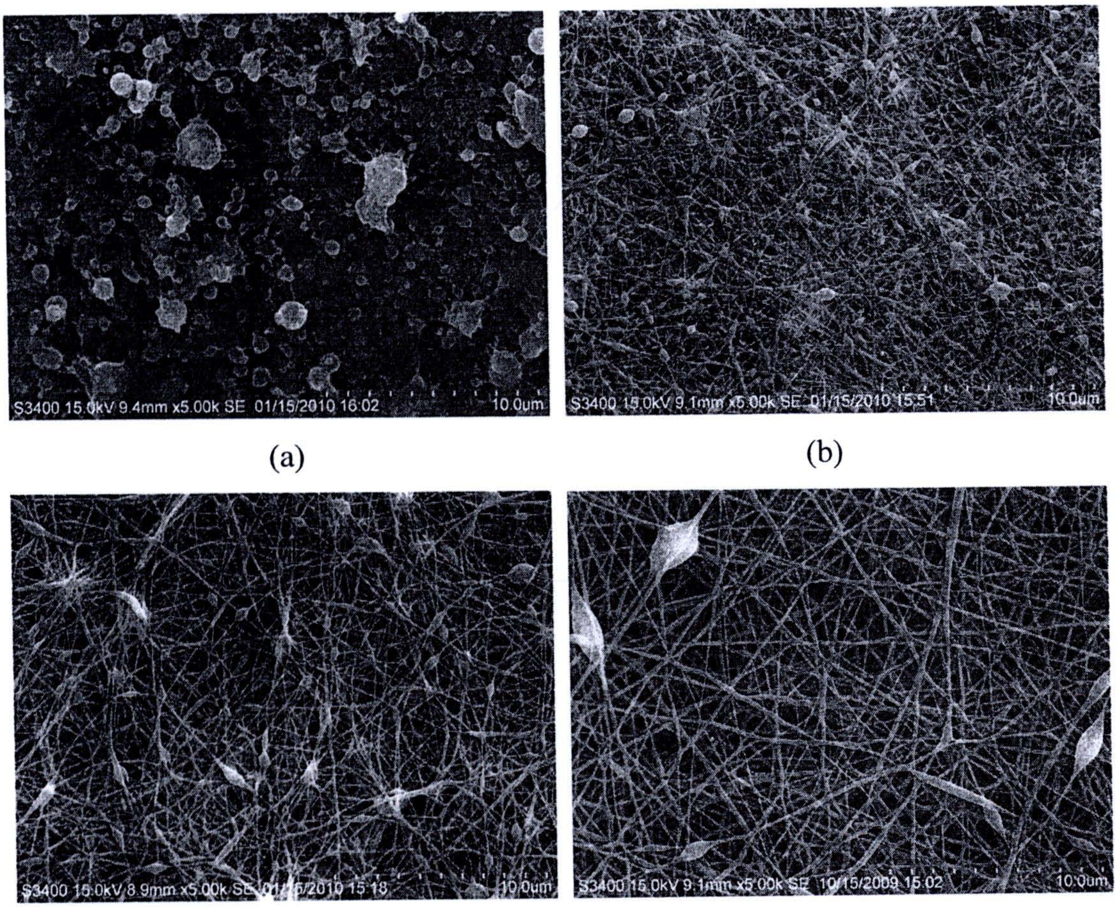
Figure 4.7 SEM micrographs of nanofibers formed from electrospinning of hydrolyzed chitosan dissolved in 90% (w/v) acetic acid. The hydrolysis time was varied from 0 h (a), 6 h (b), 12 h (c), 24 h (d), 36 h (e) and 48 h (f), respectively.

4.3.2 Morphology of electrospun chitosan/PVA composite

The electrospinning of chitosan/PVA composite was done using the condition of 10 cm tip-to-collector distance and 25 kV of applied electric field. SEM micrographs of nanofibers formed from the solution with various chitosan/PVA blending ratio spun under the same condition are shown in Figure 4.8-4.10. It was found that, for the solution with low PVA content, the electric force could not initiate the formation of fibers, in the similar manner as that for pure chitosan. The fibers could be seen at moderated or high content of PVA. The fact that the spinning solution with high chitosan content could not be electrospun into nanofibers may be the result from the interaction between polycationic group of chitosan that prevented molecular entanglement of polymer chains needed in the formation of continuous fibers. Nevertheless, as the charge density was increased, a higher elongation forces were imposed to the jet of the solution formed by the electrical field. Since the overall tension in the fibers depended upon self repulsion of excess charges on the jet, excessive charges leaded to small fibers [17, 18]. Thus, the average diameter of the fibers tended to be small with large number of bead formed when the chitosan content was increased. However, size of the beads decreased in compensation with the increased in non-uniformity of fiber diameter. The results for average fiber diameter and fraction of the product that was formed into fibers are shown in Table 4.3. For the addition of PVA as the spinning aid, the hydroxyl functional groups was also give the strong interaction of hydrogen bond between chitosan and PVA molecule in the blended solution. These behaviors were closely related to the result of viscosity and conductivity.

Table 4.3 The effect of chitosan content and molecular weight of chitosan on electrospun fiber morphology.

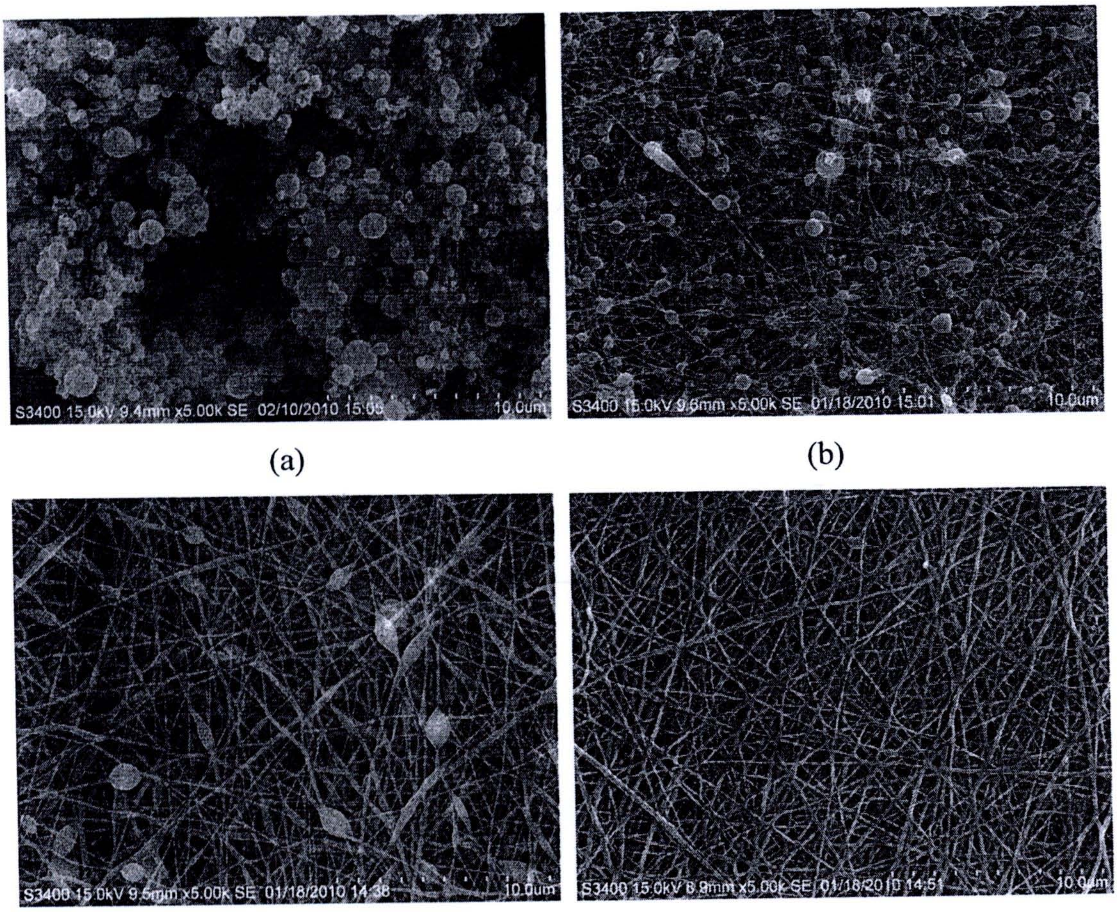
100 kDa chitosan			400 kDa chitosan			760 kDa chitosan		
Chtiosan content (%w/v)	Average diameter (nm)	Fiber percentage (%)	Chitosan content (%w/v)	Average diameter (nm)	Fiber percentage (%)	Chitosan content (%w/v)	Average diameter (nm)	Fiber percentage (%)
0.004	81.7	91.6	0.003	118.3	98.0	0.002	88.5	98.4
0.008	62	82.0	0.006	101.1	87.6	0.004	82.2	90.0
0.012	49.5	46.0	0.009	53.4	72.8	0.006	58.0	82.4
0.016	39.5	12.5	0.012	41.1	30.8	0.008	42.8	45.2



(a) (b)

(c) (d)

Figure 4.8 SEM micrographs of nanofibers electrospun from chitosan/PVA solution containing various contents of PVA: (a) 0.02, (b) 0.04, (c) 0.06 and (d) 0.08 wt%. The molecular weight of chitosan was 100 kDa.



(a) (b) (c) (d)

Figure 4.9 SEM micrographs of nanofibers electrospun from chitosan/PVA solution containing various contents of PVA: (a) 0.02, (b) 0.04, (c) 0.06 and (d) 0.08 wt%. The molecular weight of chitosan was 400 kDa.

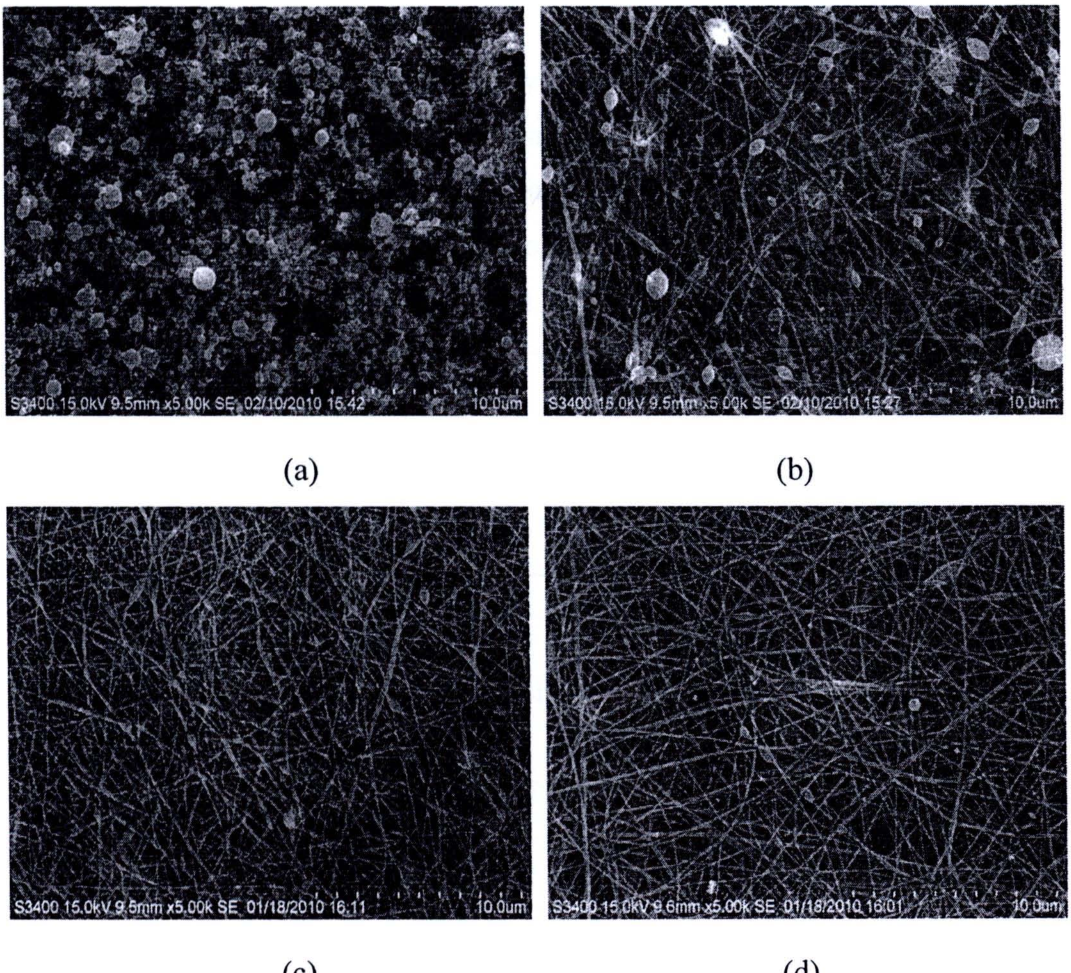


Figure 4.10 SEM micrographs of nanofibers electrospun from chitosan/PVA solution containing various contents of PVA: (a) 0.02, (b) 0.04, (c) 0.06 and (d) 0.08 wt%. The molecular weight of chitosan was 760 kDa.

In the electrospinning of nanofibers, molecular weight is also one of the most effective variables to control morphology and diameter of the fibers. It has been reported that low molecular weight polymer tends to form beads more than fibers, while high molecular weight polymer generate fibers with larger average diameter and less beads. In this study, the morphology of the fibers formed using different chitosan molecular weight were compared. In generally using 100 kDa chitosan, thin nanofibers were generated with large beads. When the molecular weight was increased to 400 kDa and 760 kDa, respectively, the average diameter of the fibers was increased. Size of the beads was decreasing in compensation with the increased and non-uniform fiber diameter. These behaviors were closely related to viscosity and spinnability of the solution.

4.4 Cell Attachment on Chitosan Nanofibers

4.4.1 Cell attachment on hydrolyzed chitosan nanofibers

4.4.1.1 Effect of incubation time

In order to investigate the effect of incubation time on the attachment of bacterial cells, chitosan hydrolyzed for 6 and 48 h were chosen. The Colony Forming Unit (CFU) was calculated from a standard curve of either *Acinetobacter baylyi* strain GFJ2 or *Brevibacillus agri* strain 13. The initial optical density (OD_{600}) of the bacterial cells solution for *Acinetobacter baylyi* strain GFJ2 and for *Brevibacillus agri* strain 13 was about 0.85 and 1.0, which was corresponding to 1.8981×10^{13} and 1.4760×10^{9} CFU, respectively. The total number of bacterial cells attached on hydrolyzed chitosan nanofibers are shown in Figure 4.11 and 4.12 for *A. baylyi* GFJ2 and *B. Agri* 13, respectively.

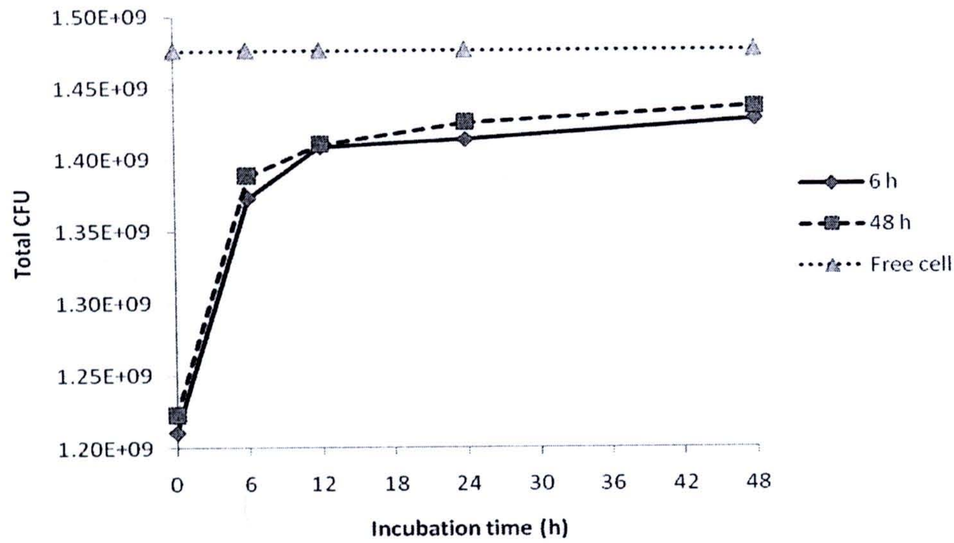


Figure 4.11 Total CFU of Gram-positive *Brevibacillus agri* strain13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 6 (____) and 48 h (.....), after various incubation time. The dotted line represents the CFU value of the free cells.

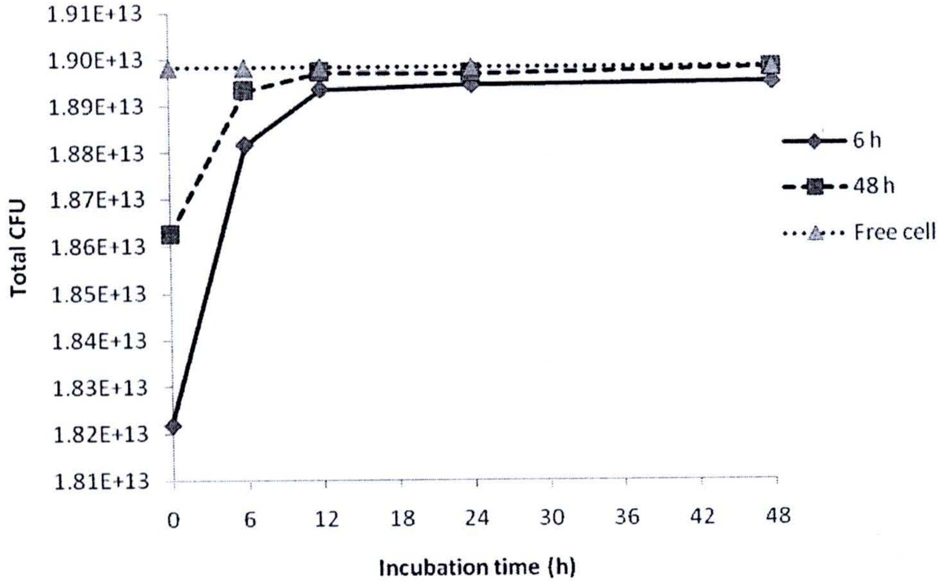


Figure 4.12 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 6 (___) and 48 h(....) after various incubation time. The dotted line represents the CFU value of the free cells.

Similar trend was found for both of Gram-negative and Gram-positive bacteria. The amount of bacterial cell attachment was increased when the incubation time was increased. At incubation time of 0 h, (i.e., right after when cells were exposed to chitosan samples) most of both bacterial cells was easily washed away. Nevertheless, some of the cells could adhere to chitosan sample even right after their explosure to chitosan. At 6 and12 h of incubation, increased bacterial cells attachment on the surface was obsearved. When the contact time between chitosan fibers and the bacteria was prolonged to 24 and 48 h, the attachment of gram-negative *Acinetobacter baylyi* strain GFJ2 bacteria on the chitosan sample had reached the stable stage, which was very closely to the CFU of the free cells. On the other hand, gram-positive *Brevibacillus agri* strain 13 bacteria continued to increase toward the value of free cells. It is possible that the microorganism may be induced to attach to chitosan by altering the physical and chemical properties by ionic attraction of bacterial cells and the chitosan surface. Chitosan is a cationic polysaccharide that acts as a glue to initiate bacterial-surface interactions. Increasing the contact time between chitosan fibers and the bacterial cells tends to increase the interaction rate.

4.4.1.2 Effect of hydrolysis time of chitosan

To compare the bacterial attachment on hydrolyzed chitosan nanofibers at various hydrolysis times, the incubation time of 12 and 24 h were chosen. The results showed that the number of both types of bacterial cells attached on the surface increased when the hydrolysis time of chitosan was increased. The results are shown in Figure 4.13-4.16. These results could be due to a significant role of a surface area-to-volume ratio. As previously mentioned, the hydrolysis time of 0, 6 and 12 h could produce chitosan nanofibers, but spherical particles were generated. On the other hand, the hydrolysis time of 24, 36 and 48 h could be resulted in nanofibers. The fiber diameter decreased as the hydrolysis time was increased. The results showed that amount of bacterial cells that were able to attach on the fiber mats was greater than on particles. This was the result from high surface area-to-volume ratio of fiber mats available for the bacterial cells to attach. Moreover, hydrophilicity of chitosan was increased after chitosan was hydrolyzed. The hydrophilicity is one factor influencing the bacterial cells response to a substrate [19, 20].

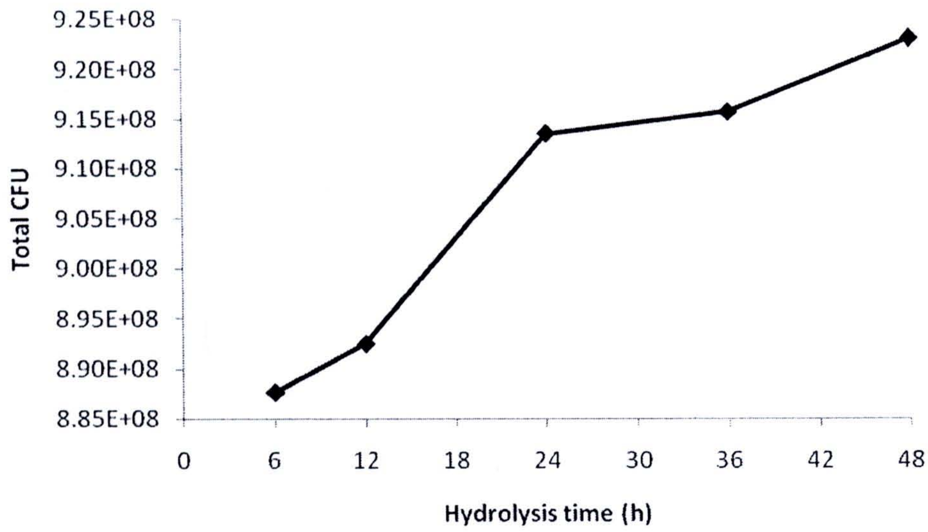


Figure 4.13 Total CFU of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan being hydrolyzed for various period of time. The incubation time of the cells was 12 h.

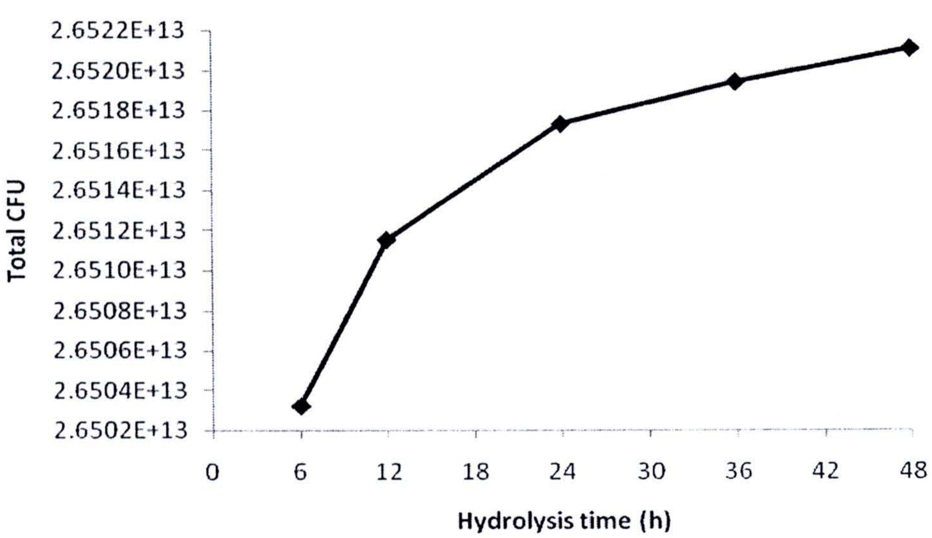


Figure 4.14 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan being hydrolyzed for various period of time. The incubation time of the cells was 12 h.

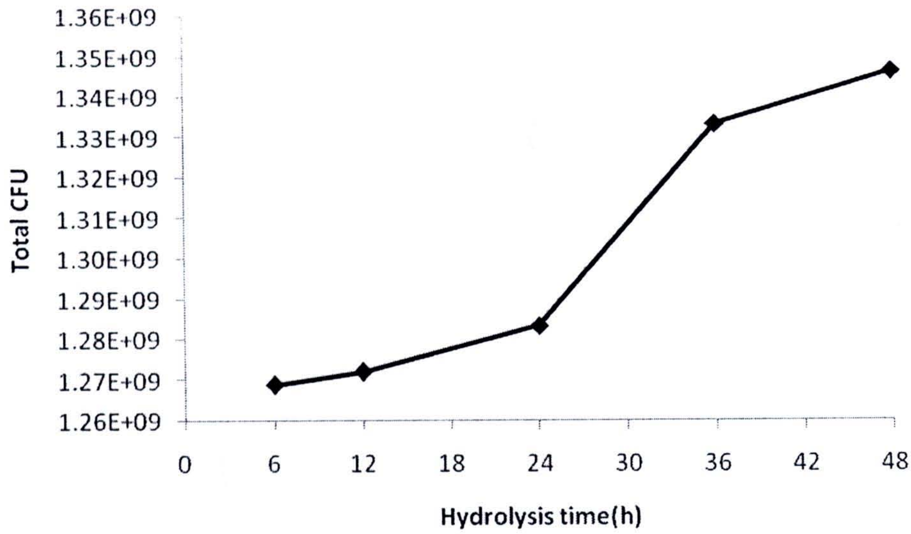


Figure 4.15 Total CFU of Gram-positive *Brevibacillus agri* strain No.13 attached onto electrospun chitosan fibers, formed from chitosan being hydrolyzed for various period of time. The incubation time of the cells was 24 h.

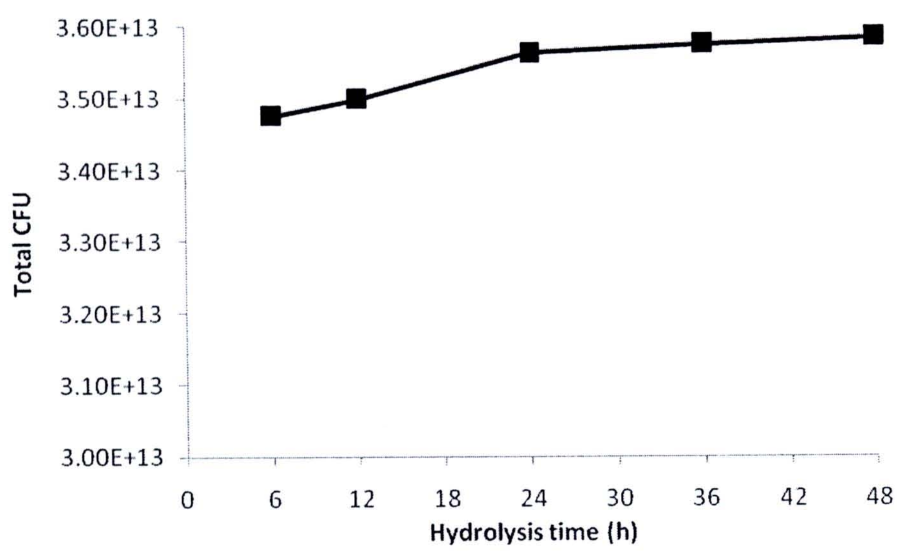


Figure 4.16 Total CFU of gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan being hydrolyzed for various period of time. The incubation time of the cells was 24 h.

To further confirm the attachment of bacterial cells onto the prepared chitosan nanofibers, the scanning electron microscopy was employed. SEM micrographs of the samples, after certain period of incubation time, are shown in Figure 4.17-4.24. At 0 and 6 h of incubation time, most cells were formed as individual cell on the fibers. Only two to three cell colonies were appeared on the SEM image. When the contacting time between chitosan fibers and the bacteria was prolonged to 12 h, the number of Gram-negative *A. baylyi* strain GFJ2 bacteria observed on the sample increased exponentially, in much greater extent than that of Gram-positive *B. agri* strain 13 bacteria. After the incubation time of 24 and 48 h, both types of bacteria were found fully covering the chitosan. These results suggested that the interaction between bacteria cells and chitosan was related to the chemical and physical properties of cell wall. It has been known that chitosan easily carries more positive-charged amino groups in more acidic solution or when the degree deacetylation of chitosan is high [21]. The hydrolysis process could significantly increase the degree of deacetylation of chitosan, which probably lead to increased positive charge (NH_3^+) of polymer chains. Increasing of NH_3^+ group of chitosan resulted in increased free amino group to interact with cell wall via negatively charge of phospholipid components of cell membranes [22]. However, Gram-positive cell wall does not posses a lipid outer membrane but Gram-negative cell wall posses an outer membrane consisting of various lipid complexes [23]. This difference is responsible to different attaching behavior of Gram-positive and Gram-negative cells to chitosan.

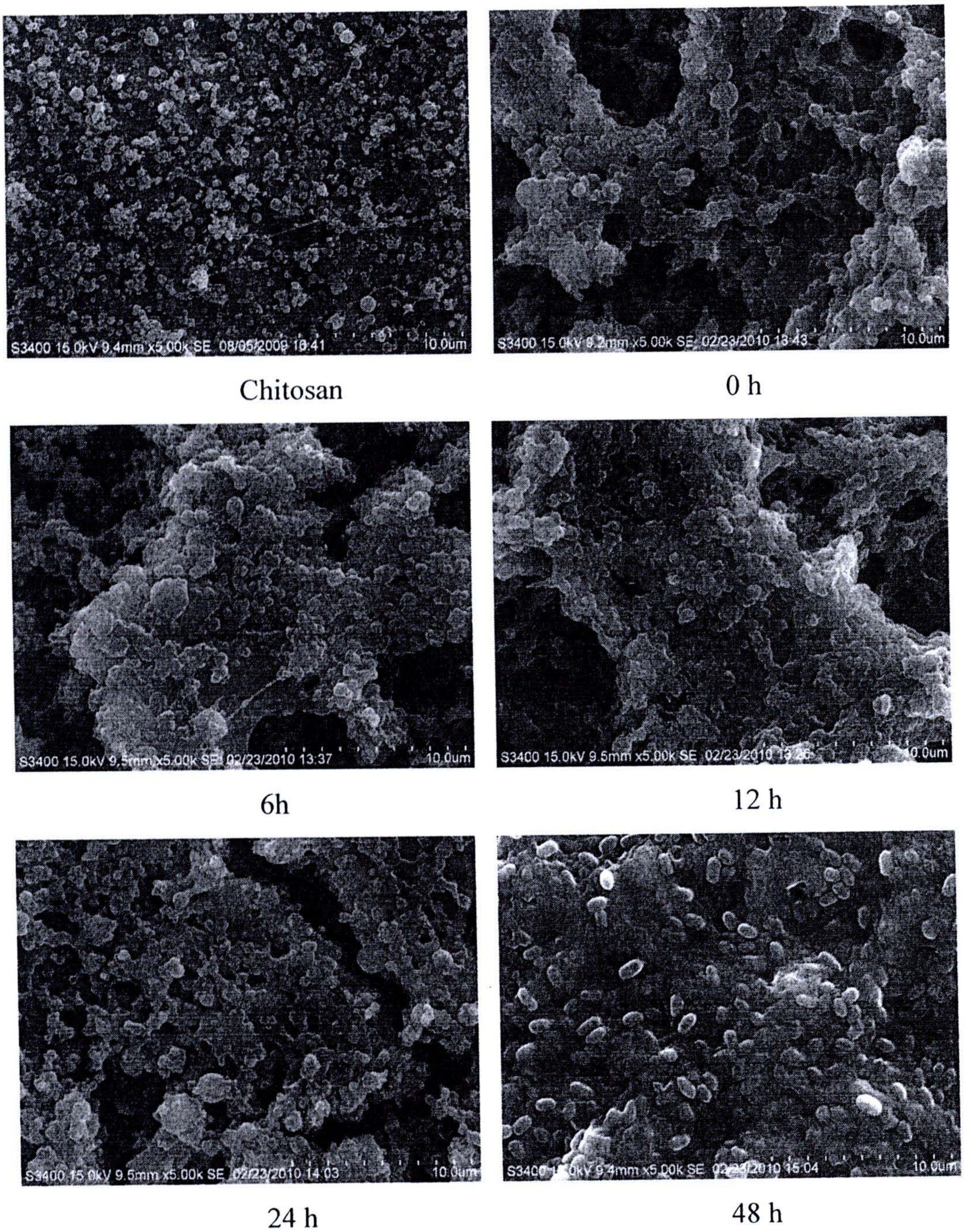


Figure 4.17 SEM micrographs of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 6 h, after various incubation times.

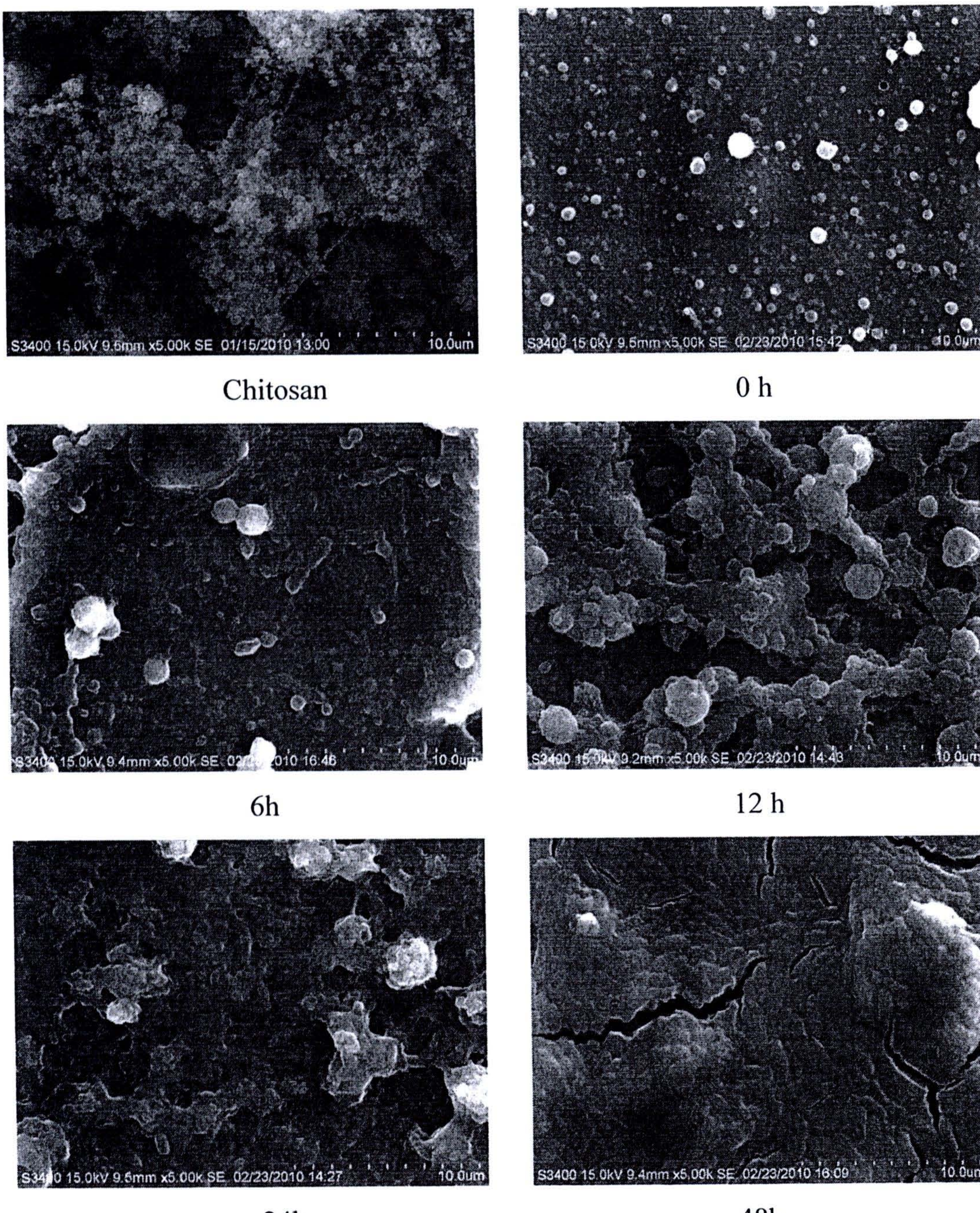


Figure 4.18 SEM micrographs of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 12 h, after various incubation times.

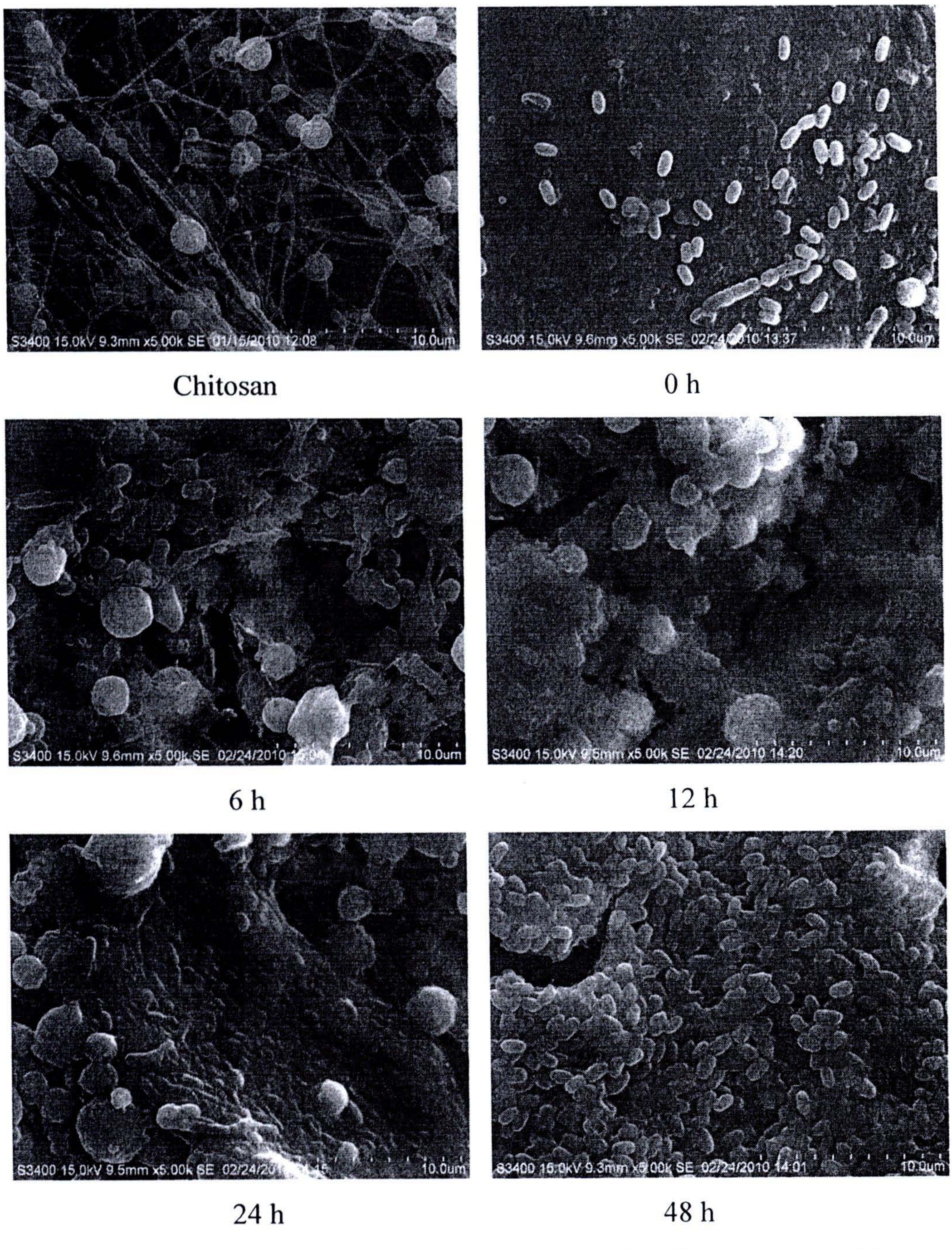


Figure 4.19 SEM micrographs of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 24 h, after various incubation times.

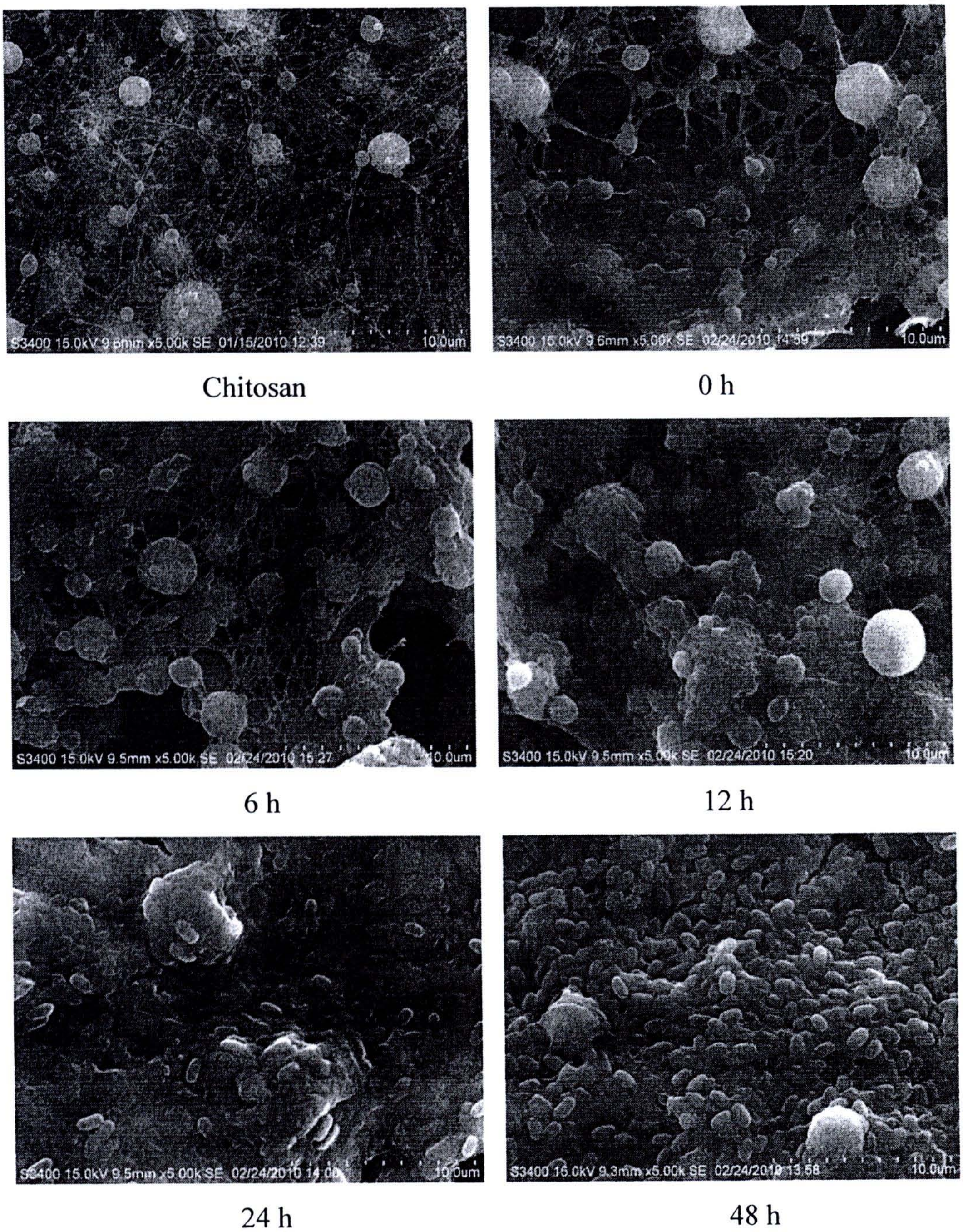
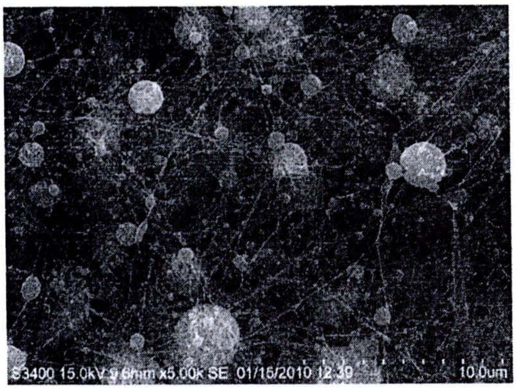
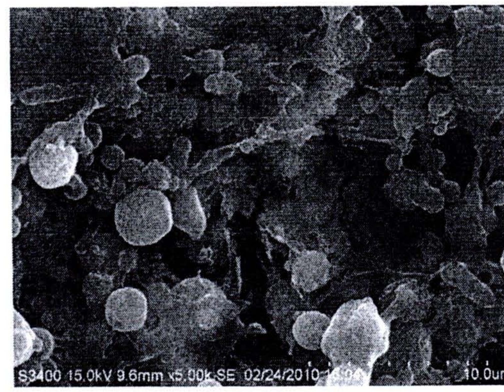


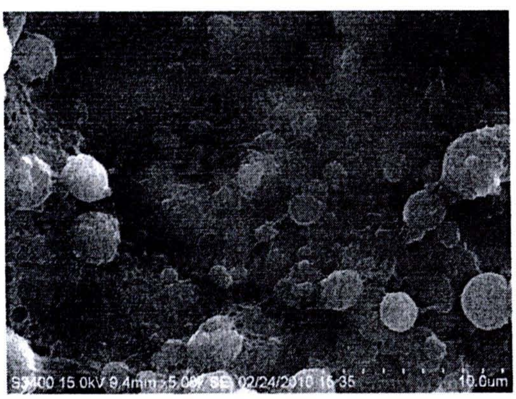
Figure 4.20 SEM micrographs of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 36 h, after various incubation times.



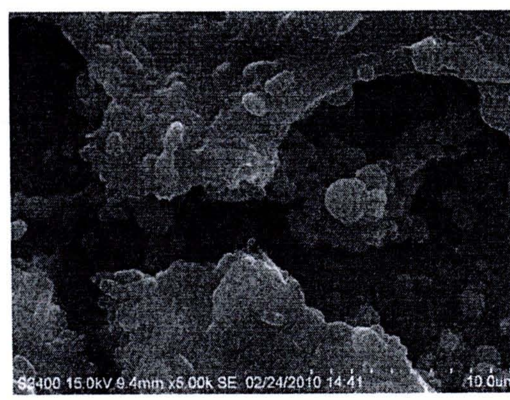
Chitosan



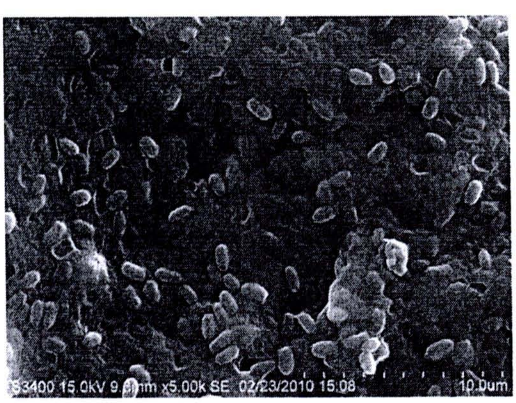
0 h



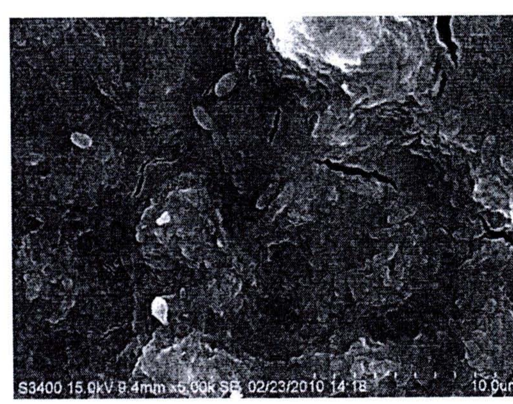
6 h



12 h

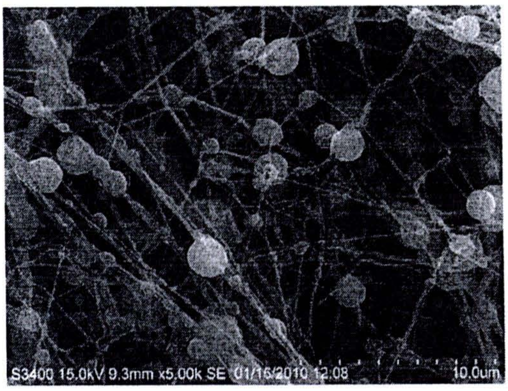


24 h

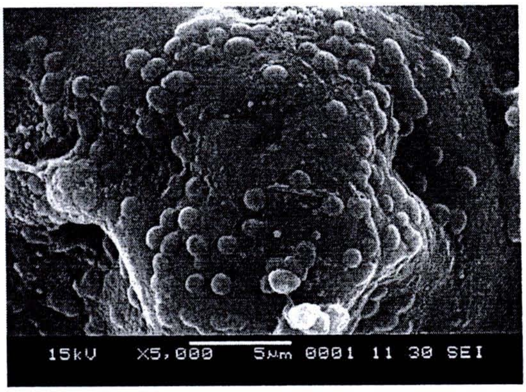


48 h

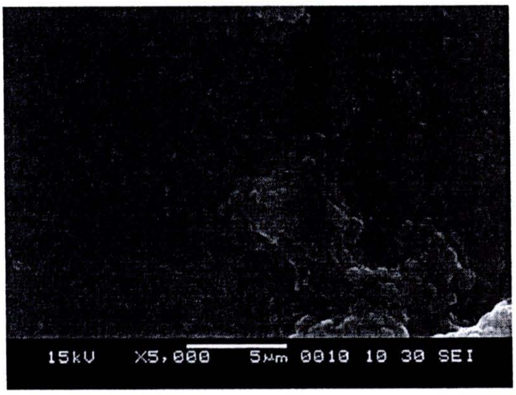
Figure 4.21 SEM micrographs of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 48 h, after various incubation times.



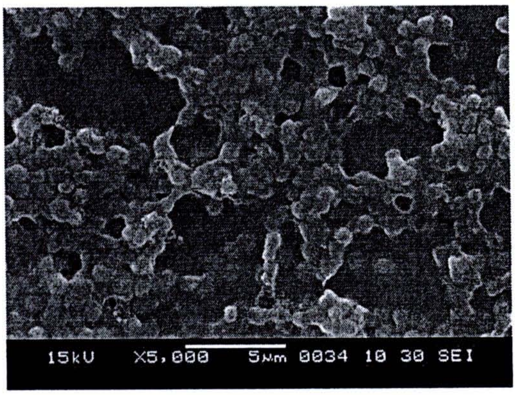
Chitosan



12 h



24 h



48 h

Figure 4.22 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 24 h, after various incubation times.

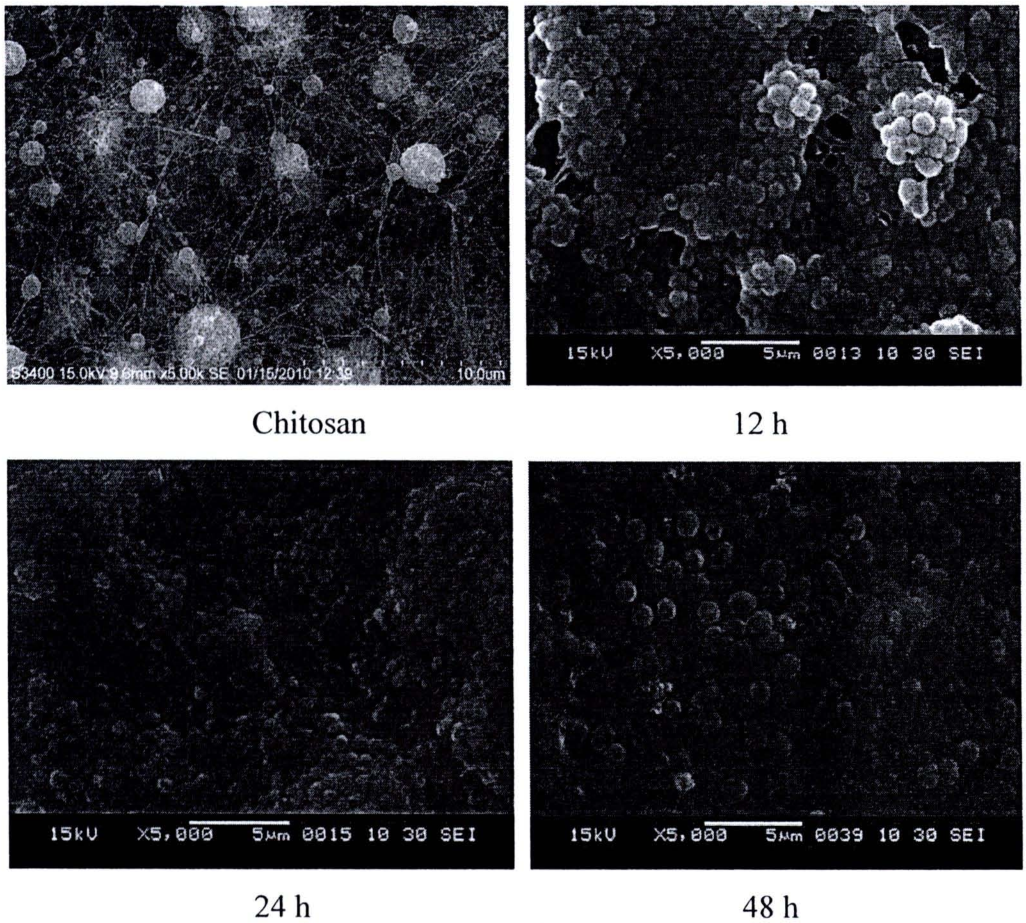
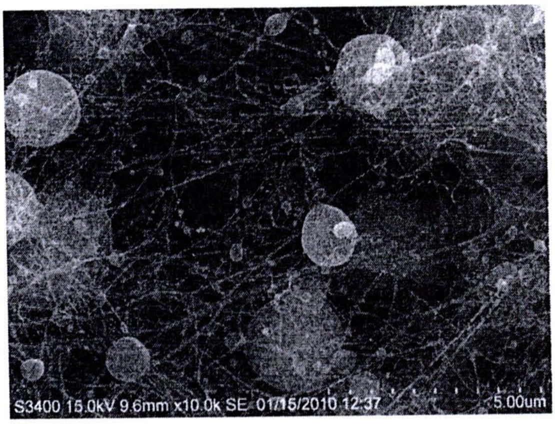
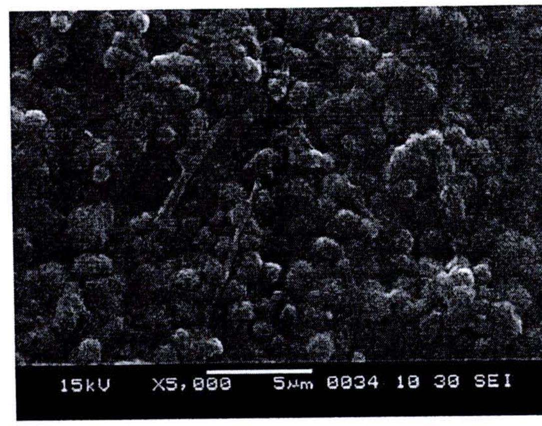


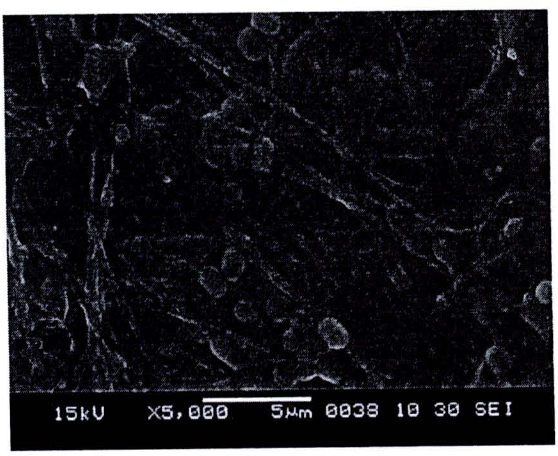
Figure 4.23 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 36 h, after various incubation times.



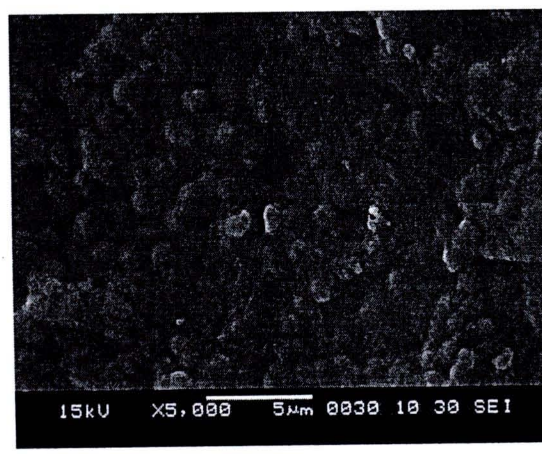
Chitosan



12 h



24 h

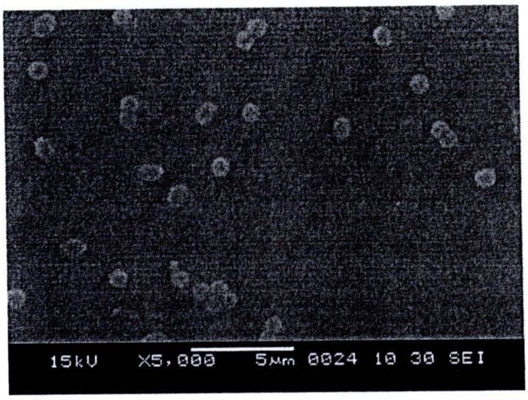


48 h

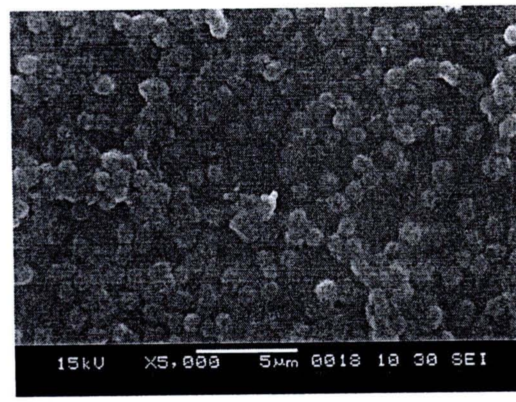
Figure 4.24 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 48h, after various incubation times.

4.4.2 Cell attachment on hydrolyzed chitosan film

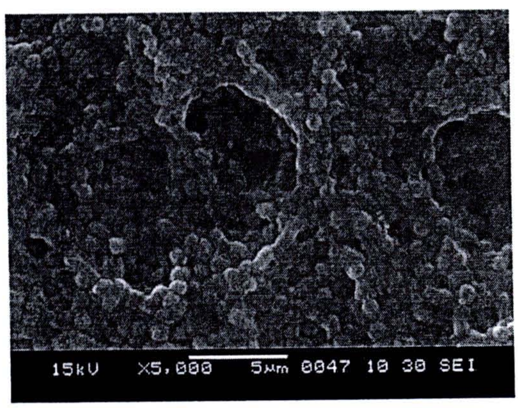
To verify the advantage of chitosan in form of nanofibers, it was compared, in term of cell attachment, with the hydrolyzed chitosan film. Both Gram-negative and Gram-positive bacteria were investigated. At 0 and 6 h of incubation time, most cells were loss with the washing, which indicated poor attachment to the films. Only two to three cell colonies were appeared on the surface. When the contact time between chitosan films and the bacteria was prolonged to 12 h, increased number of Gram-negative *A. baylyi* strain GFJ2 bacteria and Gram-positive *B. agri* strain 13 were found attached on the sample, but not yet fully covered the surface. After the incubation time of 24 and 48 h, the number of both types of bacteria cells were increased to the point that the whole chitosan films were covered with cells. SEM micrograph of *A. baylyi* strain GFJ2 and *B. agri* strain 13 attached on chitosan films that were prepared from chitosan hydrolyzed for various period of time are shown in Figure 4.25-4.30.



12 h

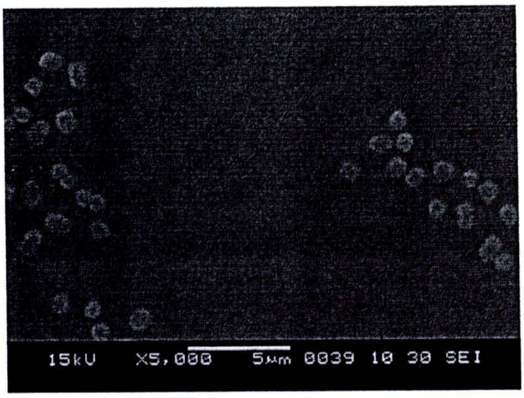


24 h

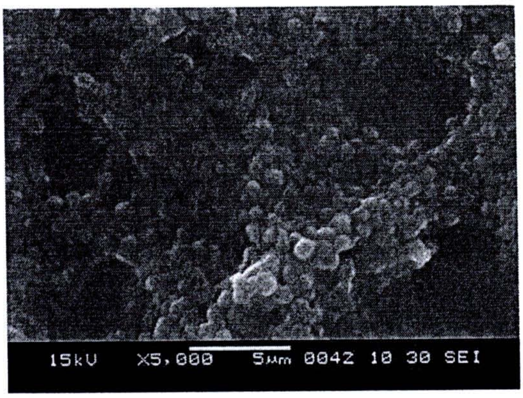


48 h

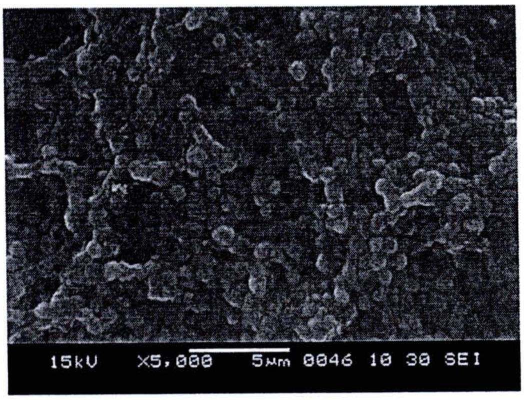
Figure 4.25 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan film, formed from chitosan hydrolyzed for 24 h, after various incubation times.



12 h

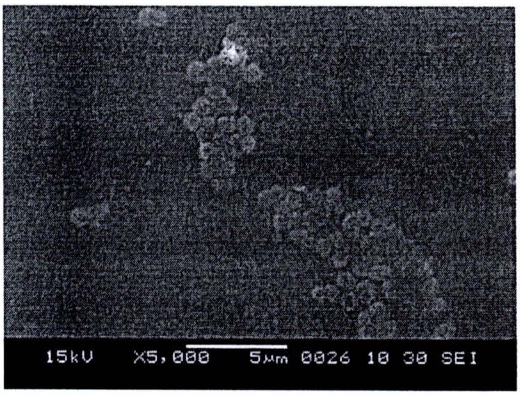


24 h

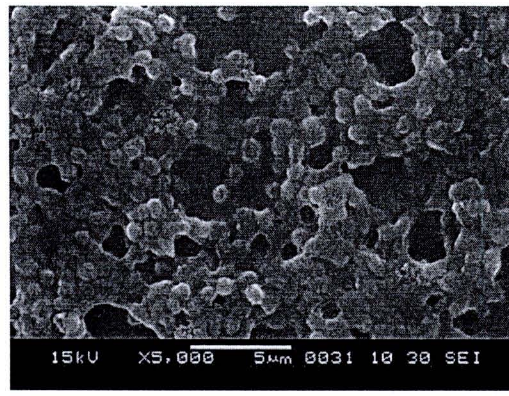


48 h

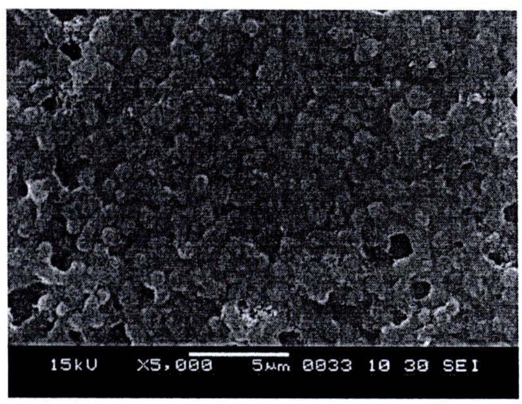
Figure 4.26 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan film, formed from chitosan hydrolyzed for 36 h, after various incubation times.



12 h



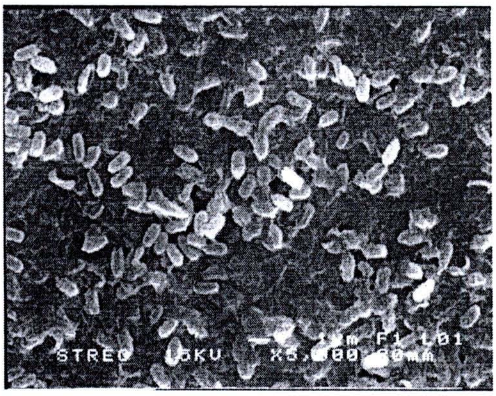
24 h



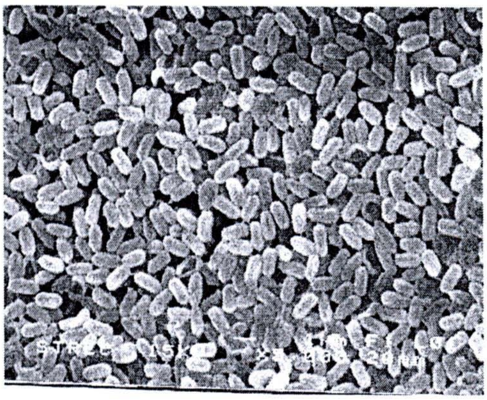
48 h

Figure 4.27 SEM micrographs of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan film, formed from chitosan hydrolyzed for 48 h, after various incubation times.

12 h



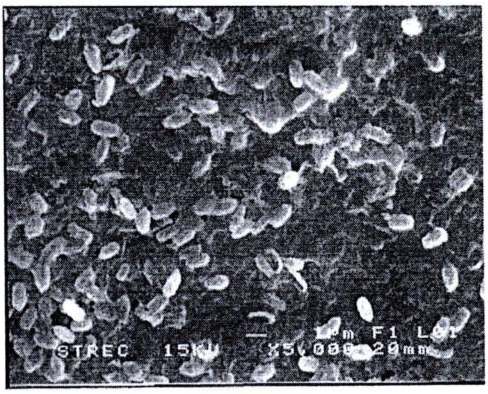
24 h



48 h

Figure 4.28 SEM micrographs of Gram-possitive *Brevibacillus agri* strain 13 attached onto chitosan film, formed from chitosan hydrolyzed for 24 h, after various incubation times.

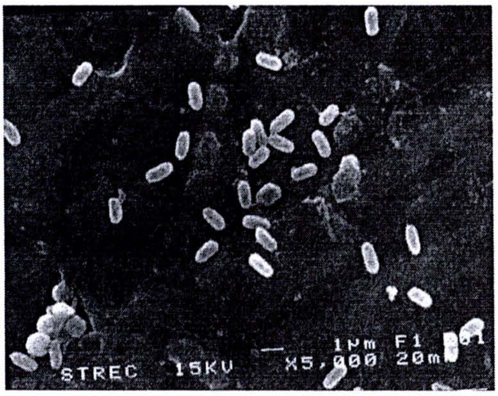
12 h



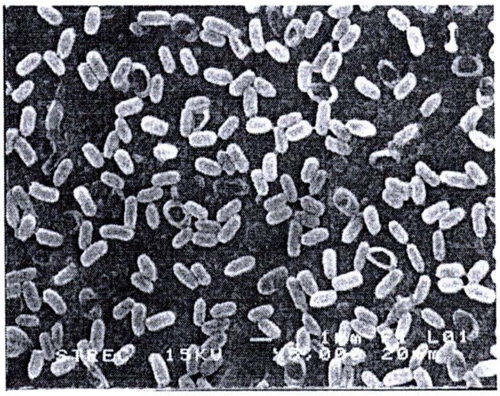
24 h

48 h

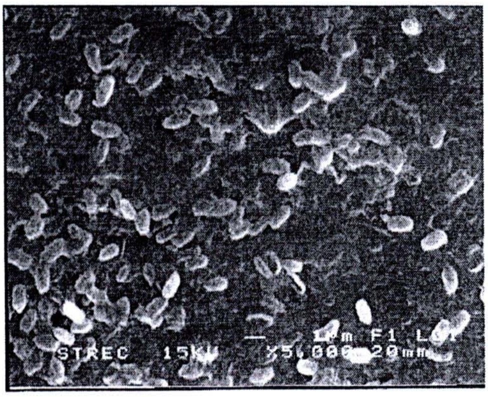
Figure 4.29 SEM micrographs of Gram-possitive *Brevibacillus agri* strain 13 attached onto chitosan film, formed from chitosan hydrolyzed for 36 h, after various incubation times.



12 h



24 h



48 h

Figure 4.30 SEM micrographs of Gram-possitive *Brevibacillus agri* strain 13 attached onto chitosan film, formed from chitosan hydrolyzed for 48 h, after various incubation times.

In order to compare the amount of bacterial cell attached on chitosan films and nanofibers, the incubation time of 12 and 24 h were chosen. The comparison results are shown in Figure 4.31-4.34. Similar trend for both of Gram-negative and Gram-positive was found that the chitosan nanofibers provided more cell attachment than chitosan film. These results could be explained by the role of surface area-to-volume ratio for cell attachment. The structure of chitosan nanofibers has higher surface area-to-volume ratio than chitosan film.

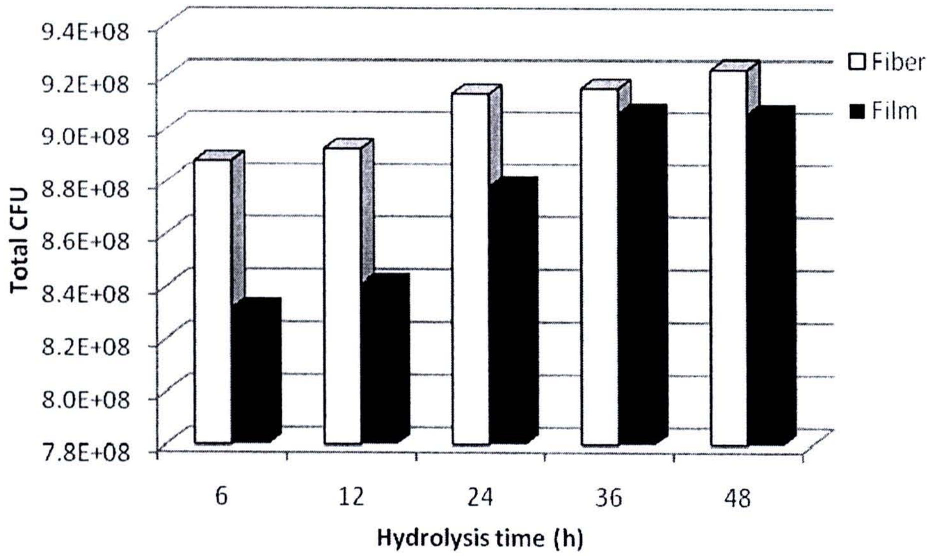


Figure 4.31 Total CFU of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers and chitosan films, at 12 h of incubation time, formed from chitosan hydrolyzed for various periods of time.

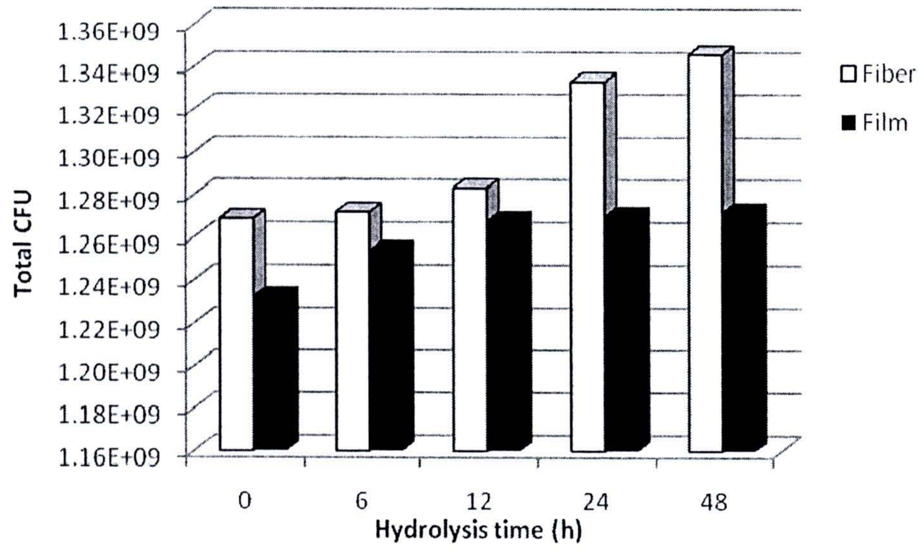


Figure 4.32 Total CFU of Gram-positive *Brevibacillus agri* strain 13 attached onto electrospun chitosan fibers and chitosan films, at 24 h of incubation time, formed from various chitosan hydrolyzed for various periods of time.

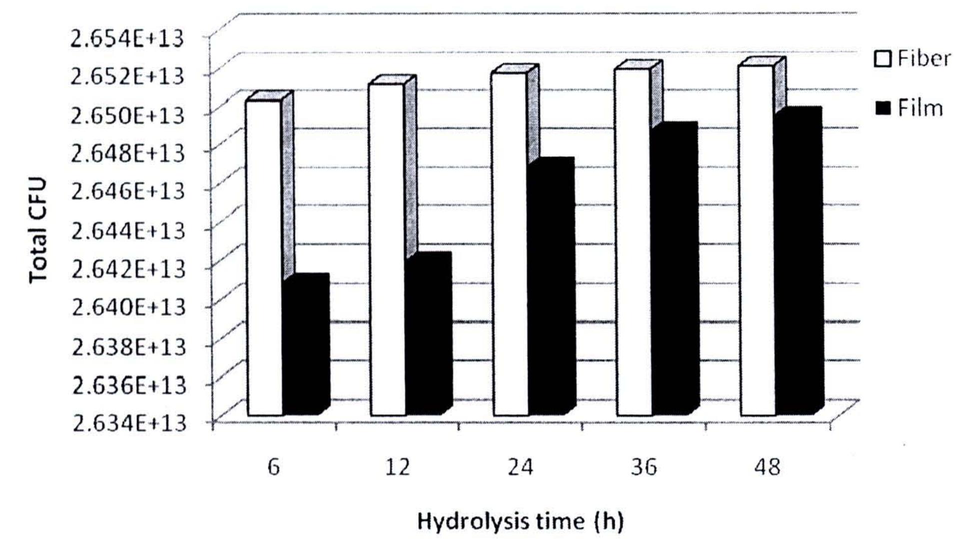


Figure 4.33 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers and chitosan films, at 12 h of incubation time, formed from chitosan hydrolyzed for various periods of time.

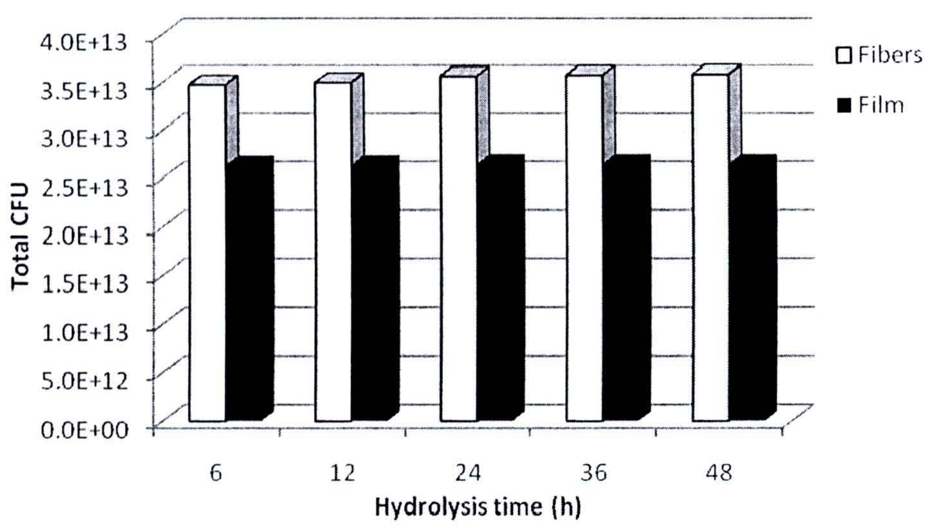


Figure 4.34 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers and chitosan films, at 24 h of incubation time, formed from various chitosan hydrolyzed for various periods of time.

4.4.3 Cell attachment on chitosan/PVA nanofibers

The capability of cells attachment on chitosan/PVA nanofibers, as a function of chitosan content, was investigated by using molecular weight of chitosan of 760 kDa. The incubation time of 12 and 24 h were chosen for investigation of cell attachment for both Gram-negative and Gram-positive bacteria. The results, regarding the number of cells attached to the nanofibers, are shown in Figure 4.35-4.38.

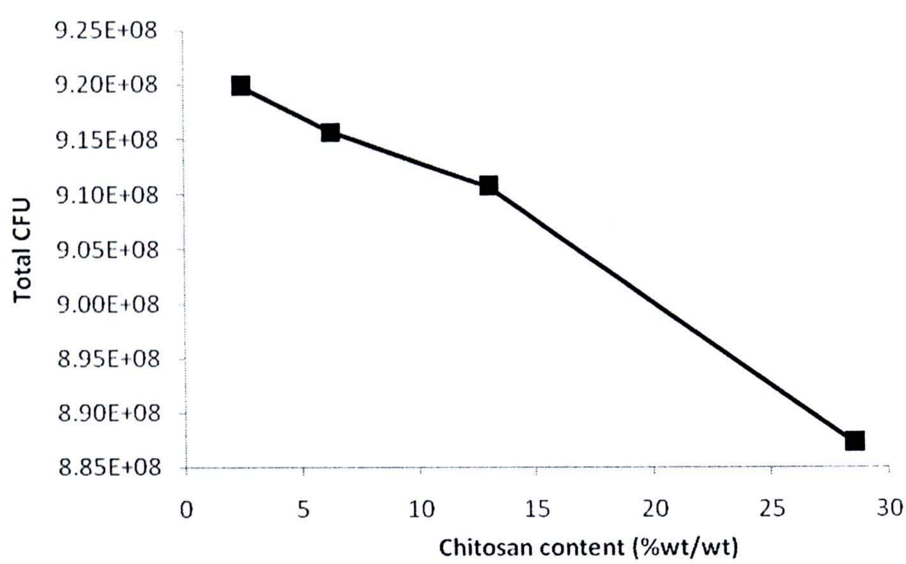


Figure 4.35 Total CFU of Gram-positive *Brevibacillus agri* strain 13 attached onto chitosan/PVA nanofibers with various chitosan contents, at 12 h of incubation time.

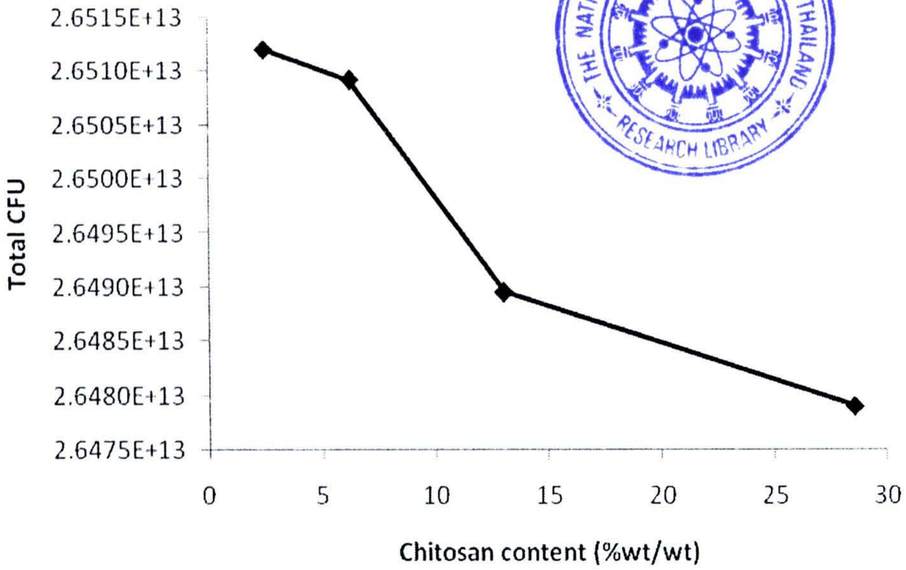


Figure 4.36 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan/PVA nanofibers with various chitosan contents, at 12 h of incubation time.

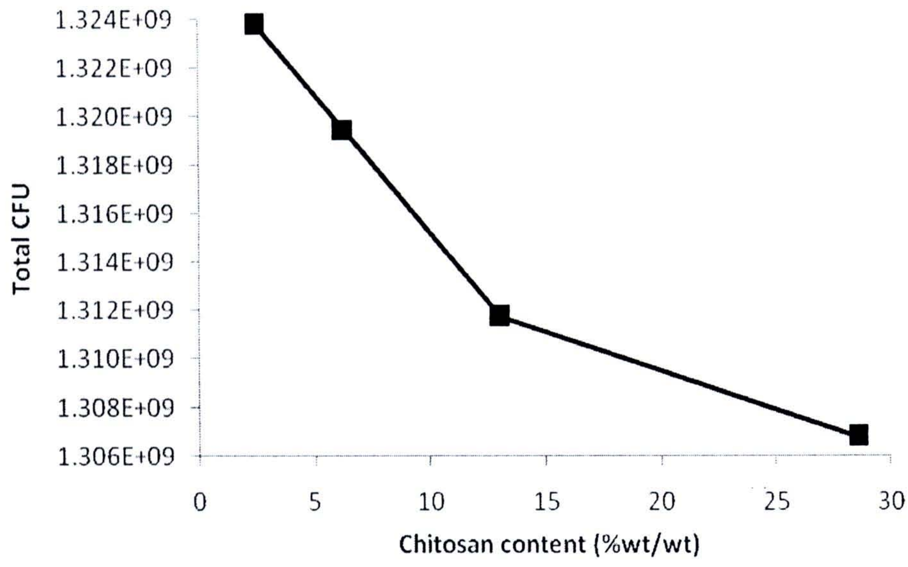


Figure 4.37 Total CFU of Gram-positive *Brevibacillus agri* strain 13 attached onto chitosan/PVA nanofibers with various chitosan contents, at 24 h of incubation time.

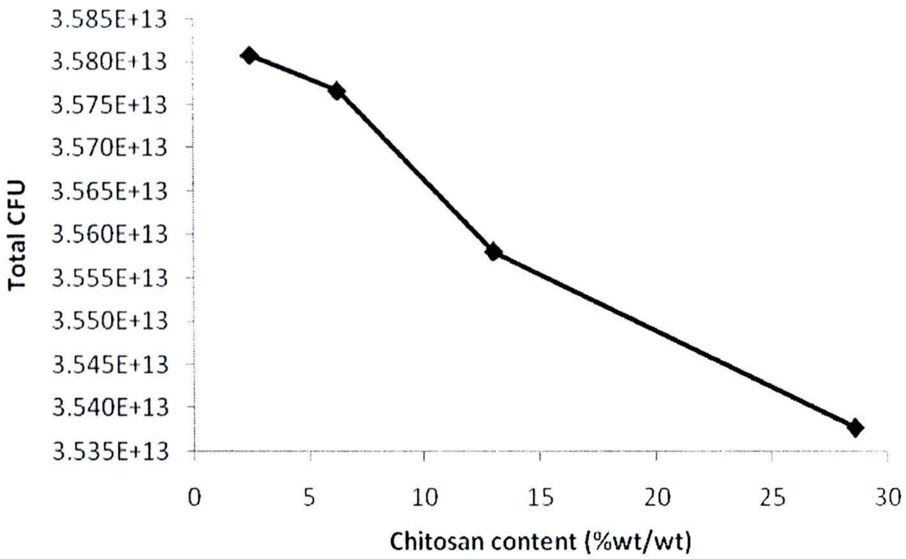


Figure 4.38 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan/PVA nanofibers with various chitosan contents, at 24 h of incubation time.

At 2.44 %wt/wt of chitosan content in the fibers, it was found that most of bacterial cells, for both types of bacteria, were attached on surface. When the chitosan contents was increased to 6.25, 13.04 and 28.57 %wt/wt, the number of bacterial cell attached on the surface was decreased. These results could be explained by the role of chitosan content and molecular weight of chitosan. As previously discussed, at high chitosan contents, the electrospinning yielded not only fibers but also beads. Decreasing chitosan contents in the electrospinning solutions could generate more fibers and less beads, resulting in increased surface area-to-volume ratio. High surface area-to-volume ratio of the fiber mats made it available for the bacterial cells to attach. Moreover, as it has been known that the positively charged chitosan interacts with negatively charged cell surface, the amino groups (NH_3^+) as the active functional group was found to be essential factor affecting the bacterial cells attachment [22-24]. Chitosan is a cationic polysaccharide with amino group at the C2 position. The addition of chitosan contents would also increase the number of NH_3^+ groups leading to the free amino group that could alter cell permeability. Due to outer membrane damage, it involves changes in the hydrophilicity and charge density of the cell surface.

To prove the effect of molecular weight on chitosan/PVA nanofibers, the chitosan with molecular weight 100 kDa, 400 kDa and 760 kDa were investigated, using 24 h for incubation time and Gram-negative *Acinetobacter baylyi* strain GFJ2 bacteria. The results are shown in Figure 4.39.

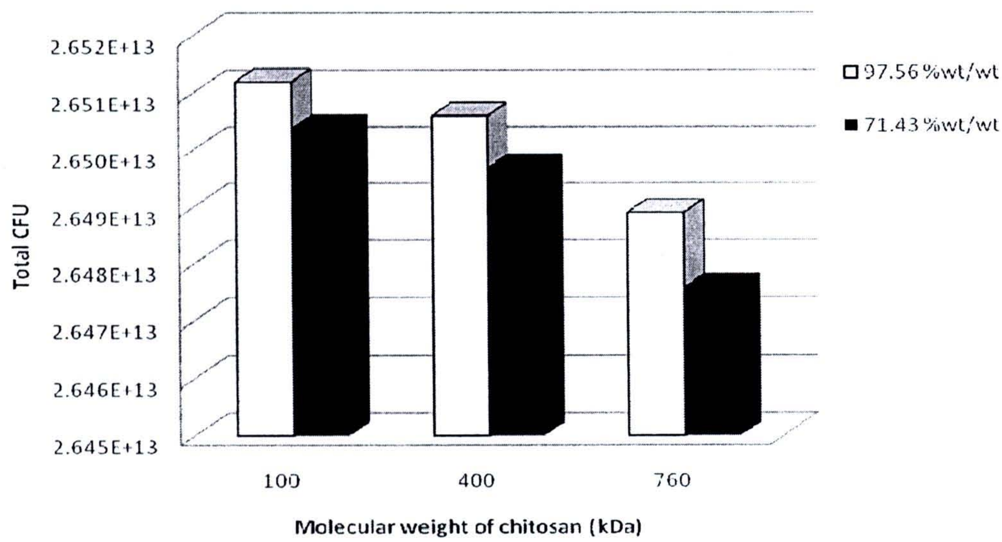


Figure 4.39 Total CFU of Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto chitosan/PVA nanofibers, at 24 h incubation time, formed from various PVA contents (%wt/wt) and molecular weight of chitosan.

These results could be confirmed that molecular weight of chitosan affected bacterial cells attachment. Closely relating to viscosity and spinability, molecular weight of chitosan affected the behavior of chitosan such as chain conformation, solubility and degree of substitution. High molecular weight chitosan could have block arrangement of acetylated and deacetylated units. Intermolecular interactions may reduce available sites on the chitosan molecule. The number of amino groups (NH_3^+) as the active functional group was low and resulted in reduced available sites for the bacterial cells to attach on the chitosan surfaces.

4.5 Viability of Bacterial Cells Attaching on Chitosan Nanofibers

In the immobilization by attachment, the bacterial cells were bounded to the carrier material via reversible surface interactions. The forces involved are ionic and H-bonding interaction as well as hydrophobic forces. Due to low amount of free amino groups on its surface, chitosan could be considered as neutral and there is a low possibility of interaction through H-bonding or ionic forces. As the amount of free amino groups were increased, the ionic and H-bonding forces becomes more relevant. These behaviors are closely related to cells viability on chitosan. In this sections, the viability of attaching on chitosan prepared by various is presented.

4.5.1 Viability of bacterial cells attaching on hydrolyzed chitosan nanofibers

4.5.1.1 Effect of incubation time

In order to determine the bacterial viability on hydrolyzed chitosan nanofibers at various incubation times, the hydrolysis time of 6 and 48 h were chosen. The initial optical densities (OD_{600}) of bacterial cells solution (i.e., free cells) for *A. baylyi* strain GFJ2 and *B. agri* strain 13 were 0.85 and 1.0, which were corresponding to the colony forming unit of 1.8981 x 10^{13} and 1.4760 x 10^{9} CFU, respectively. The percentage of live cells attaching on hydrolyzed chitosan nanofibers is shown in Figure 4.40-4.41. The florescence images of dyed live cells (green) and dead cells (red) on hydrolyzed chitosan are shown in Figure 4.42-4.45.

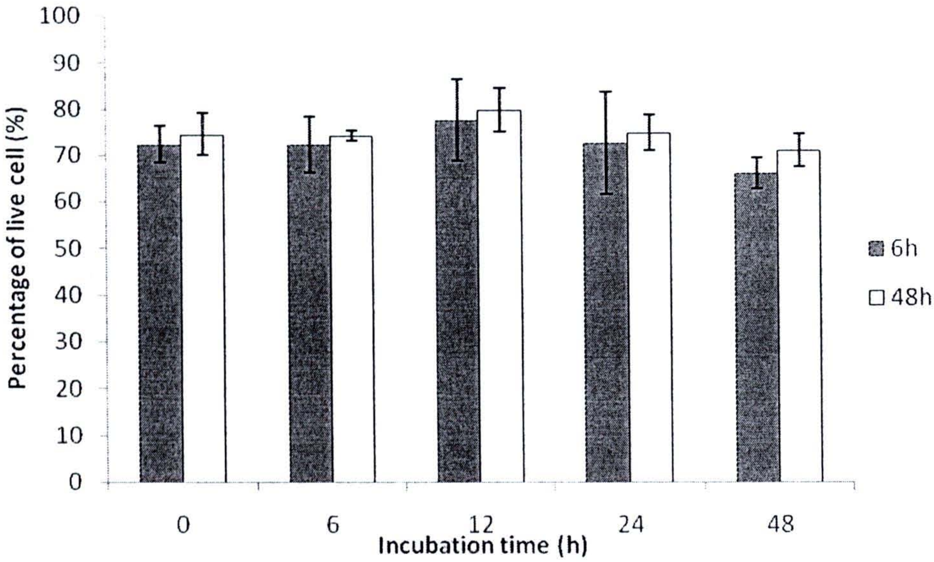


Figure 4.40 Percentage of live cells for Gram-positive *Brevibacillus agri* strain No.13 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 6 and 48 h, after various incubation times.

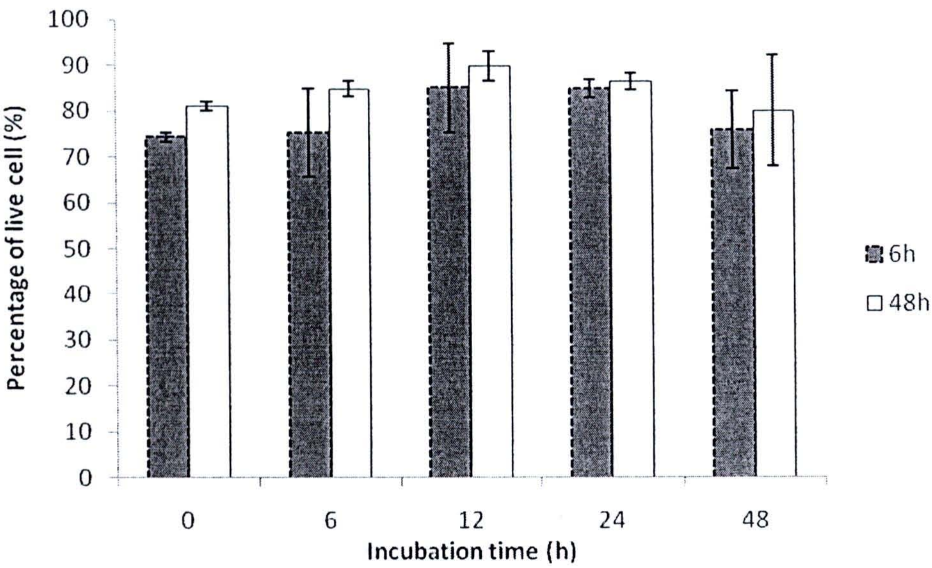
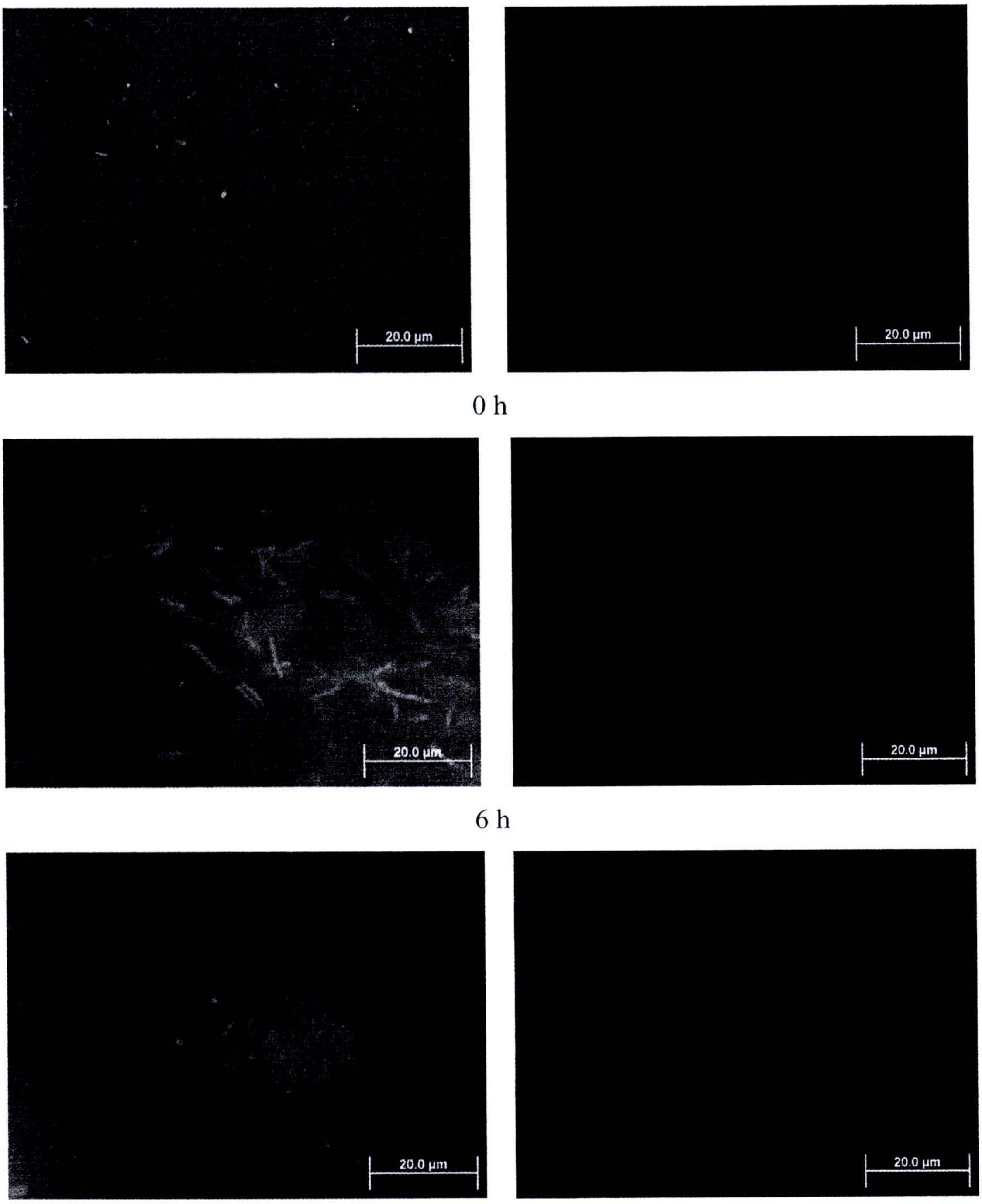


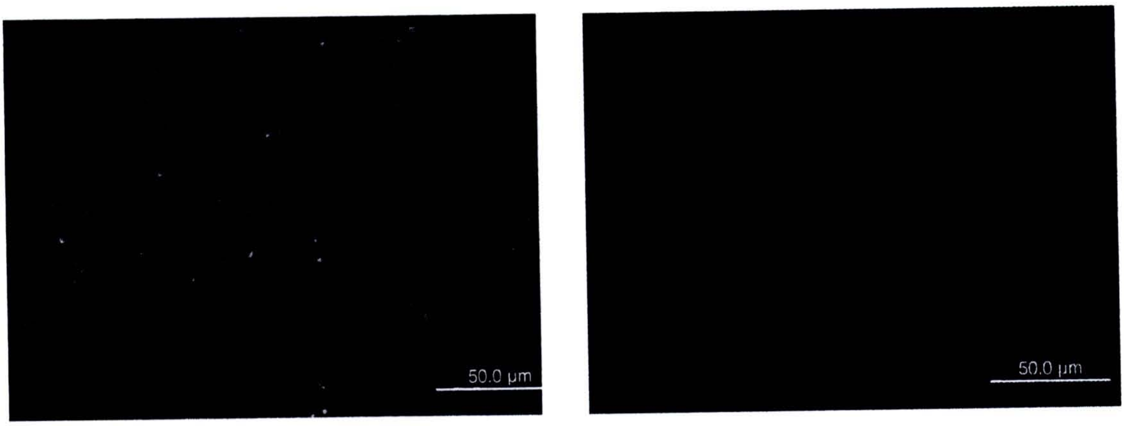
Figure 4.41 Percentage of live cells for Gram-negative *Acinetobacter baylyi* strain GFJ2 attached onto electrospun chitosan fibers, formed from chitosan hydrolyzed for 6 and 48h, after various incubation times.

Similar trend for both of Gram-negative and Gram-positive was found. The percentage of live cells attaching on the hydrolyzed chitosan was slightly increased when the incubation time was increased to 12 h. When the incubation time between chitosan fibers and the bacteria cells was prolonged to 24 h, the percentage of live cells attaching on the sample was slightly decreased. Nevertheless, the fraction of live cells was relatively high. It is possible that the microorganism may be induced to attach by altering the physical and chemical properties according to ionic attraction of bacterial cells and the chitosan surface. Chitosan is a cationic polysaccharide that acts as a glue to initiate bacterial-surface interactions. Increasing the contact time between chitosan fibers and the bacterial cells tends to increase the interaction rate. However, the carbon sources could be presumed to limit in this study. When the incubation time was prolonged to 12 h, much of the carbon sources was still available for cells. After the incubation time up to 24 h, the carbon source for bacterial cells was decreased. In addition to the comparison of the percentage of viable cells for both Gram-positive and Gram-negative, it was found that the survival of Gram-positive bacteria on chitosan was less than Gram-negative. This result could be explained by the difference in chemical properties of their cell walls. Gram-positive cell walls do not posses a lipid outer membrane while Gram-negative cell walls posses an outer membrane consisting of various lipid complexes [25, 26]. When the positive charges on amino groups of chitosan interacted with negatively charged bacterial cells wall, it leaded to the leakage of intracellular constituents.

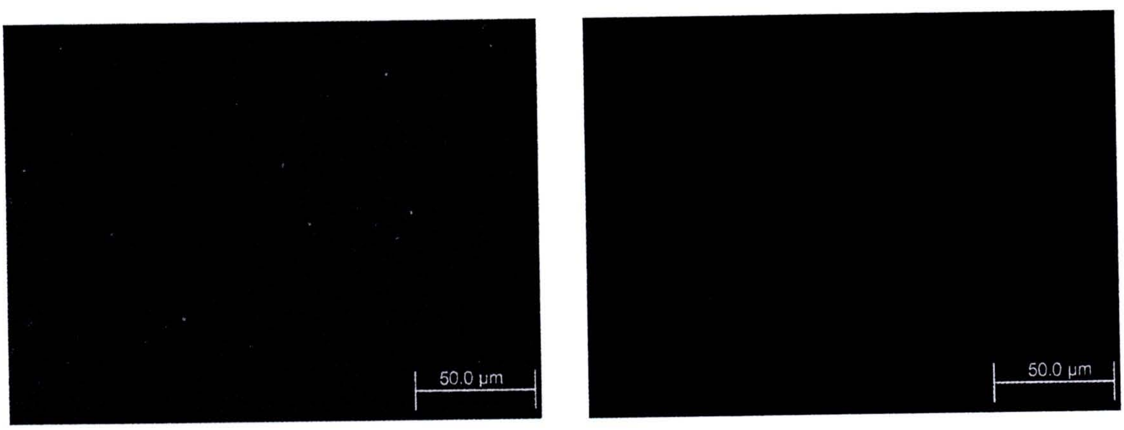


12 h

Figure 4.42 Fluorescence micrograph of Gram- positive *B. agri* strain 13 attaching on the electrospun chitosan nanofibers, that were prepared from chitosan hydrolyzed for 6 h, after various incubation time. The green fluorescence indicated live cells, while the red fluorescence indicated dead cells.



24 h



48 h

Figure 4.42 (continued).

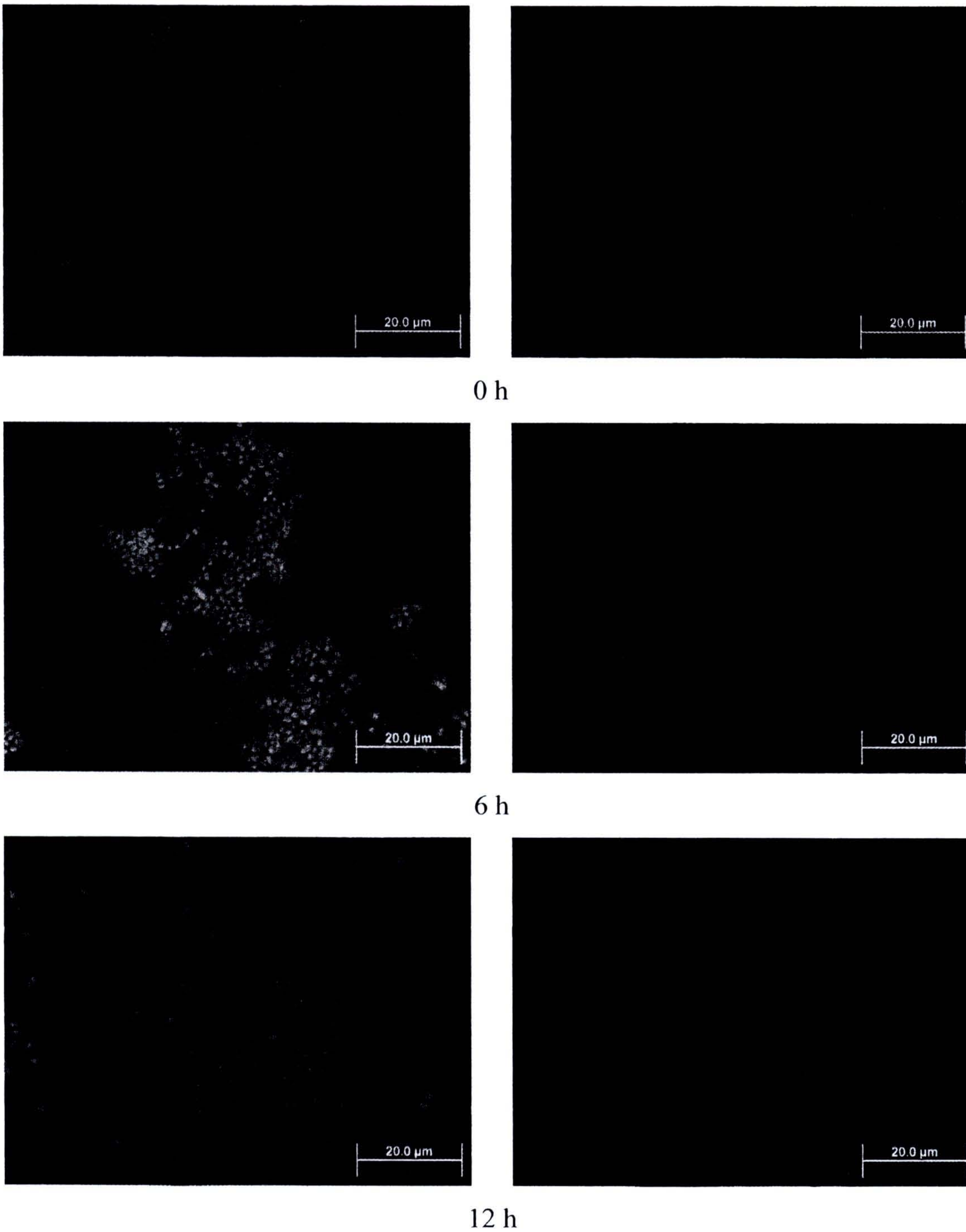
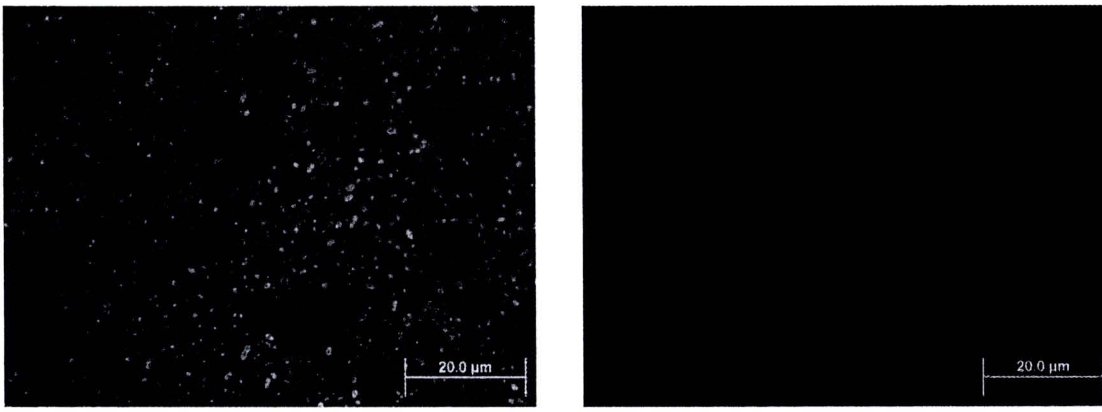
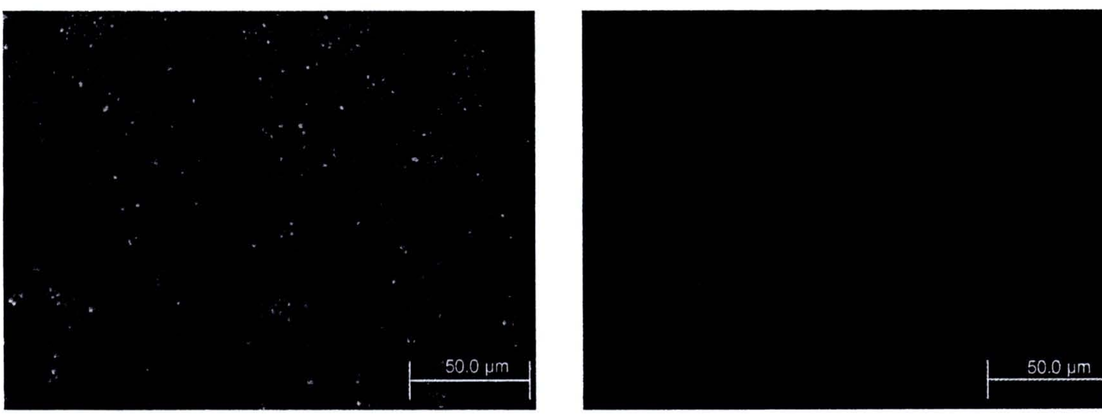


Figure 4.43 Fluorescence micrograph of Gram- negative *A. baylyi* strain GFJ2 attaching on the electrospun chitosan nanofibers, that were prepared from chitosan hydrolyzed for 6 h, after various incubation time. The green fluorescence indicated live cells, while the red fluorescence indicated dead cells.

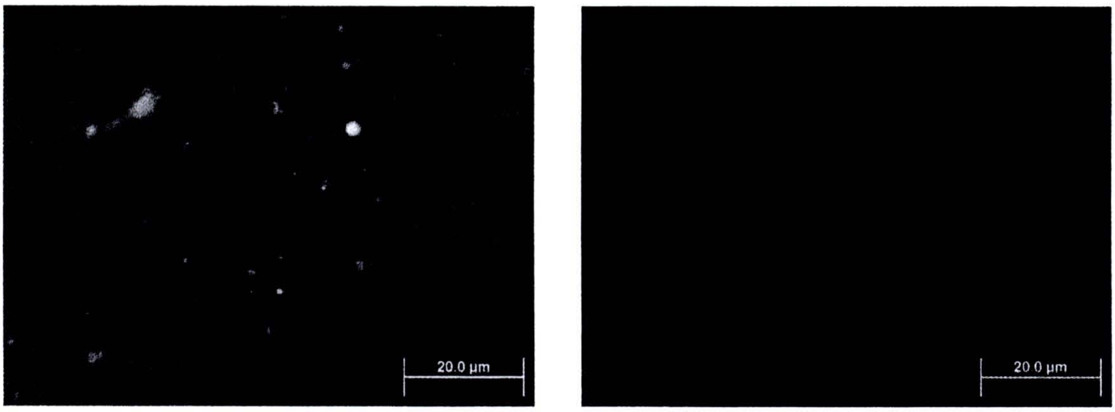


24 h

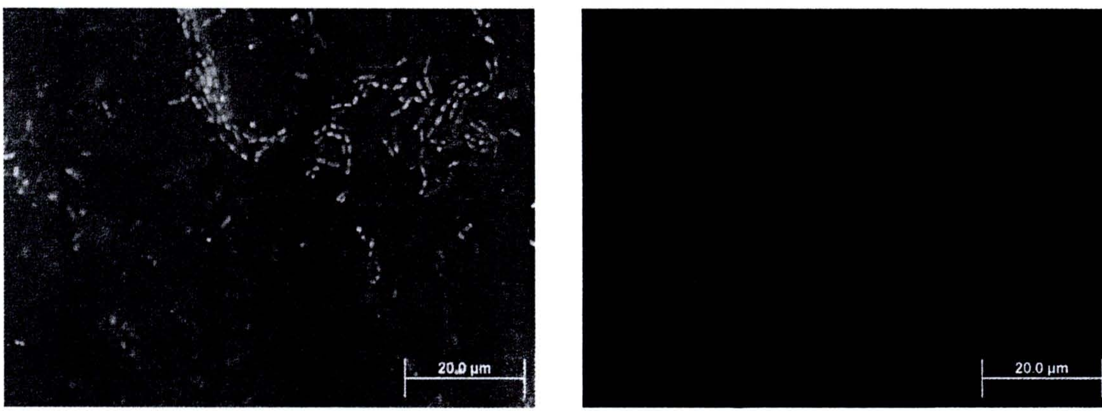


48 h

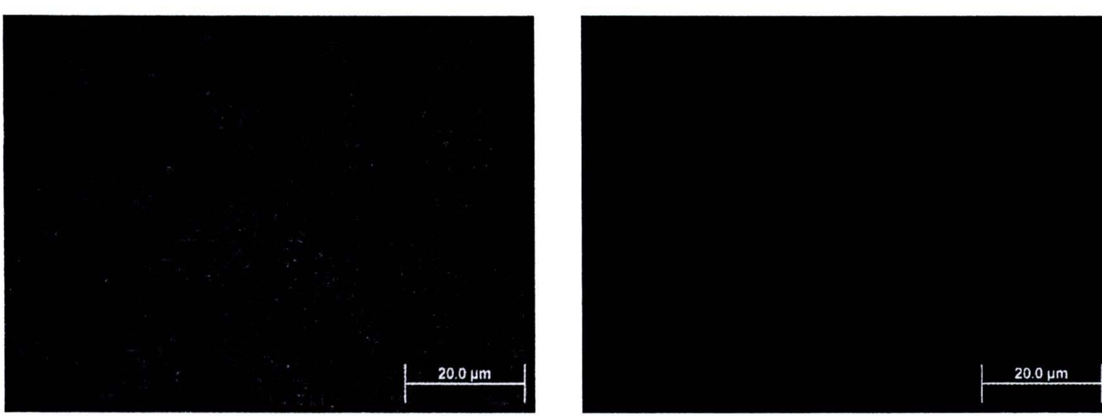
Figure 4.43 (continued).



0 h



6 h



12 h

Figure 4.44 Fluorescence micrograph of Gram- positive *B. agri* strain 13 attaching on the electrospun chitosan nanofibers, that were prepared from chitosan hydrolyzed for 48 h, after various incubation time. The green fluorescence indicated live cells, while the red fluorescence indicated dead cells.

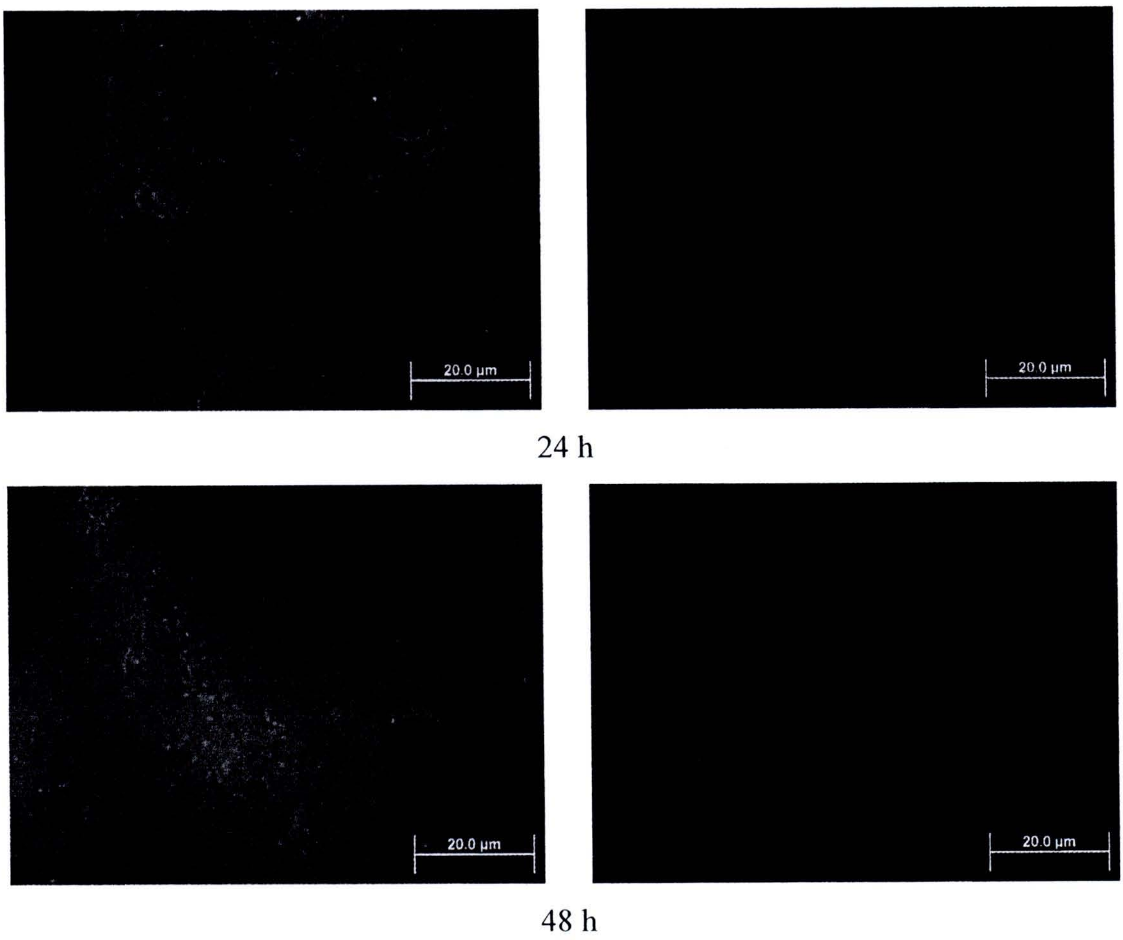


Figure 4.44 (continued).

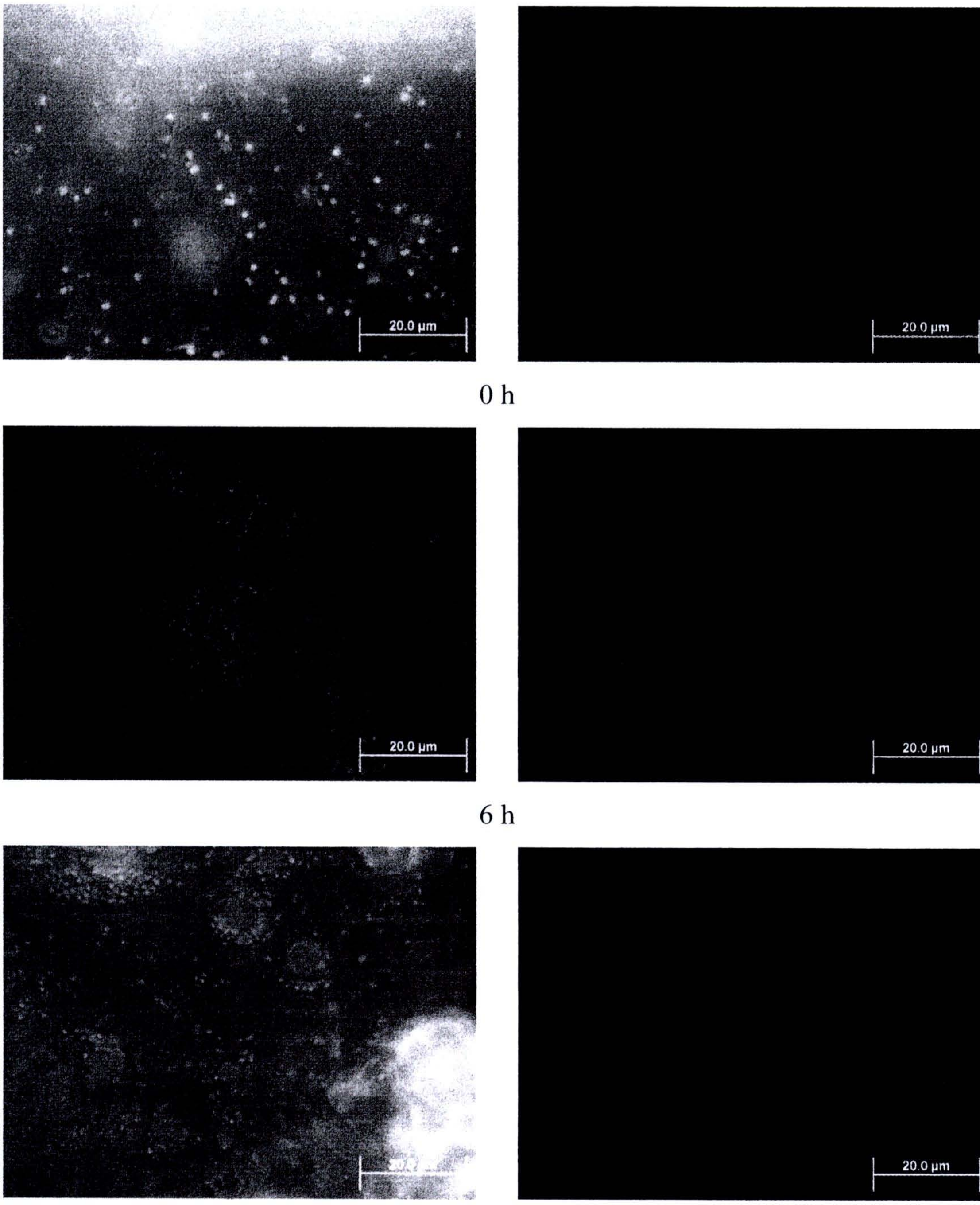
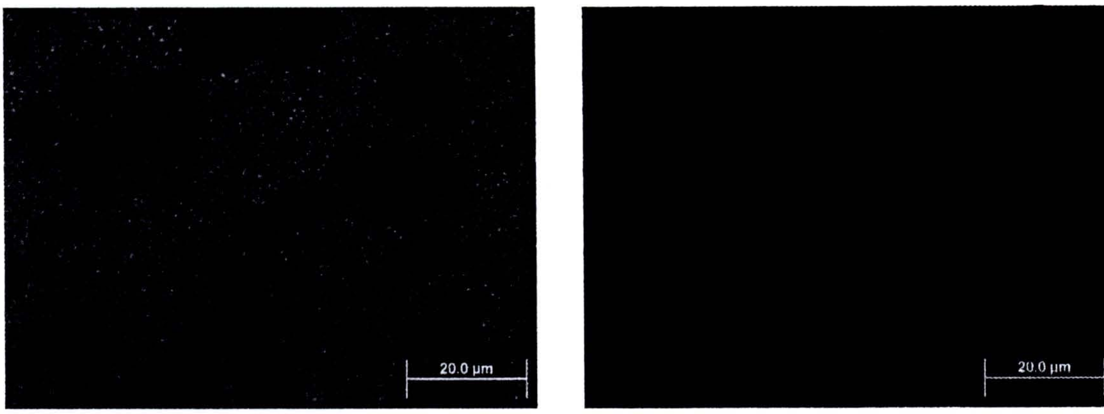
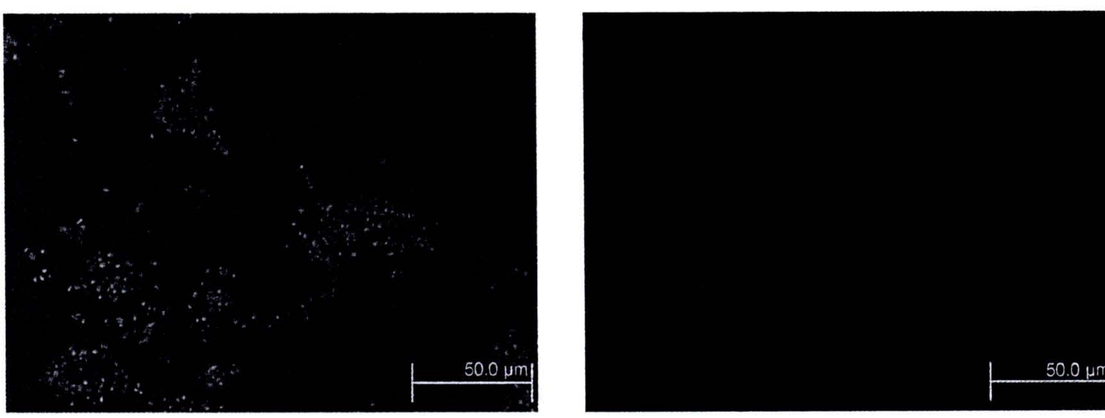


Figure 4.45 Fluorescence micrograph of Gram- negative *A. baylyi* strain GFJ2 attaching on the electrospun chitosan nanofibers, that were prepared from chitosan hydrolyzed for 48 h, after various incubation time. The green fluorescence indicated live cells, while the red fluorescence indicated dead cells.



24 h



48 h

Figure 4.45 (continued).

4.5.1.2 Effect of hydrolysis time of chitosan

To compare the effect of hydrolysis time of chitosan on cells viability, the incubation time of 12 and 24 h were chosen. It was found that the percentage of live cells for both types of bacteria attaching on the surface increased when the hydrolysis time of chitosan was increased. The results are shown in Figure 4.46-4.47. The trend of percentage of live cells with respect to the hydrolysis time was similar for both incubation times. Increasing of hydrolysis time of chitosan resulted in increased cells viability. The results are shown in Figure 4.48-4.49.

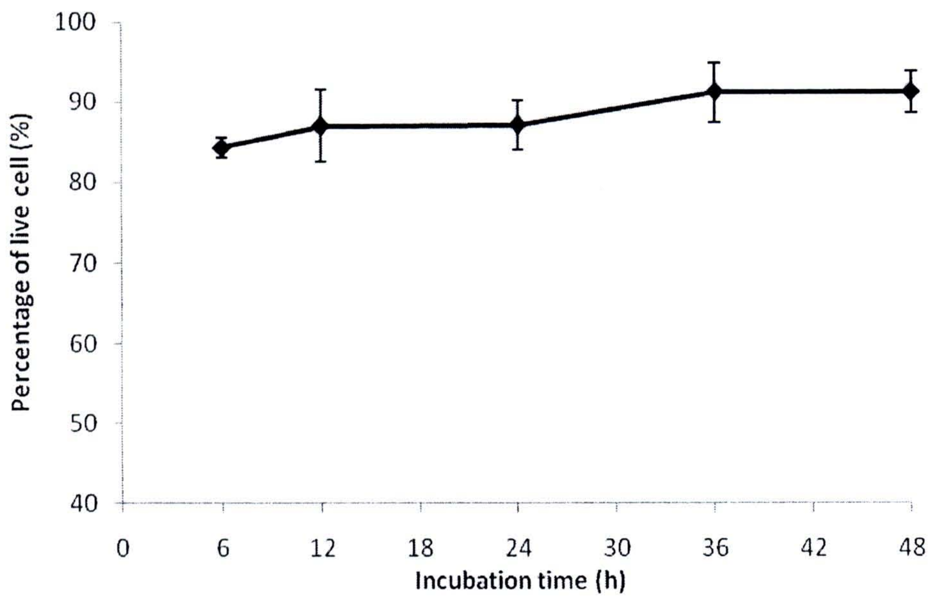


Figure 4.46 Percentage of live cells for Gram-positive *Brevibacillus agri* strain No.13 attaching on electrospun chitosan fibers, prepared from chitosan being hydrolyzed for period time, after the incubation time of 12 h.

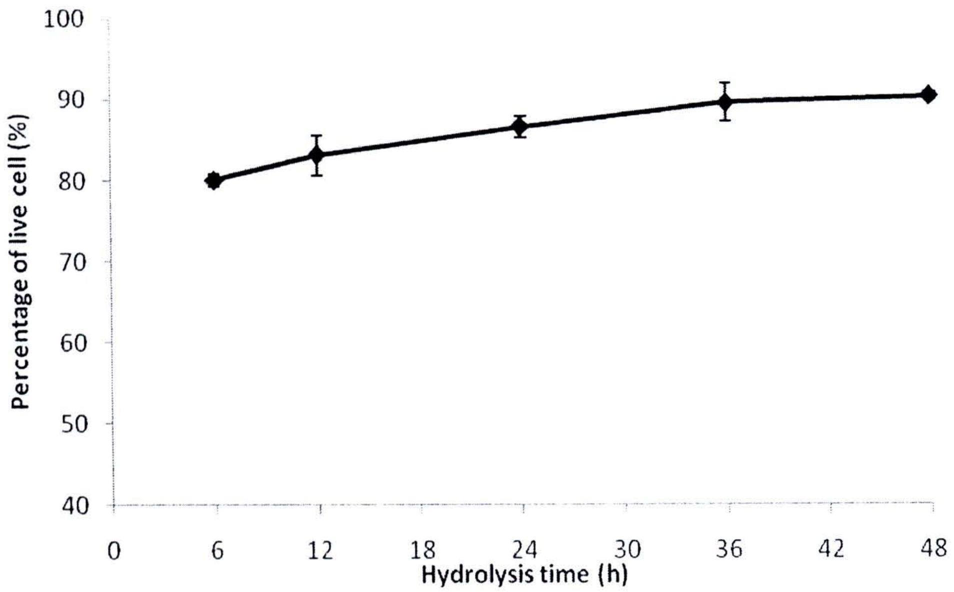


Figure 4.47 Percentage of live cell for Gram-positive Brevibacillus agri strain No.13 attaching on electrospun chitosan fibers, prepared from chitosan being hydrolyzed for period time, after the incubation time of 24 h.

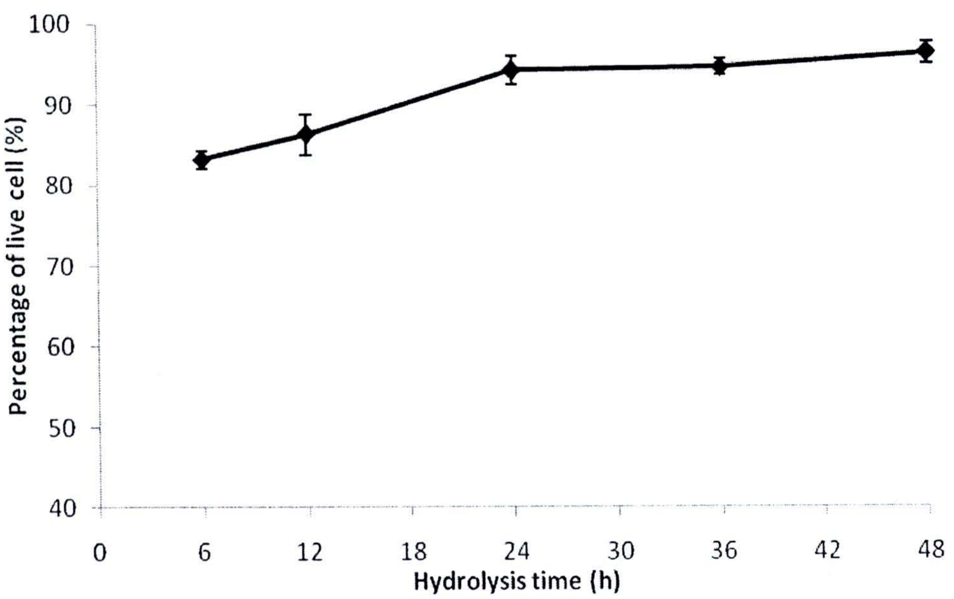


Figure 4.48 Percentage of live cell for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on electrospun chitosan fibers, prepared from chitosan being hydrolyzed for period time, after the incubation time of 12 h.

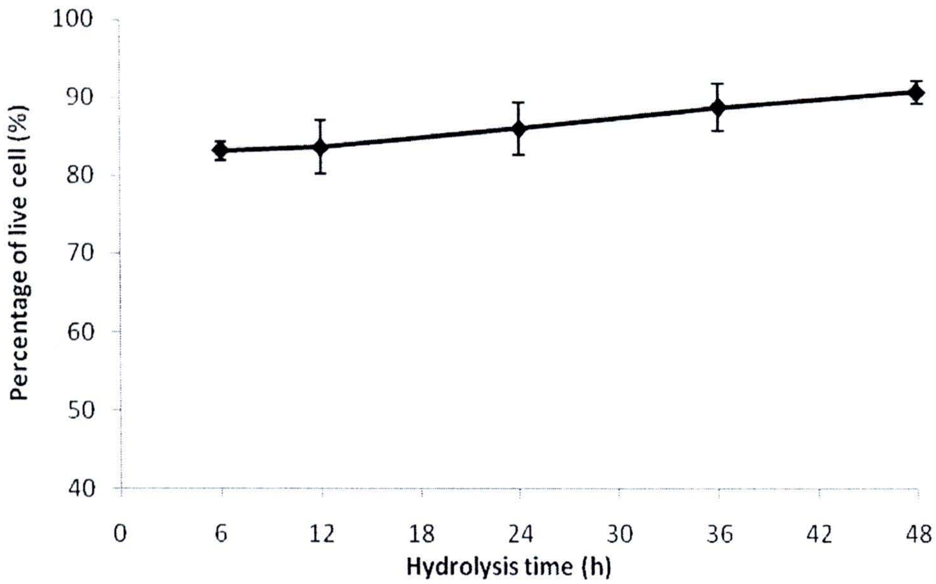


Figure 4.49 Percentage of live cell for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on electrospun chitosan fibers, prepared from chitosan being hydrolyzed for period time, after the incubation time of 12 h.

The results were not only explained by the surface area-to-volume ratio available for cells attachment on the surface but also due to a significant role of the cationic behavior of chitosan. Although the degree of deacetylation is the one of the most important chemical characteristics of chitosan, the influence of molecular weight on the viscosity of chitosan solution also played a significant role.

To clearly explain the hydrolysis of chitosan, the FT-IR spectrum of chitosan after being hydrolyzed for various period of time shown in Figure 4.50. Chitosan shows a broad O-H stretching at wave number between 2800-2900 cm^{-1} and the absorbtion corresponding to polysaccharide structure at wave number between 1155-850 cm^{-1}. Another major absorption band at 1600 cm^{-1} represents the free amino group ($-NH_2$) at C2 position of glucosamine. The peak at 1641 cm^{-1} and 1324 cm^{-1} corresponds to a CO-NH of amide I and amide II deformation to CH_2 group [27-30]. Noted that, for chitosan, the peak at 1600 cm^{-1} has larger intensity than at 1641 cm^{-1} and 1324 cm^{-1} which suggests effective deactylation. Decreasing of the peak at 1641 cm^{-1} and 1324 cm^{-1} corresponding to higher deactylation. From the results, at 6, 12 , 24, 36 and 48 h of hydrolysis, it was indicated that there was an intensification of the peak at 1600 cm^{-1} while the peak at 1641 cm^{-1} of CO-NH appeared only after 48 h for hydrolysis time. However, the difference was shown at the peak of 1324cm^{-1} which indicated that when the hydrolysis time was prolonged to 48 h, it resulted in the increased deacetylation. It is possible due to the propagation of the hydrolysis reaction. Nevertheless, all of hydrolyzed chitosan is not fully deacetylated. The interaction between chitosan and anionic surface active species of phospholipids from cell wall depended on the amino group at C2 position. The presence of free amino groups along the chitosan chain allows this macromolecule to dissolve in diluted acidic solution. Increasing free amino groups, which also related to the increased chitosan solubility, leaded to the increase in positive charge of the polymer chains for cell to attach on the surface.

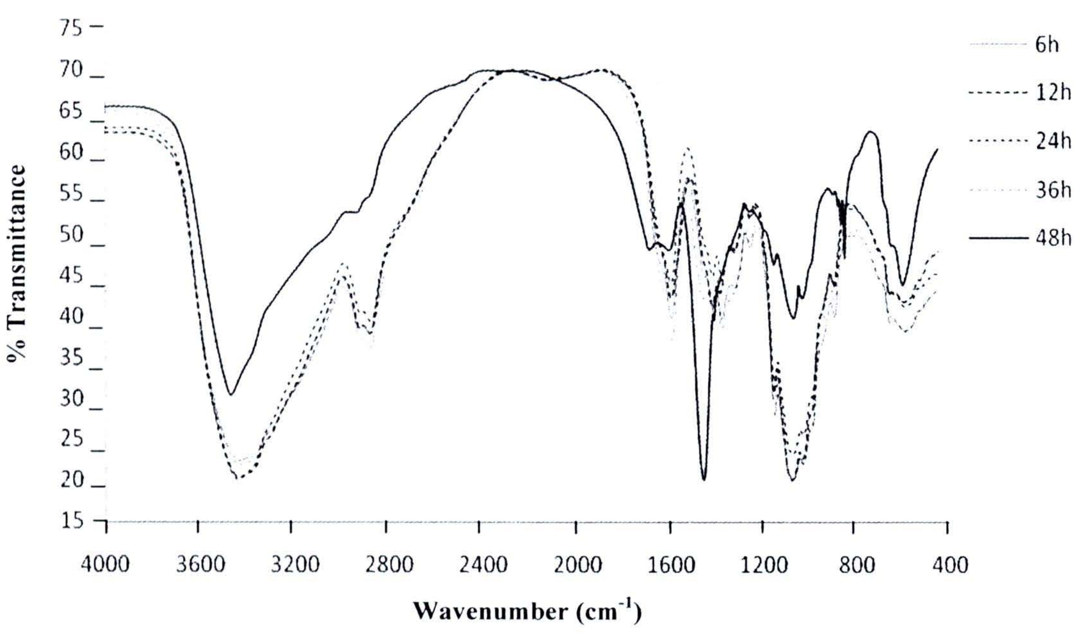


Figure 4.50 FT-IR spectra of chitosan being hydrolyzed for various period of time.

In fact, the highest of cells viability was found on chitosan hydrolyzed for 48 h. It is possible to presume in regard of biocompatibility that decreasing the molecular weight of chitosan was able to decrease a toxicity toward bacteria. To confirm the effect of chitosan molecular weight on cell viability, the FT-IR spectrum of chitosan obtained at different hydrolysis times were analyzed. The peak of polysaccharide structure at wave number between 896-1155 cm^{-1} was found. It was found that when the hydrolysis time was increased, the intensity of the polysaccharide group was decreased and the peak corresponding to ring breathing at 849-901 cm^{-1} appeared. This result indicated that the saccharide structure of the molecule was opened by dehydration of saccharide rings and cut into the polymer chain by hydroxyl groups of NaOH. It is attributed to a complex process including dehydration of sacharide rings, depolymerization and decomposition of the acetylated and deacetylated units of polymers that could reduce the polymer chain length and the molecular weight of chitosan [31]. In addition, the solubility of the hydrolyzed chitosan was increased due to the decreased molecular weight [32]. The live cells and dead cells are shown in Figure 4.51-4.54.

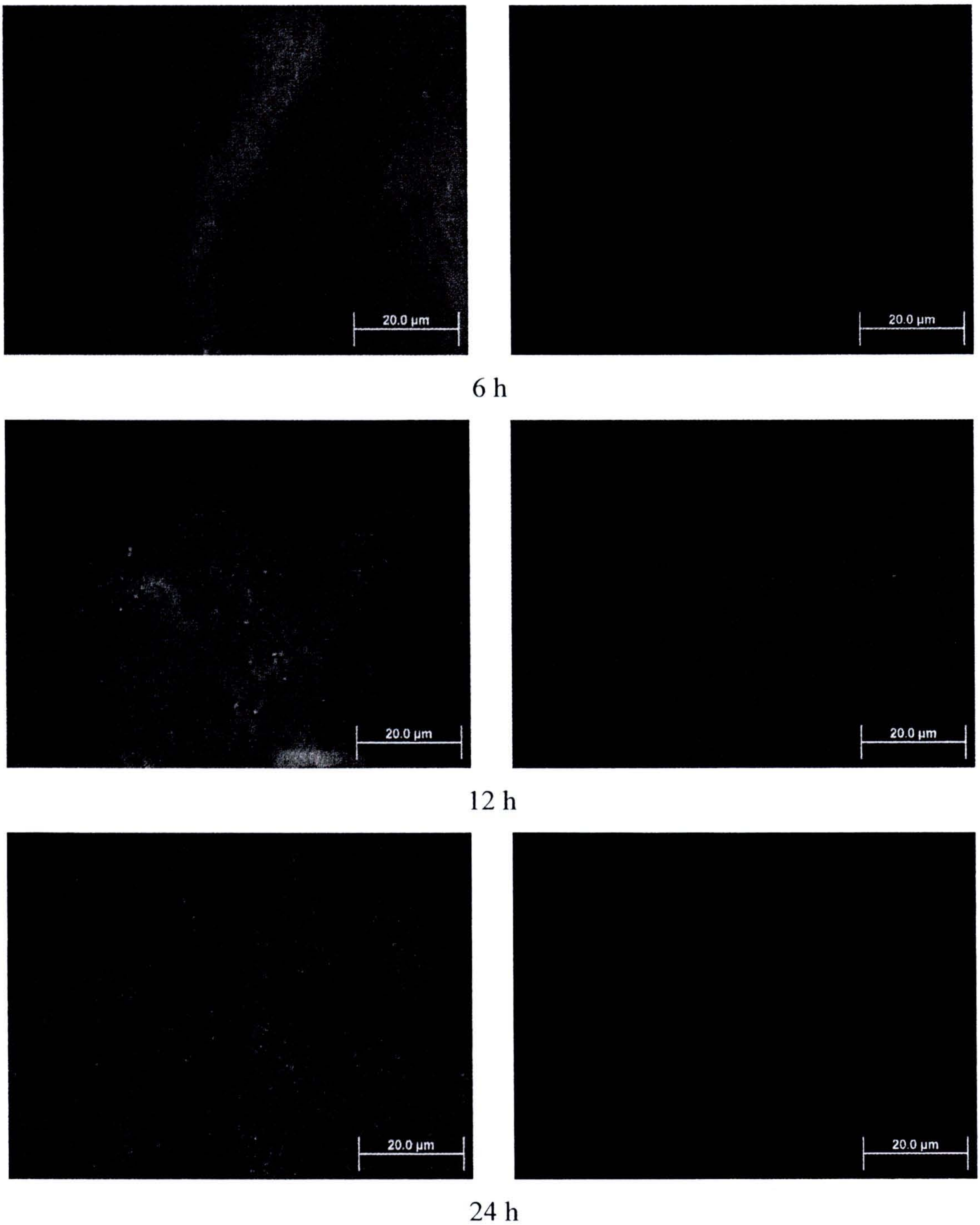
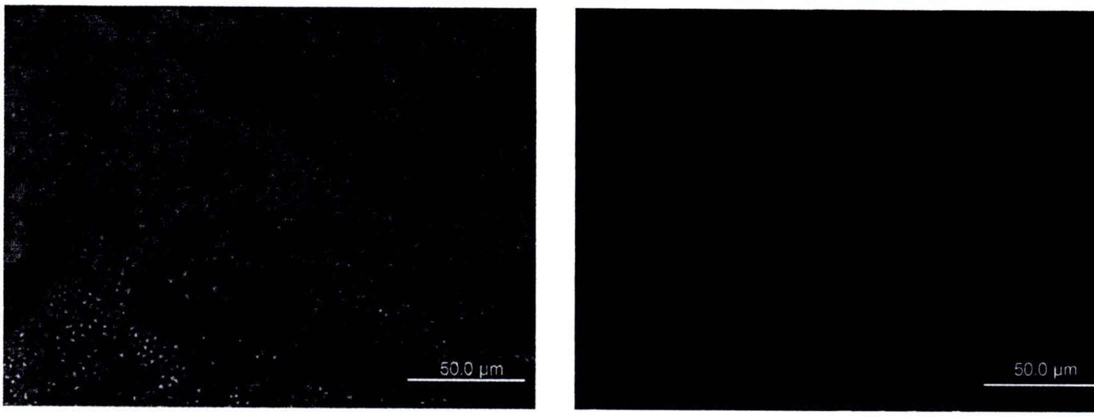
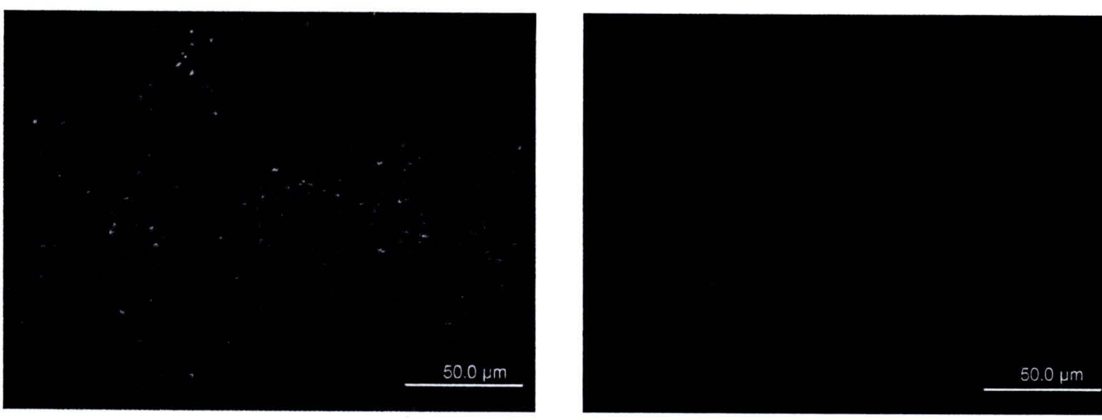


Figure 4.51 Fluorescence micrographs of Gram-positive *B. agri* No.13 attached on electrospun chitosan nanofibers that were prepared from chitosan hydrolyzed for various period of time, after the incubation time of 12 h.



36 h



48 h

Figure 4.51 (continued)

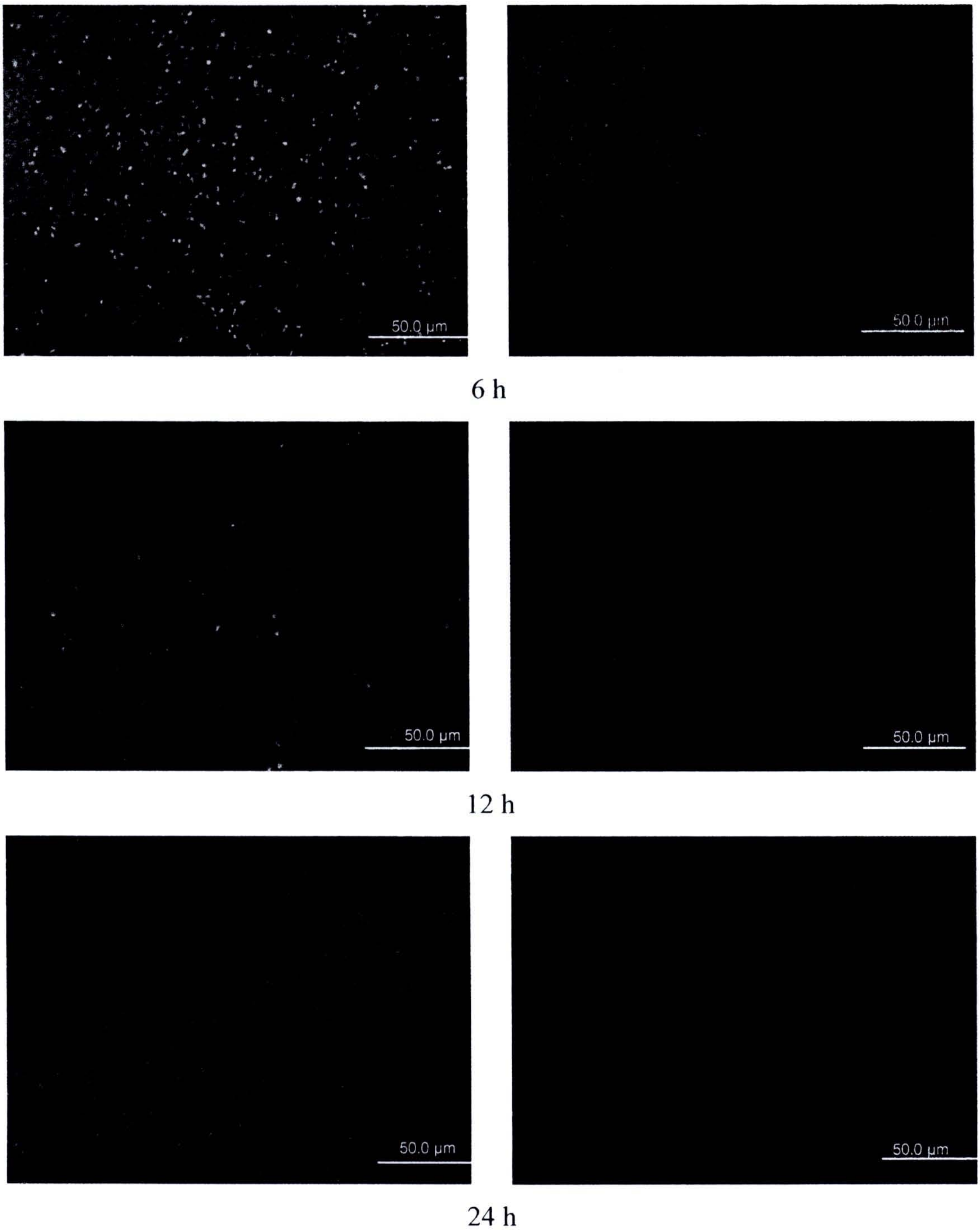
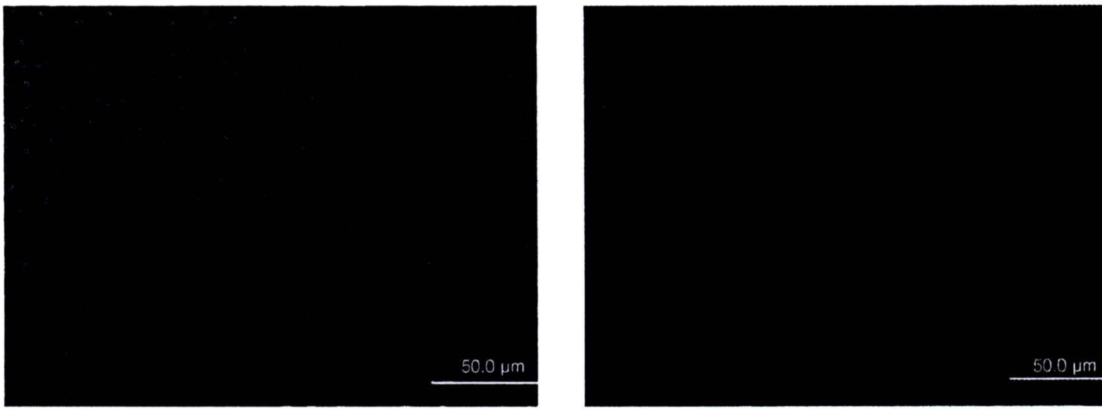
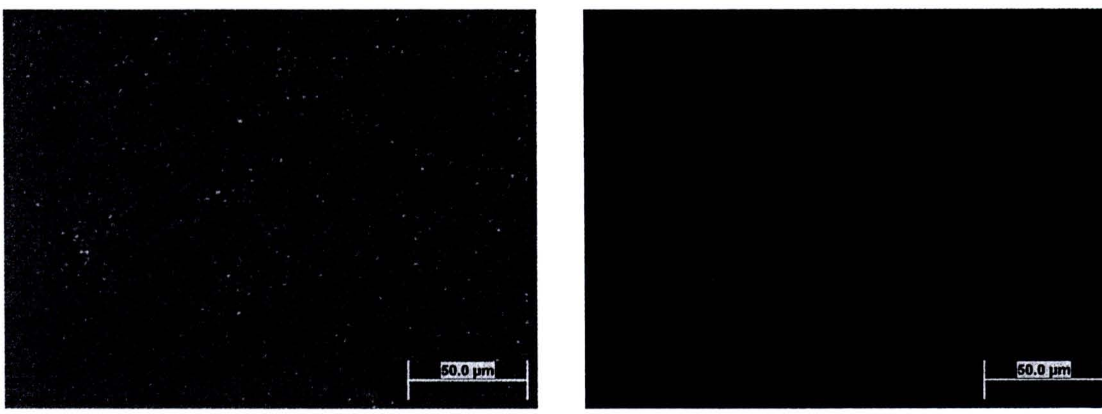


Figure 4.52 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attached on electrospun chitosan nanofibers that were prepared from chitosan hydrolyzed for various period of time, after the incubation time of 12 h.



36 h



48 h

Figure 4.52 (continued)

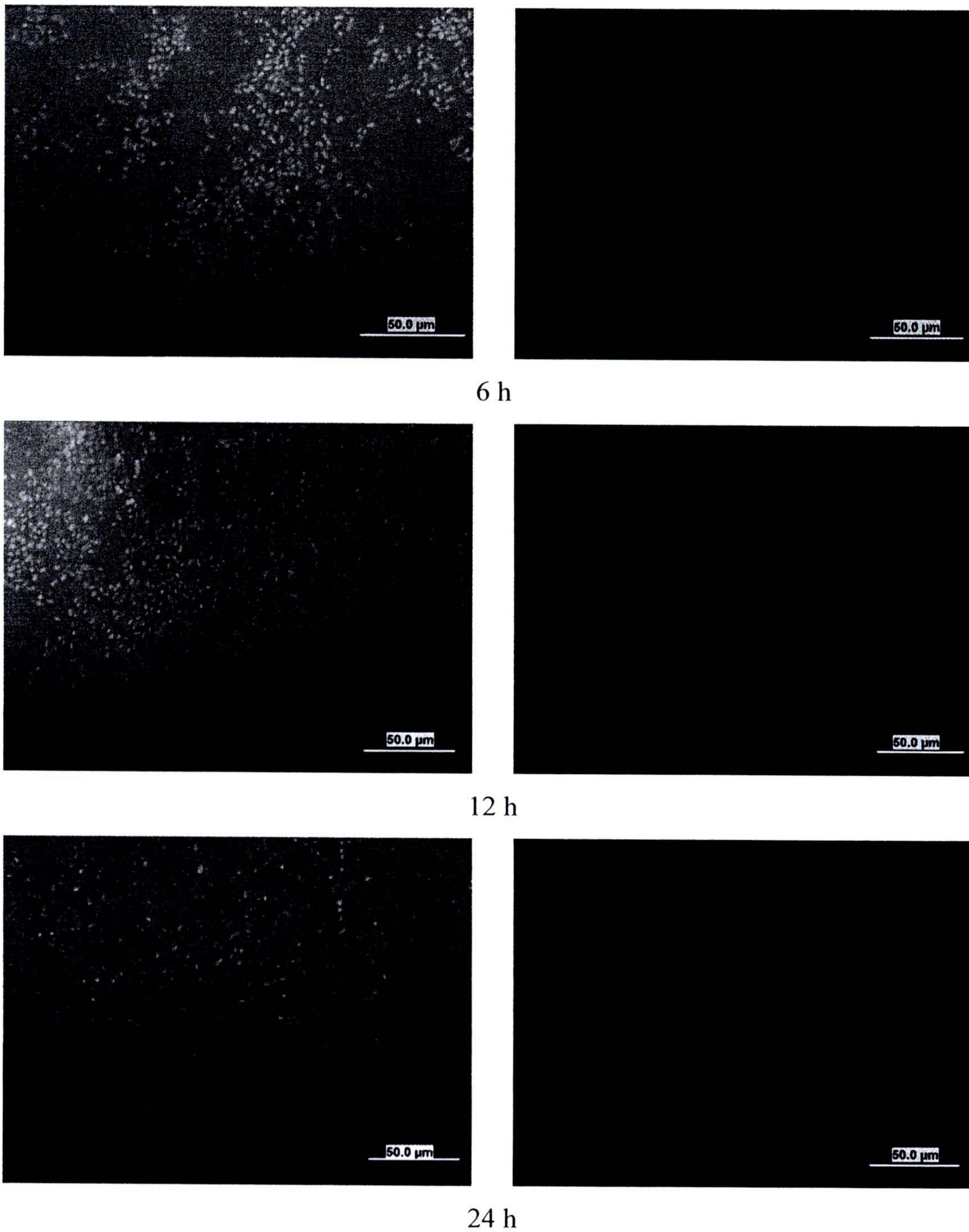
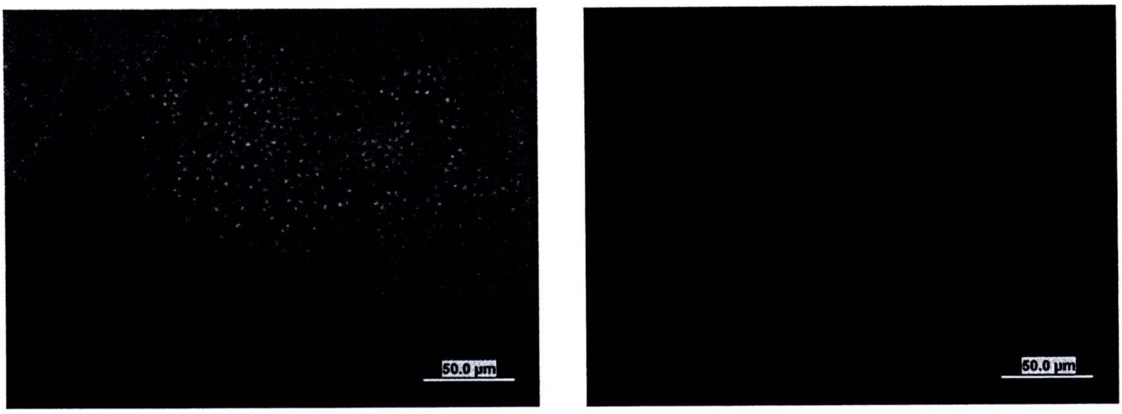


Figure 4.53 Fluorescence micrographs of Gram-positive *B. agri* No.13 attached on electrospun chitosan nanofibers that were prepared from chitosan hydrolyzed for various period of time, after the incubation time of 24 h.

36 h



48 h

Figure 4.53 (continued)

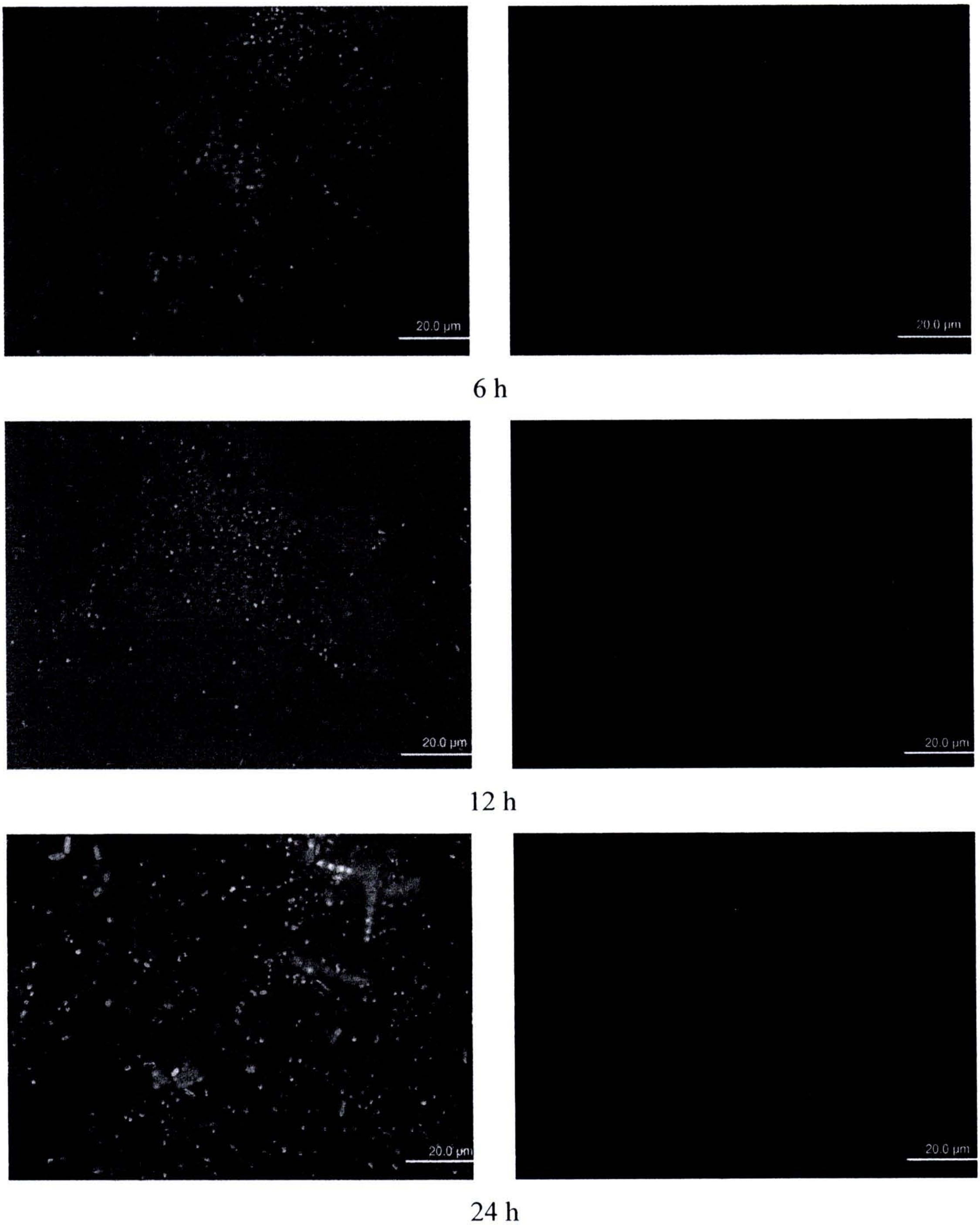
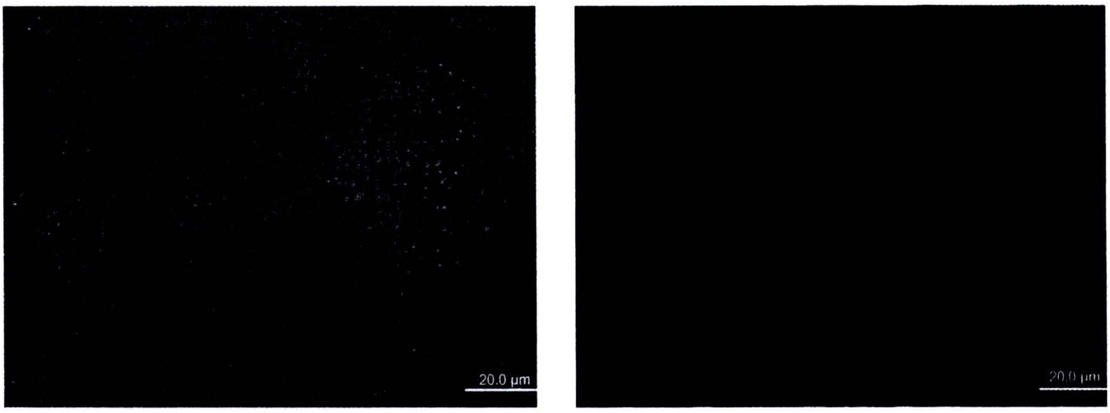
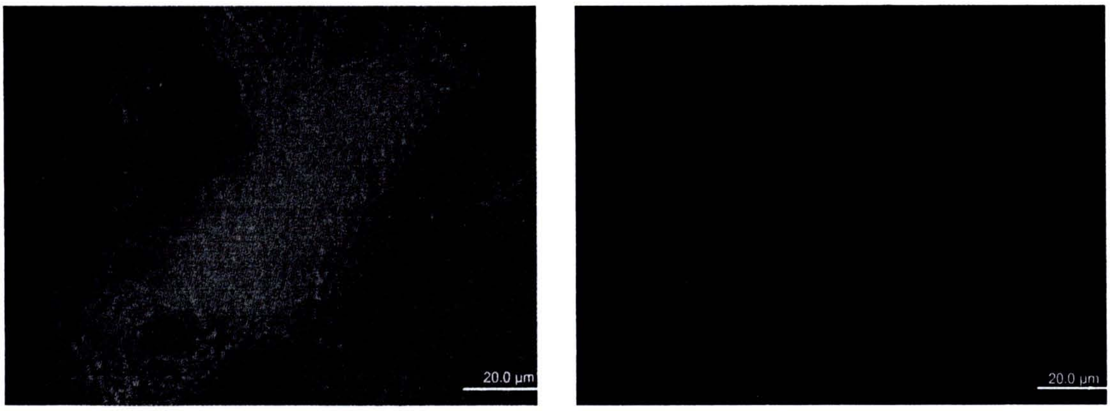


Figure 4.54 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attached on electrospun chitosan nanofibers that were prepared from chitosan hydrolyzed for various period of time, after the incubation time of 24 h.



36 h



48 h

Figure 4.54 (continued)

4.5.2 Viability of bacterial cells attaching on hydrolyzed chitosan film

For the comparative study of the percentage of live cells attaching on chitosan films and nanofibers, the incubation time of 12 and 24 h were chosen. Similar trend for both of Gram-negative and Gram-positive bacteria were found. Higher percentage of viable cells were attaching on chitosan fibers than those on chitosan films. Moreover, both types of bacterial cells showed the increase in the percentage of live cells attaching on the surface of chitosan being hydrolyzed for prolonged period of time. The results are shown in Figure 4.55-4.56 for Gram-negative *Acinetobacter Baylyi* strain GFJ2 and in Figure 4.57 -4.58 for Gram-positive *Brevibacillus agri* strain 13.

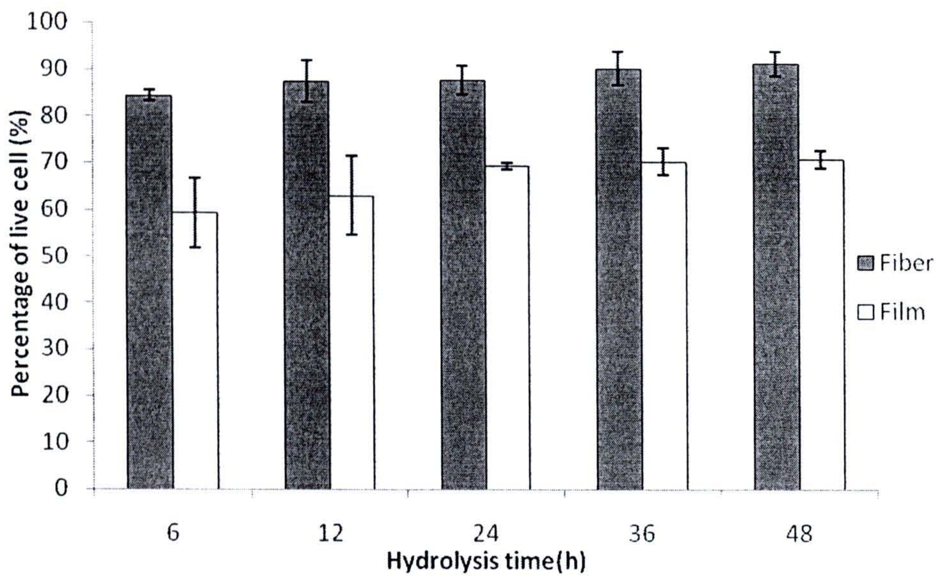


Figure 4.55 Percentage of live cells for Gram-positive *Brevibacillus agri* strain 13 attaching on electrospun chitosan fibers and chitosan films, prepared from chitosan being hydrolyzed for various periods of time, after the incubation time of 12 h.

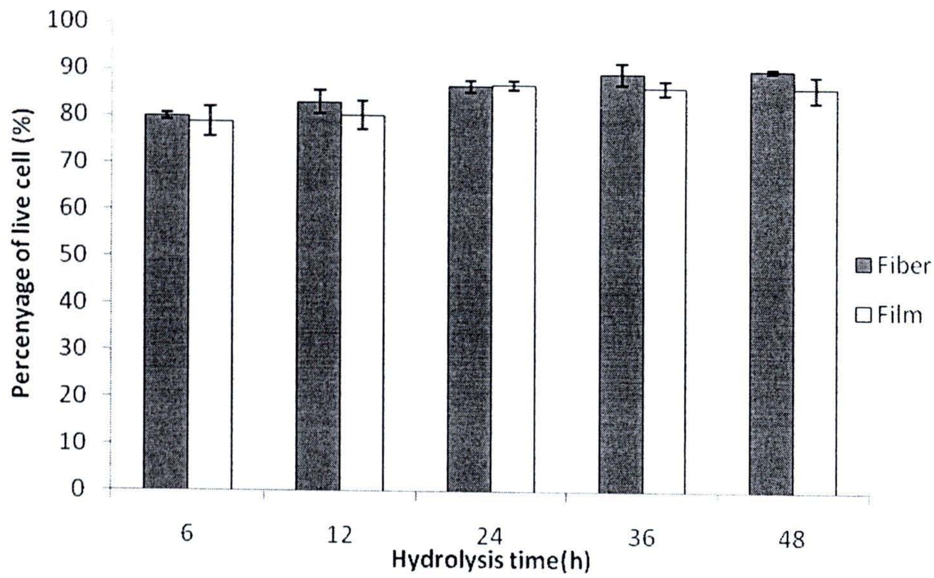


Figure 4.56 Percentage of live cells for Gram-positive *Brevibacillus agri* strain 13 attaching on electrospun chitosan fibers and chitosan films, prepared from chitosan being hydrolyzed for various periods of time, after the incubation time of 24 h.

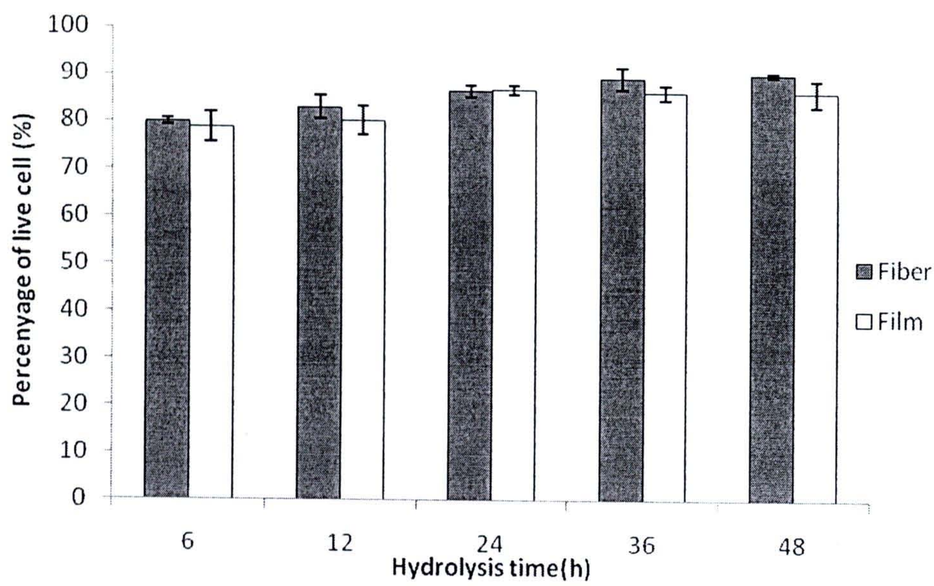


Figure 4.57 Percentage of live cells for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films, prepared from chitosan being hydrolyzed for various periods of time, after the incubation time of 12 h.

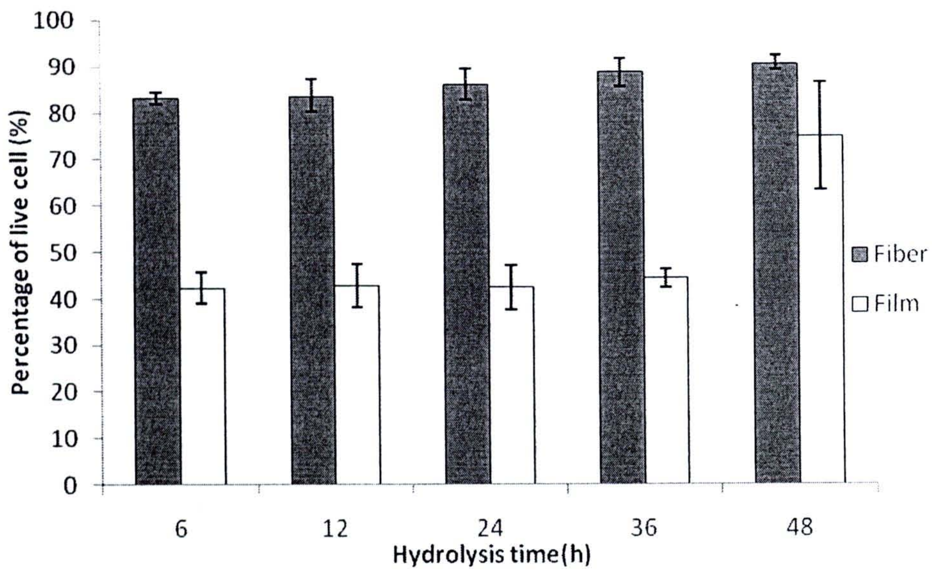


Figure 4.58 Percentage of live cell for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films, prepared from chitosan being hydrolyzed for various periods of time, after the incubation time of 24 h.

Although hydrolyzed chitosan nanofibers and films were prepared from the same solution, it was found that the chitosan in form of nanofibers resulted in higher cells viability than chitosan films. These results could be due to a significant role of a surface area-to-volume ratio. A sheet of randomly aligned fibers showed the surface morphology with grooves, ridges and highly porousity formed between the nanofibers that assisted cell attachment. The high porousity of the nanofibers also allowed cell migration, nutrient flow and oxygen transfer to immobilized microorganisms thus enhancing cell supporting capacity and consequently thier viability. Availability of oxygen is one of the most important parameters. The nanofibers which was more porous than the films allowed oxygen diffusion from the bottom of the sheet though the nanofibers sheet to the bacterial cells attaching on top of the sheet, in addition to the direct diffusion to the cells. Oxygen has transferred into chitosan support via diffusion due to difference in oxygen concentration in direction perpendicular to the chitosan sheet. It should be noted that the observation by the fluorescence microscope revealed multi-layers of cells on top of the chitosan sheet. For the cell attachment on chitosan films, most of the dead cells were observed in the bottom layer close to chitosan. It is possible that low oxygen concentration of the cells stack caused cells to die. Furthermore, negative charges of cells wall interacted with amino groups from chitosan such that it resulted in damages in outer membrane of the cells, which changed the hydrophilicity and charge density of the cell surface. The fluorescence micrographs of live cells and dead cells on chitosan fibers and chitosan films prepared at various conditions are shown in Figure 4.59-4.78.

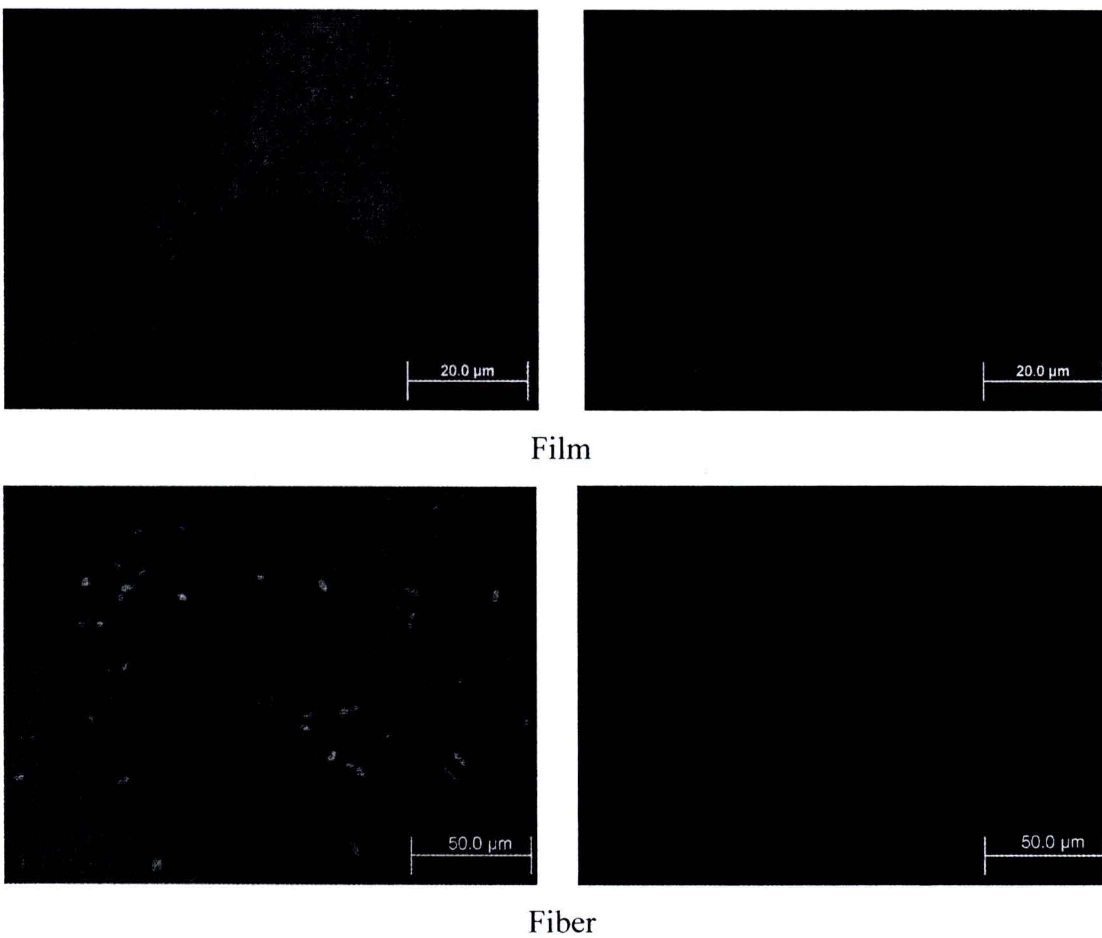
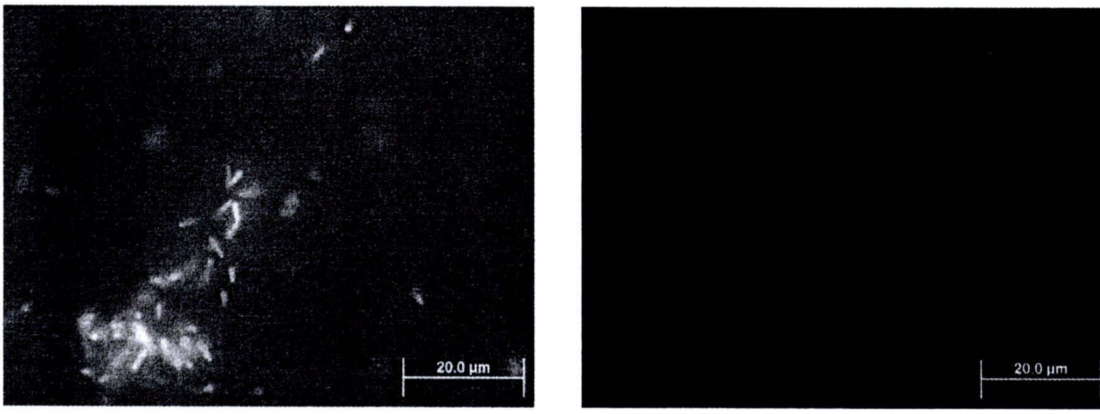
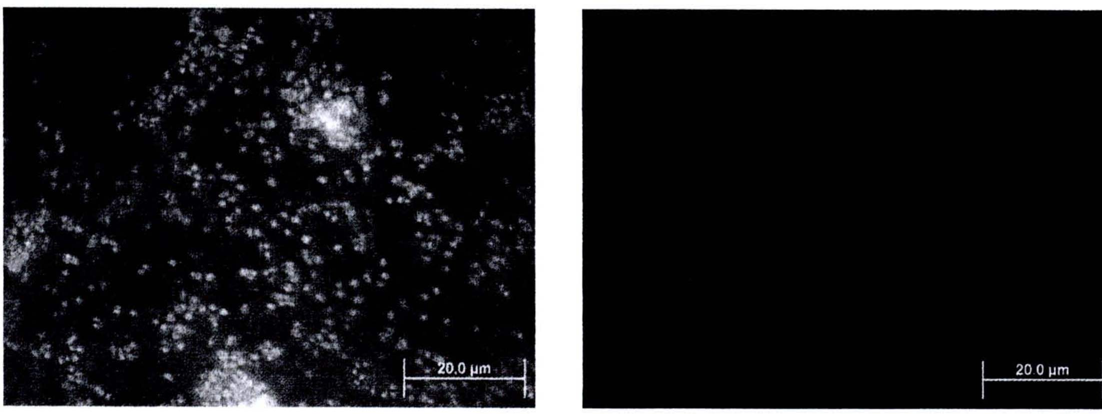


Figure 4.59 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 6 h, after 12 h of incubation.

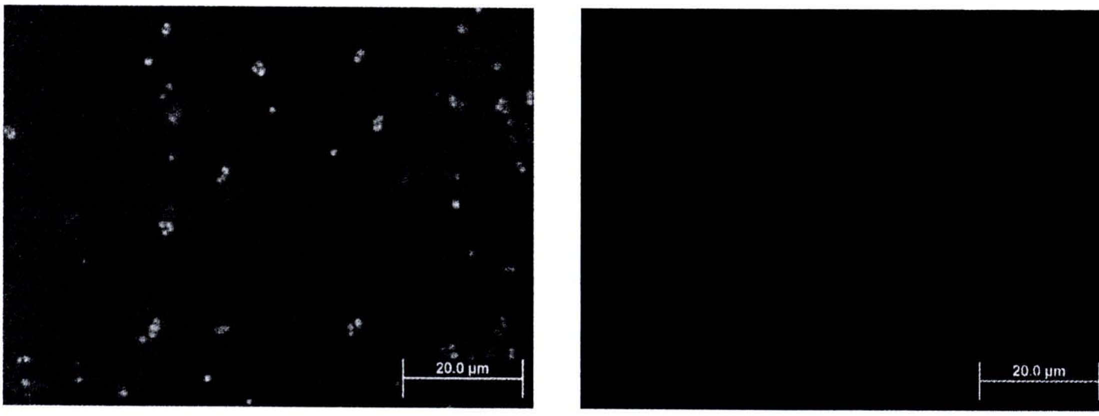


Film

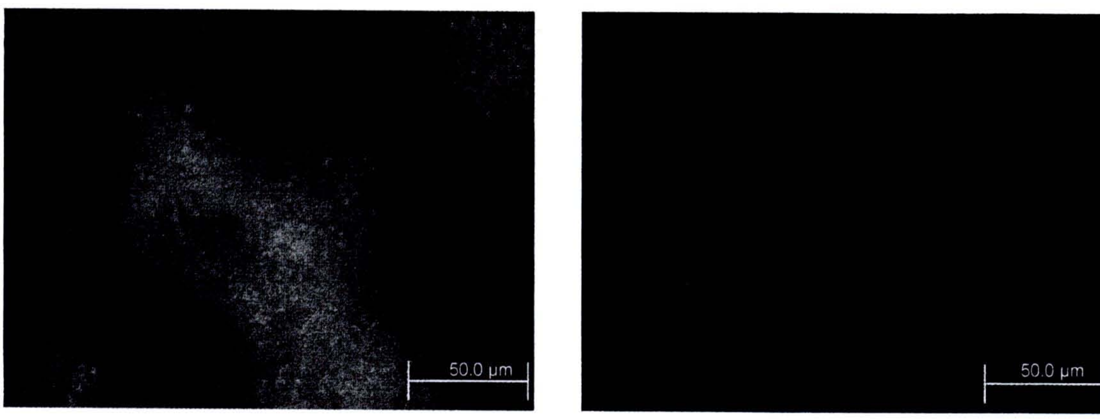


Fiber

Figure 4.60 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 12 h, after 12 h of incubation.

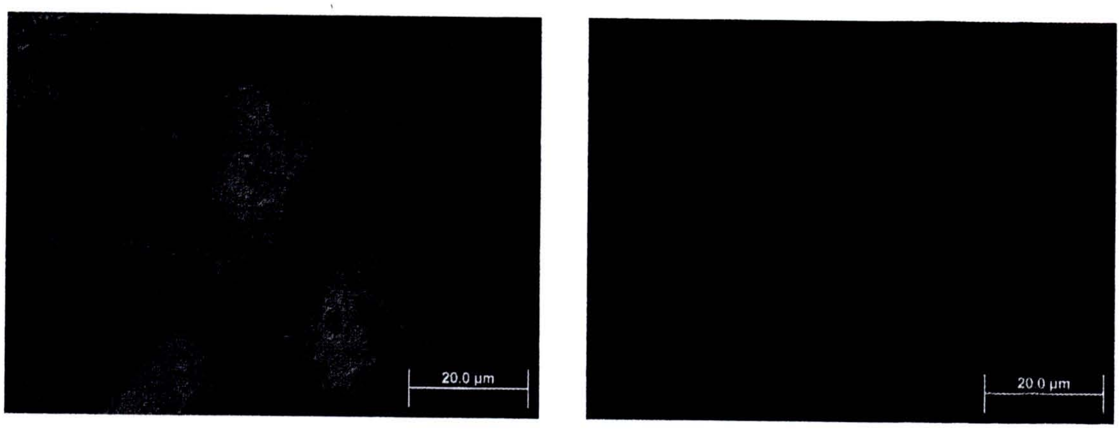


Film

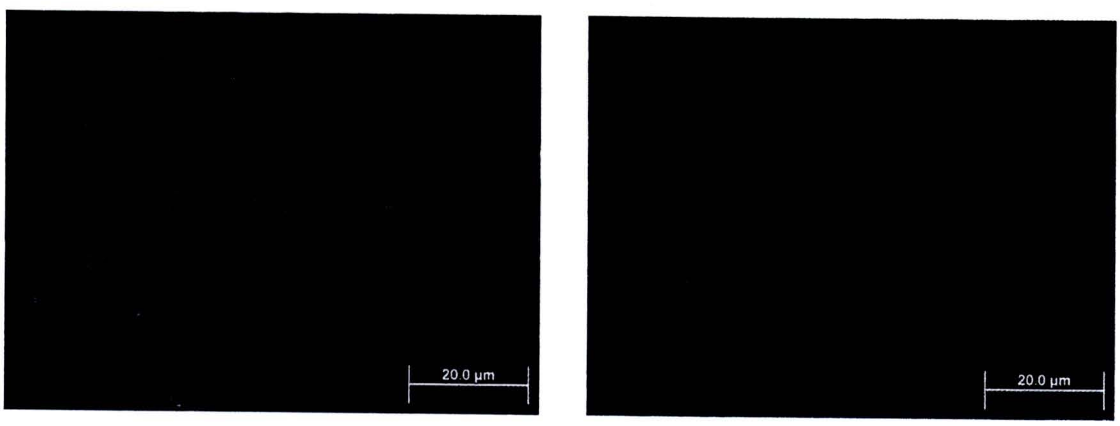


Fiber

Figure 4.61 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 24 h, after 12 h of incubation.

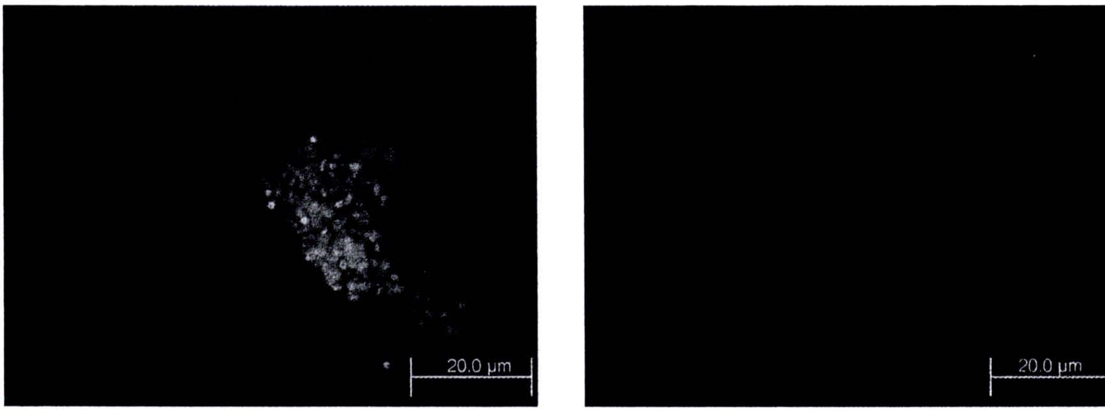


Film

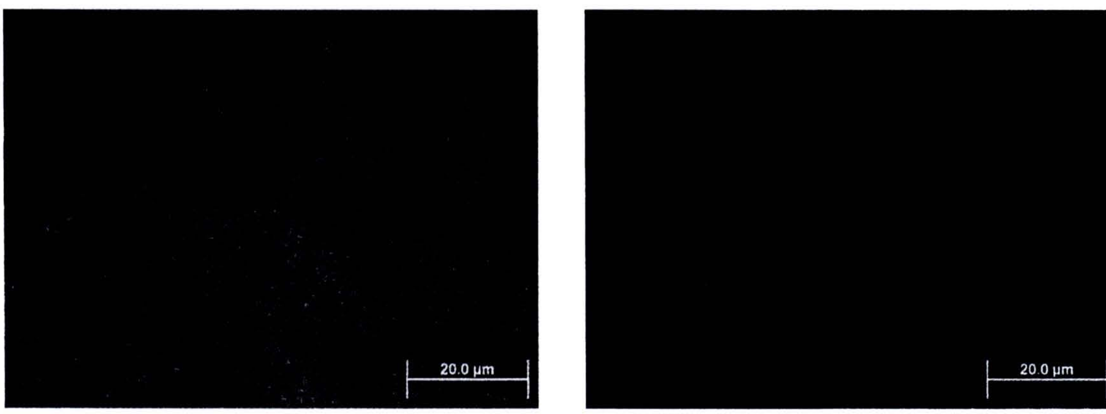


Fiber

Figure 4.62 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 36 h, after 12 h of incubation.

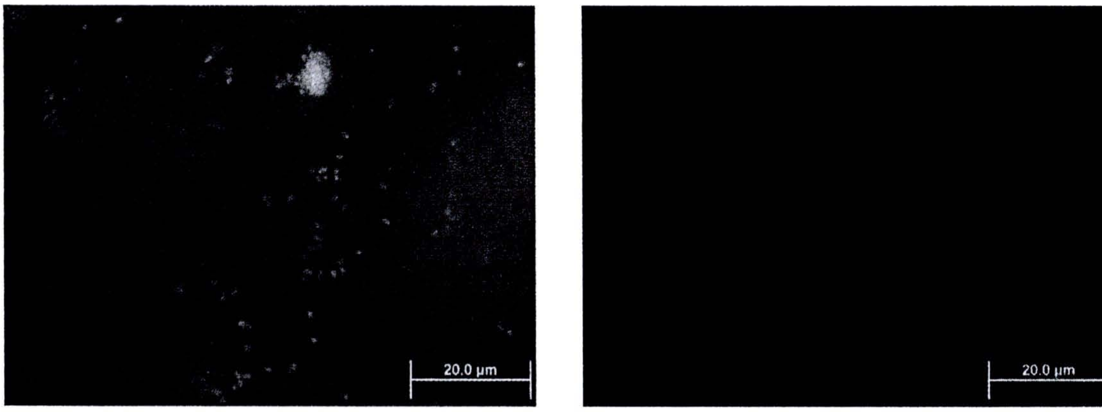


Film

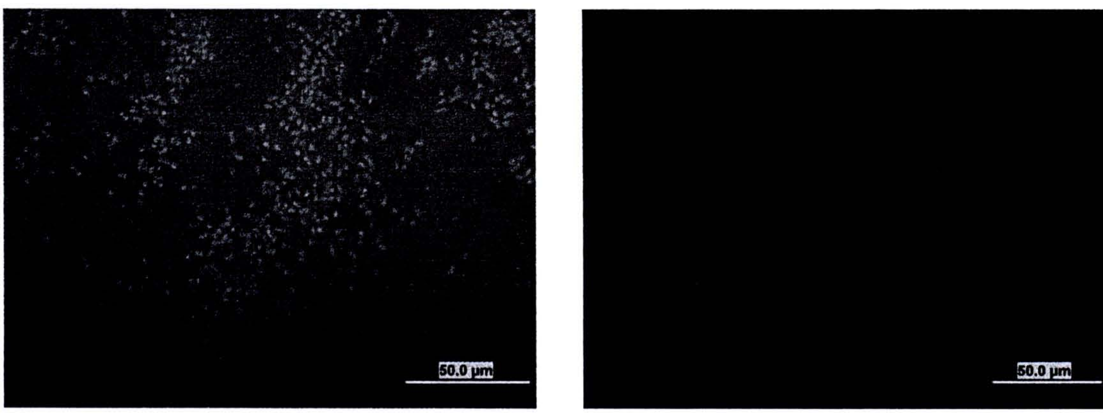


Fiber

Figure 4.63 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 48 h, after 12 h of incubation.

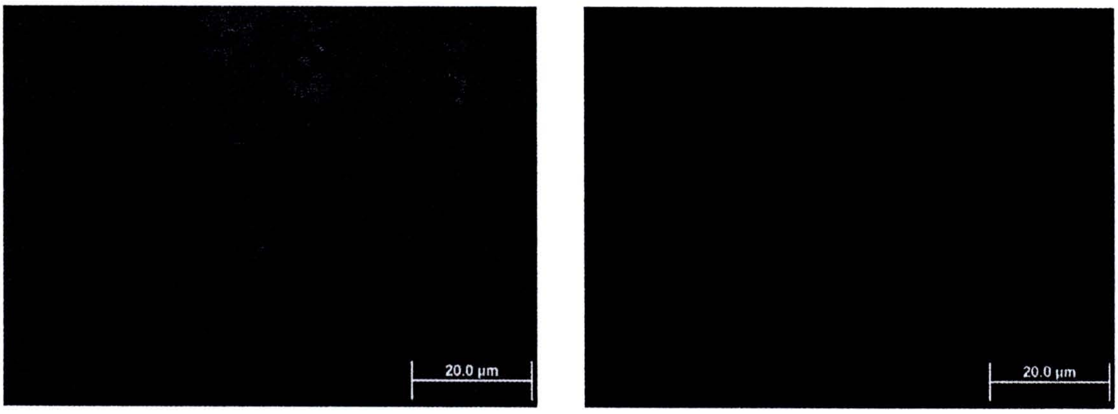


Film

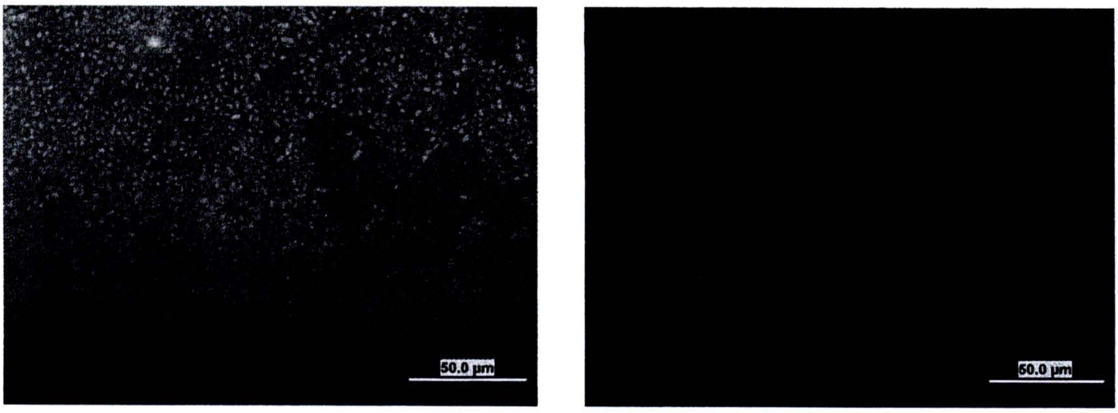


Fiber

Figure 4.64 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 6 h, after 12 h of incubation.

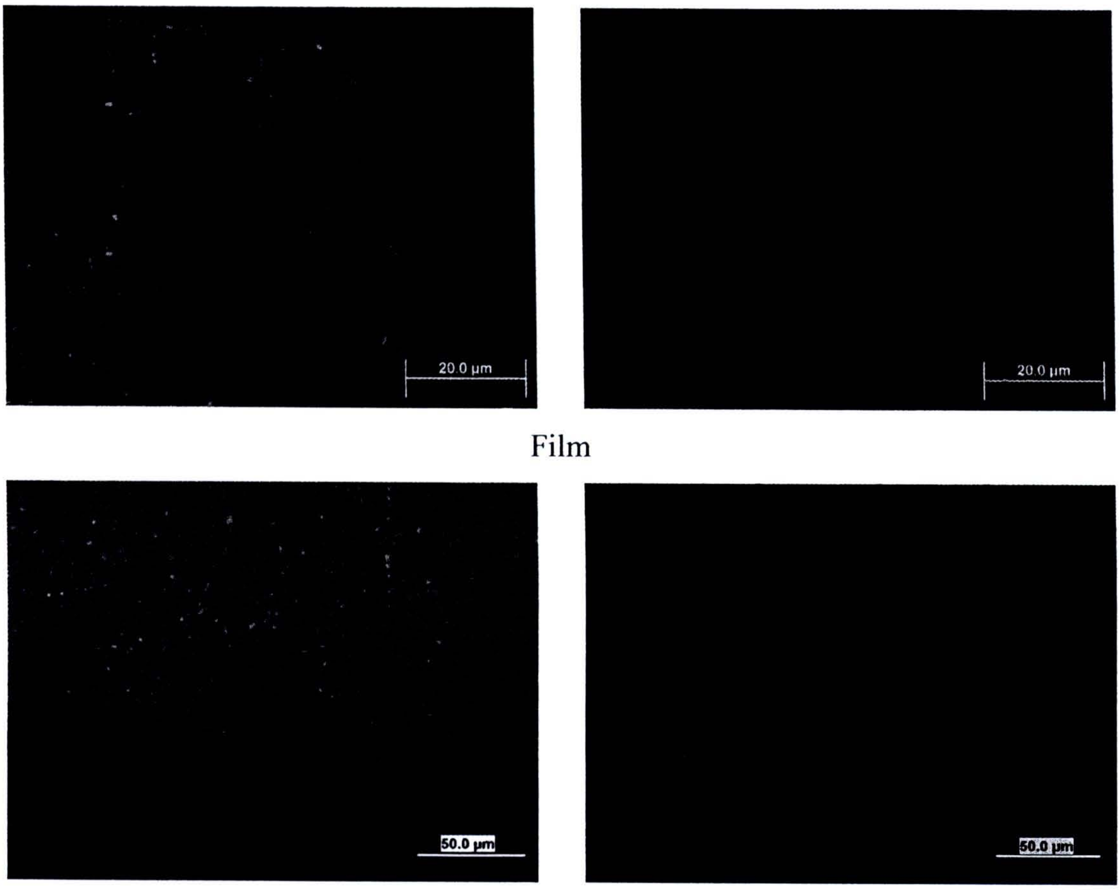


Film



Fiber

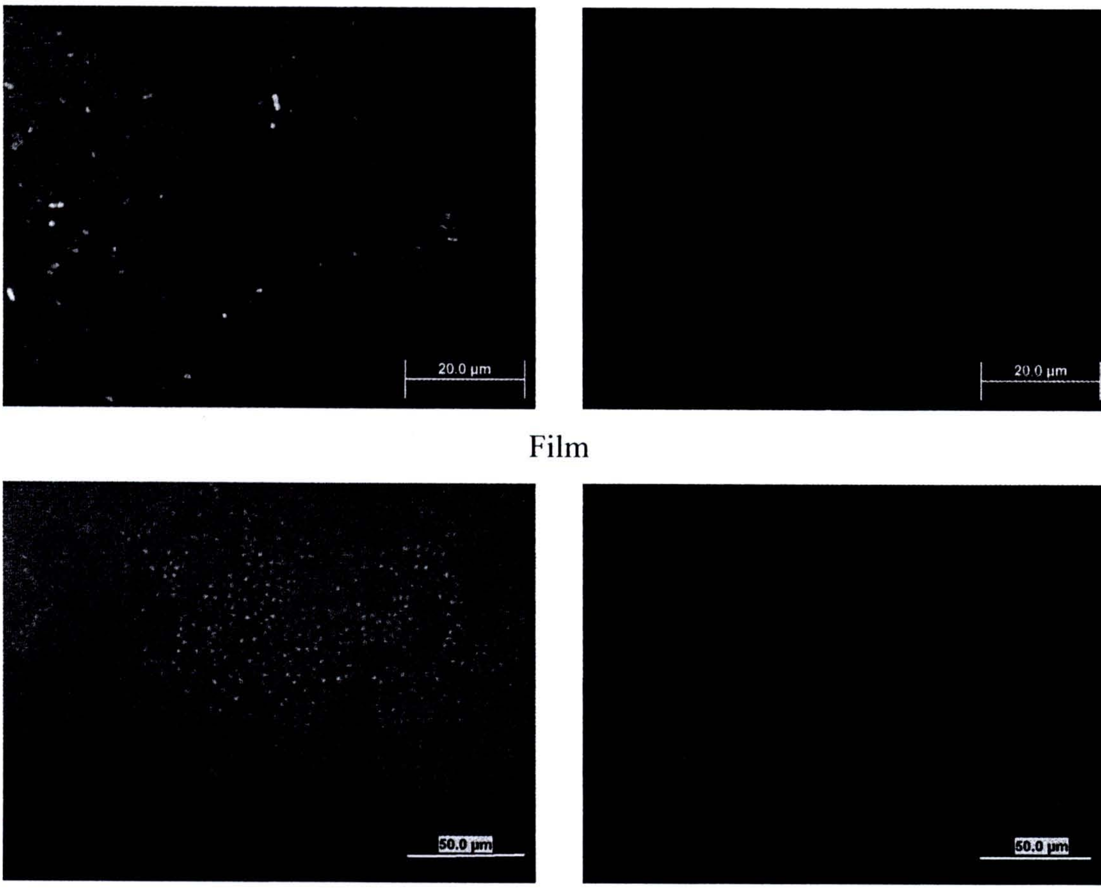
Figure 4.65 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 12 h, after 12 h of incubation.



Film

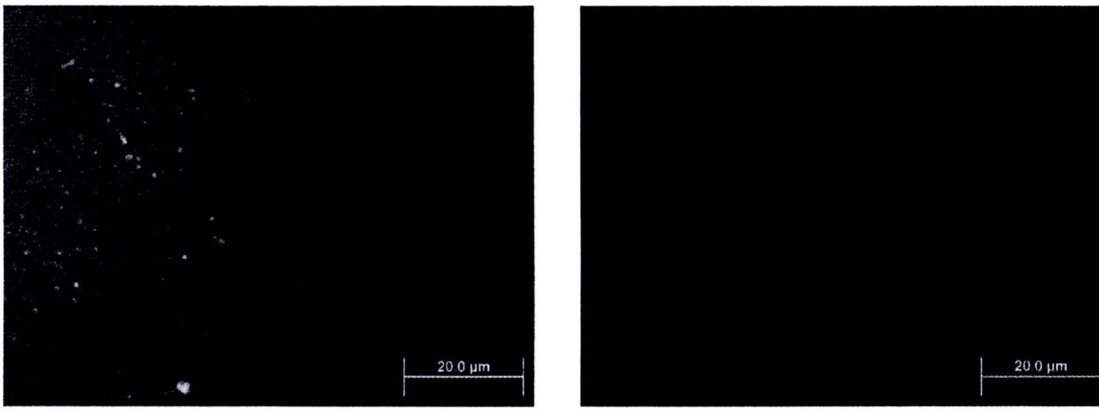
Fiber

Figure 4.66 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 24 h, after 12 h of incubation.

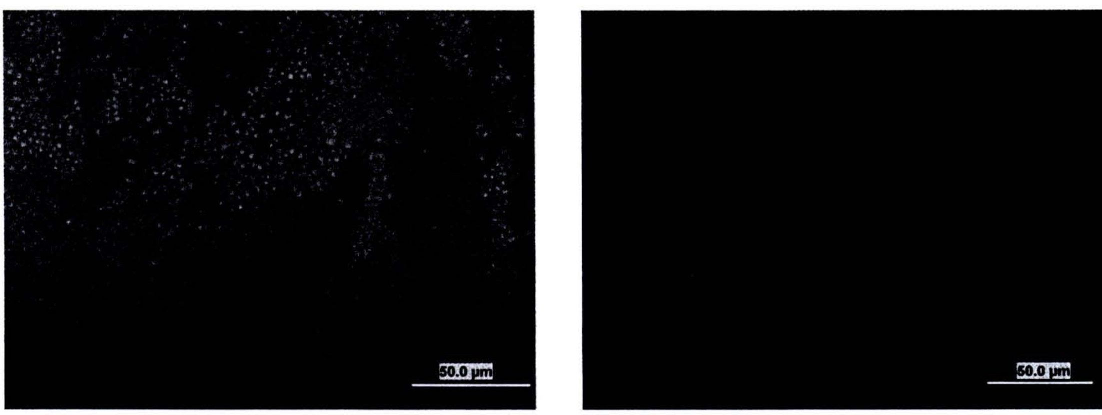


Fiber

Figure 4.67 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 36 h, after 12 h of incubation.



Film



Fiber

Figure 4.68 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 48 h, after 12 h of incubation.

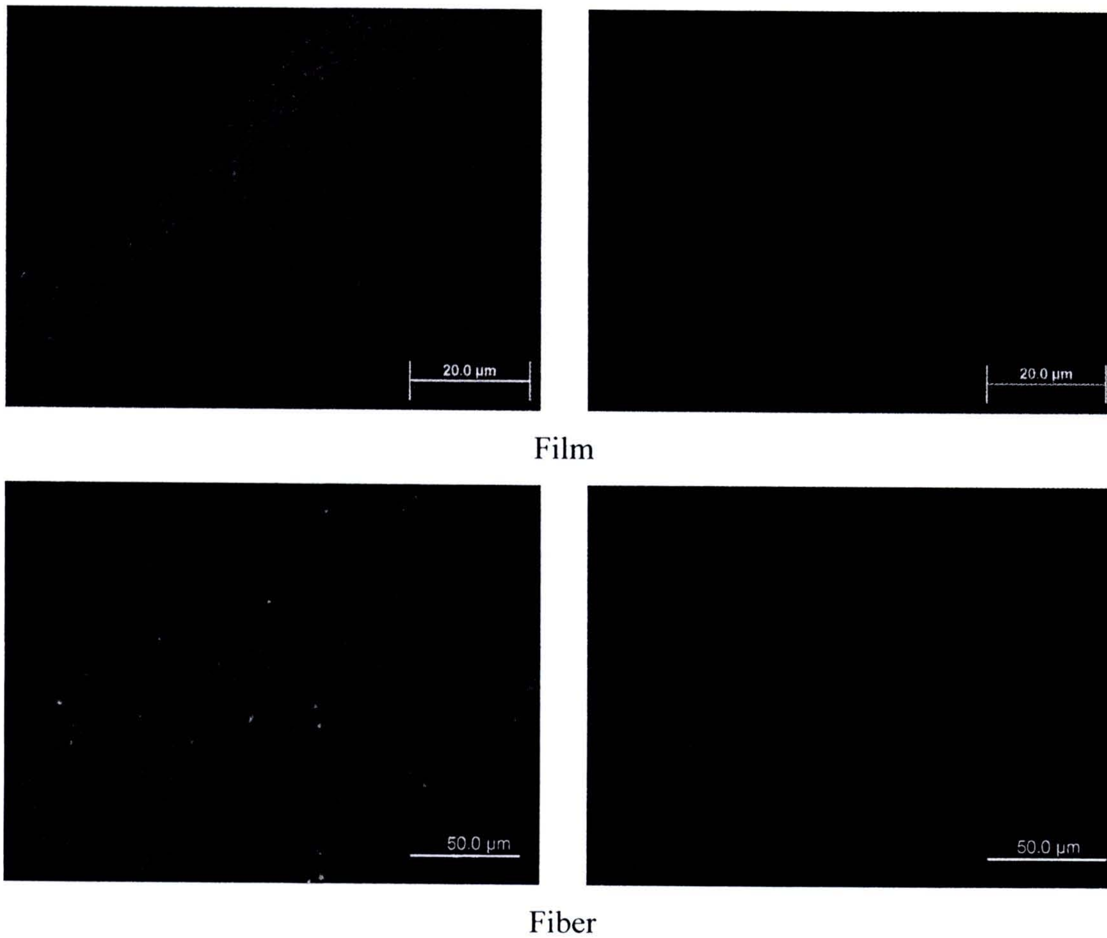
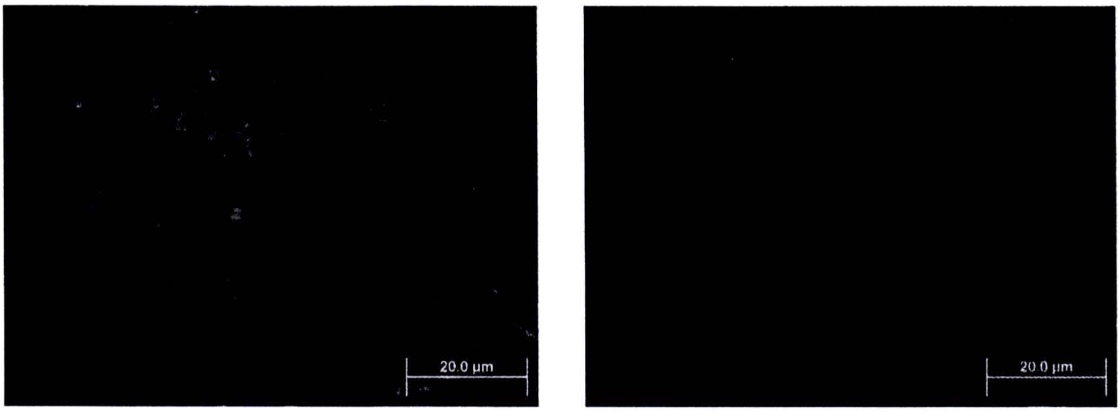
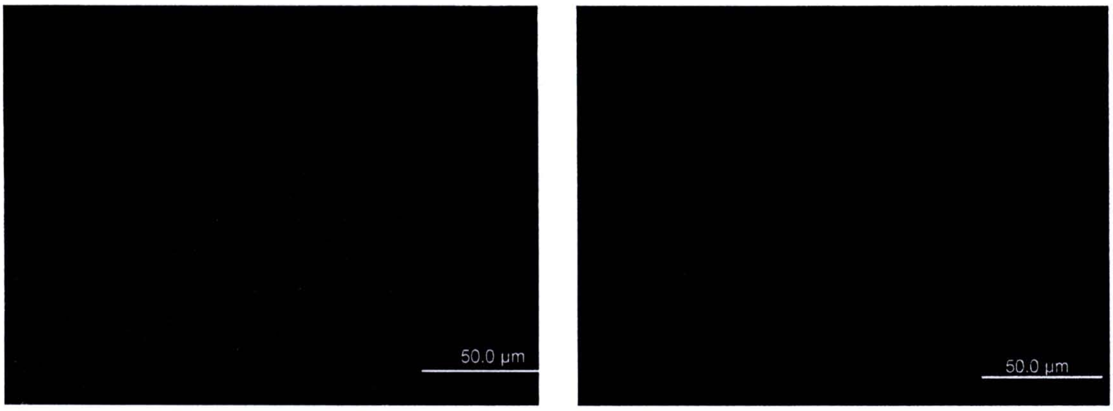


Figure 4.69 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 6 h, after 24 h of incubation.



Film



Fiber

Figure 4.70 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 12 h, after 24 h of incubation.

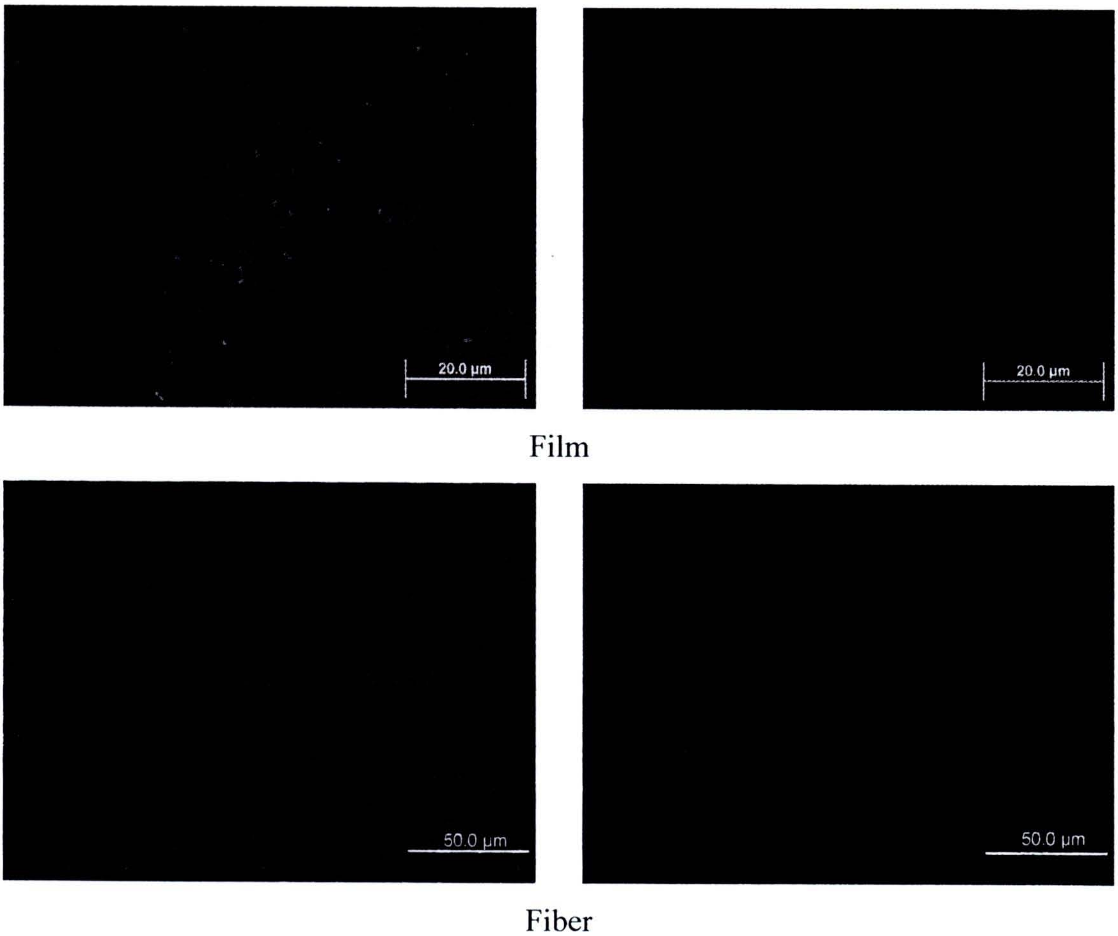
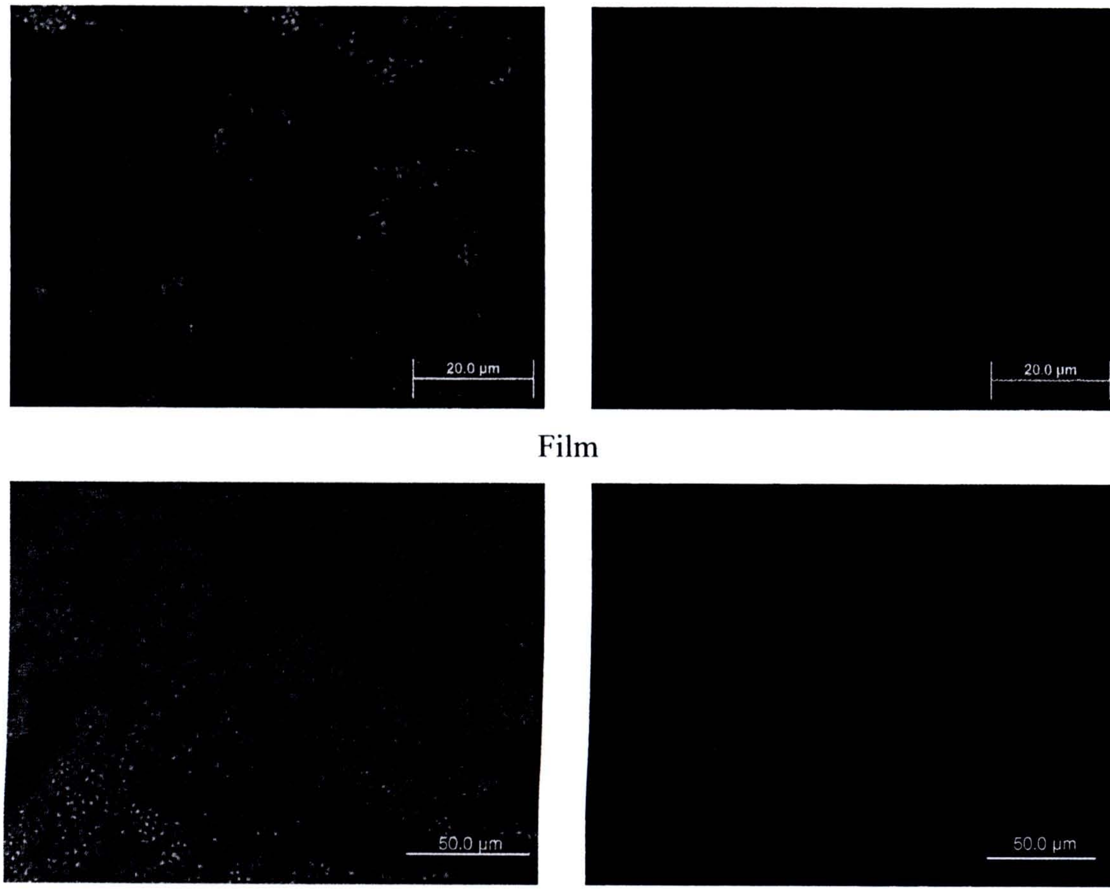
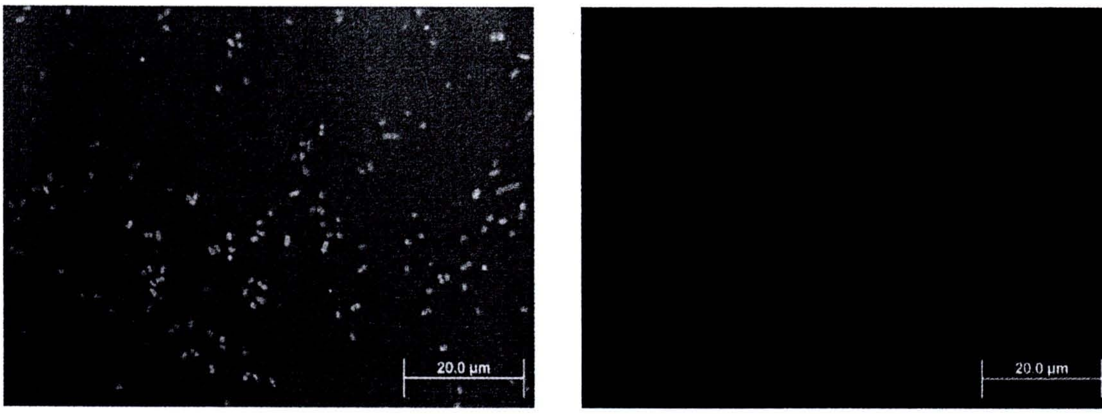


Figure 4.71 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 24 h, after 24 h of incubation.

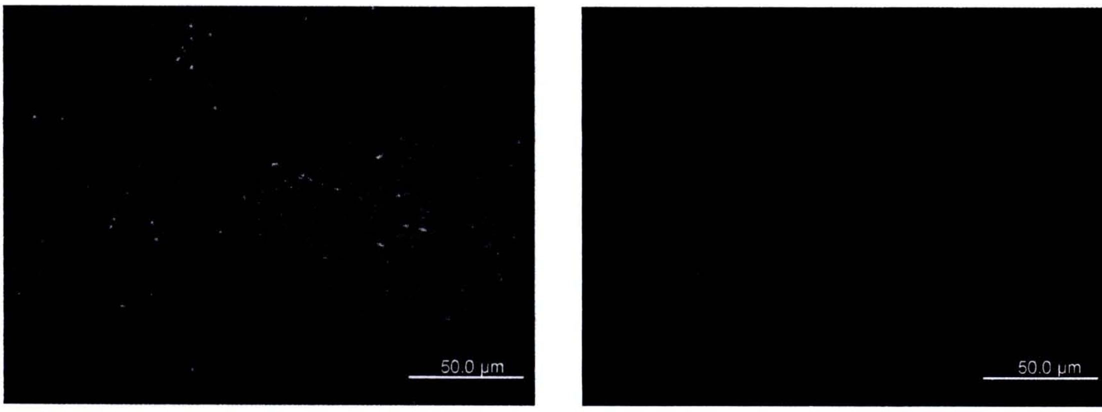


Fiber

Figure 4.72 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 36 h, after 24 h of incubation.

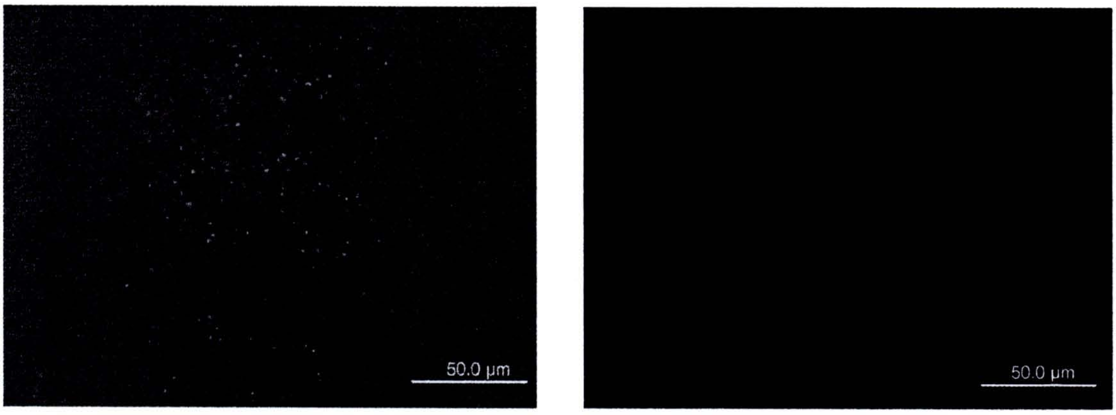


Film

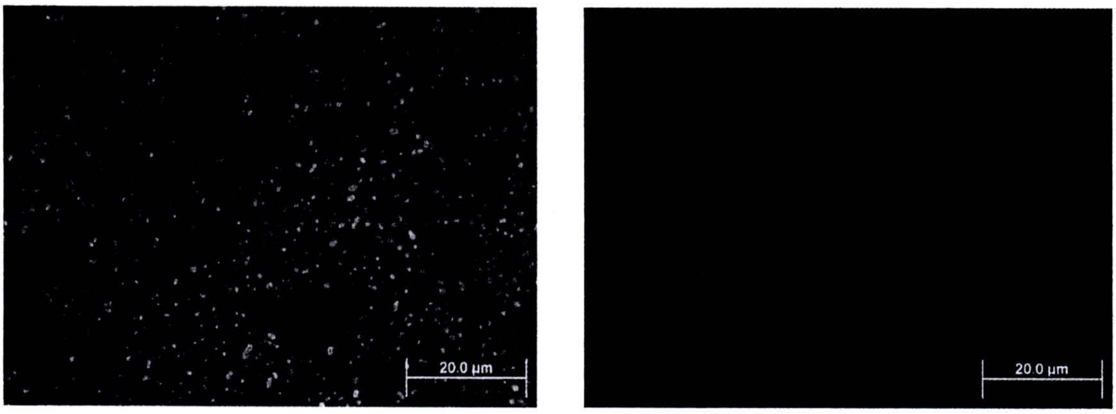


Fiber

Figure 4.73 Fluorescence micrographs of Gram-positive *B. agri* No.13 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 48 h, after 24 h of incubation.

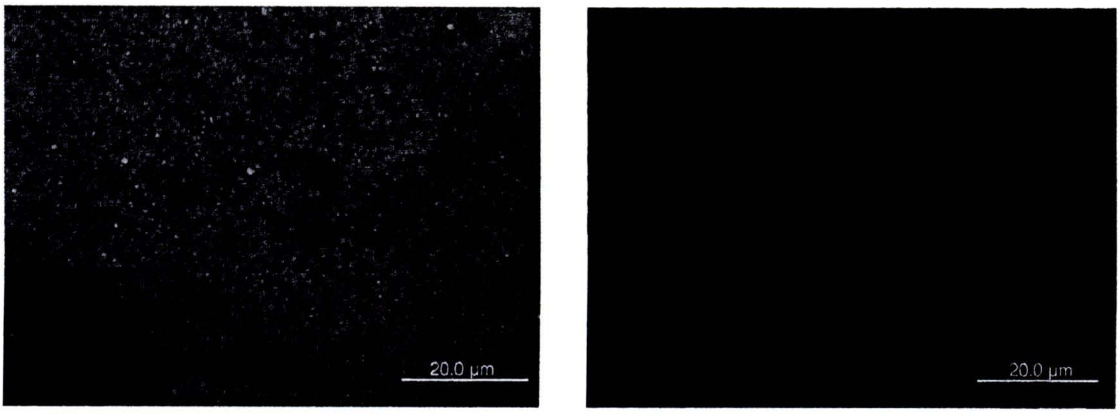


Film

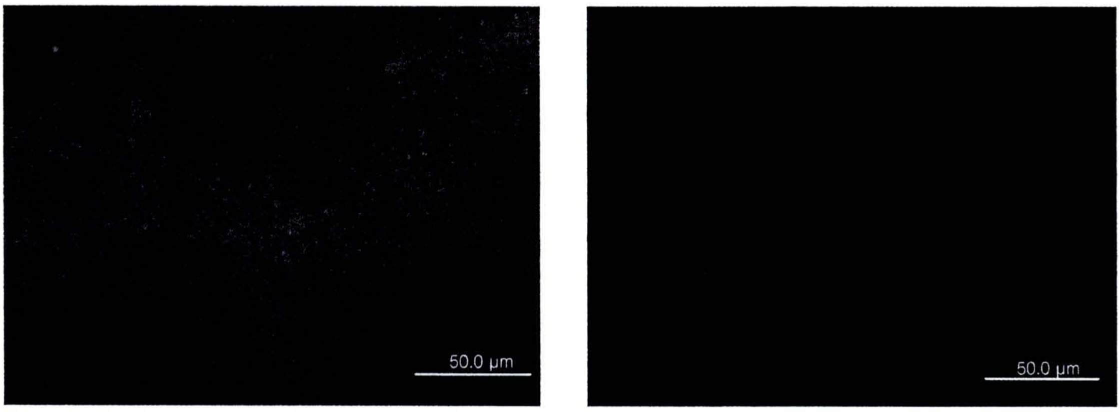


Fiber

Figure 4.74 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 6 h, after 24 h of incubation.

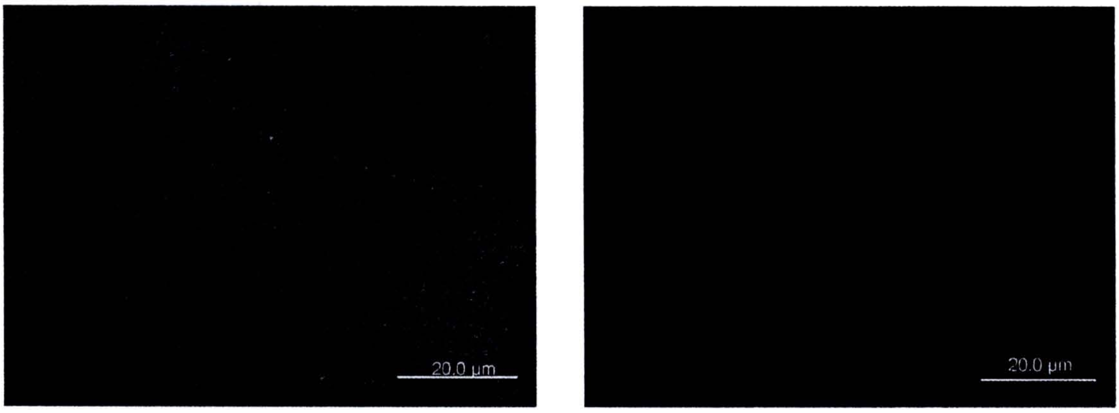


Film

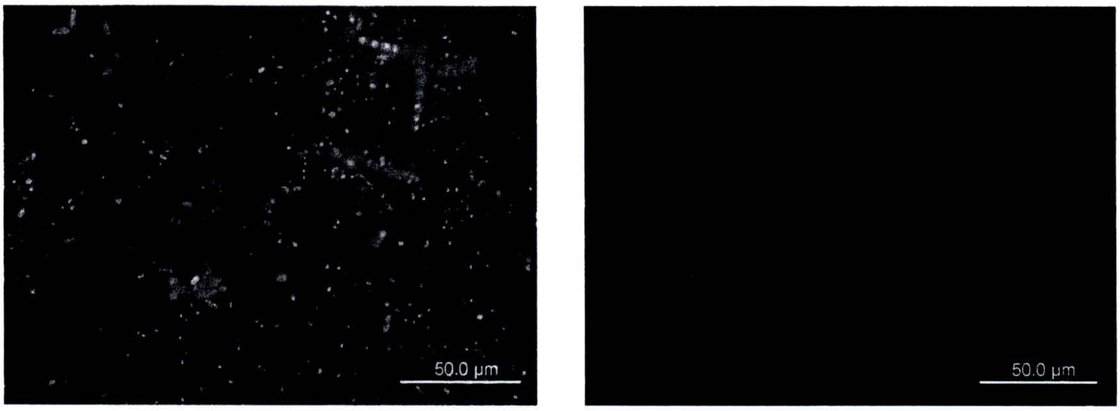


Fiber

Figure 4.75 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 12 h, after 24 h of incubation.



Film



Fiber

Figure 4.76 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 24 h, after 24 h of incubation.

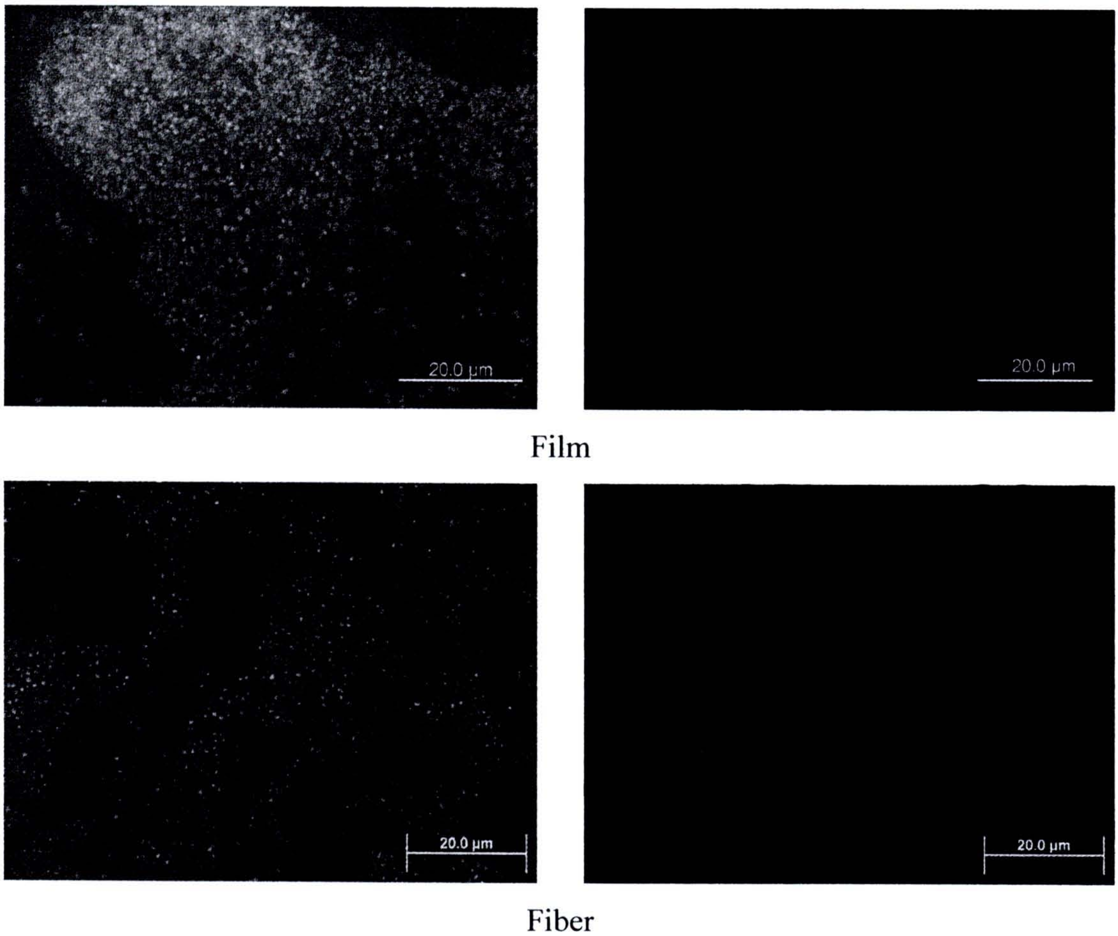
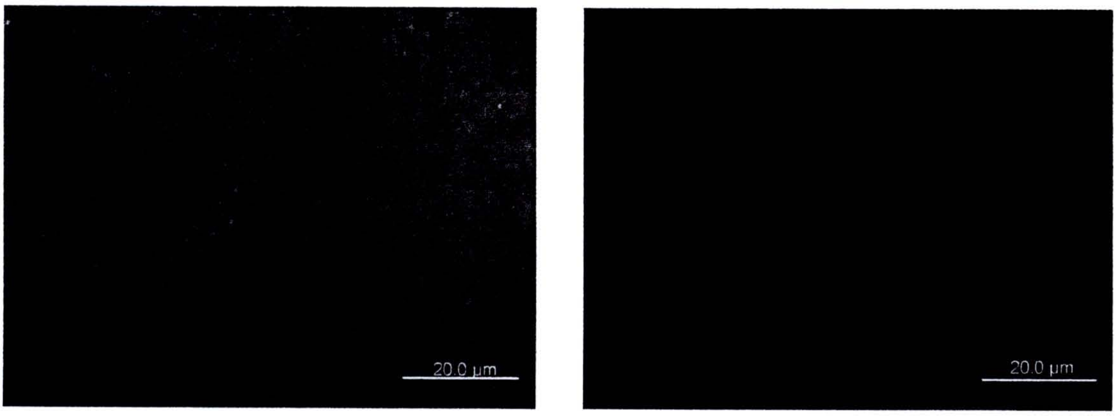
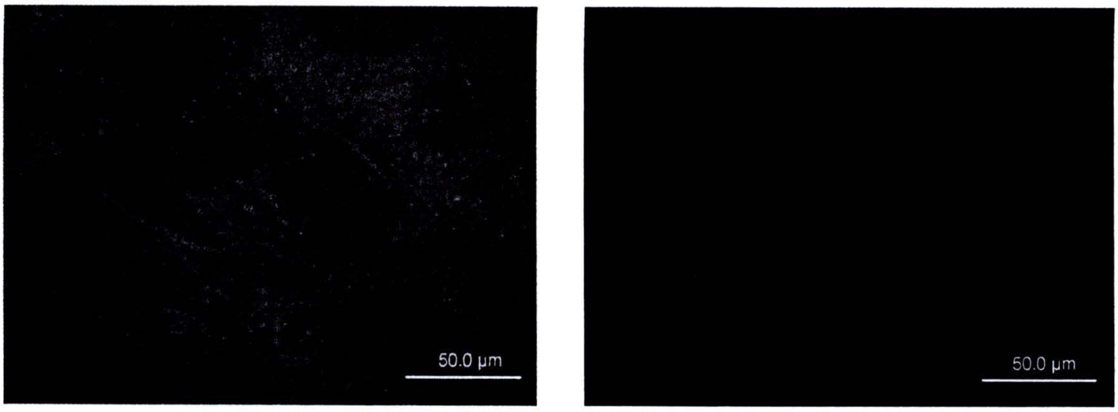


Figure 4.77 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 36 h, after 24 h of incubation.



Film



Fiber

Figure 4.78 Fluorescence micrographs of Gram-negative *A. baylyi* strain GFJ2 attaching on electrospun chitosan fibers and chitosan films that were prepared from chitosan hydrolyzed for 48 h, after 24 h of incubation.

4.5.3 Viability of bacterial cells attaching on chitosan/PVA nanofibers

In the study of cells viability on chitosan/PVA nanofibers fabricated by electrospinning, the chitosan with molecular weight 760 kDa were chosen. The cell attachment was investigated after 12 and 24 h of incubation for both Gram-negative and Gram-positive bacteria. The similar trend for both Gram-negative *Acinetobacter baylyi* strain GFJ2 and Gram-positive *Brevibacillus agri* strain 13 was observe, as shown in Figure 4.79 -4.82.

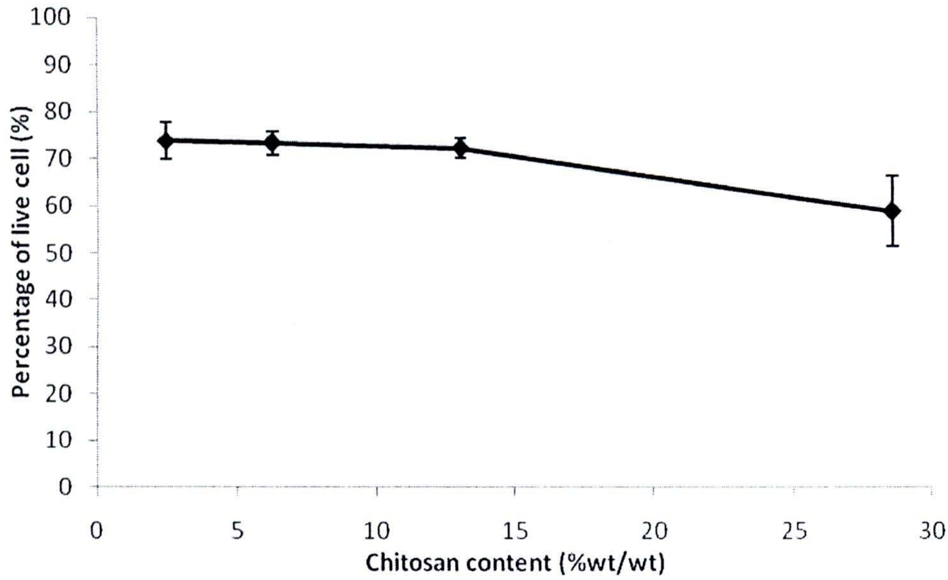


Figure 4.79 Percentage of live cells for Gram-positive *Brevibacillus agri* strain 13 attaching on chitosan/PVA nanofibers with various chitosan contents, after the incubation time of 12 h.

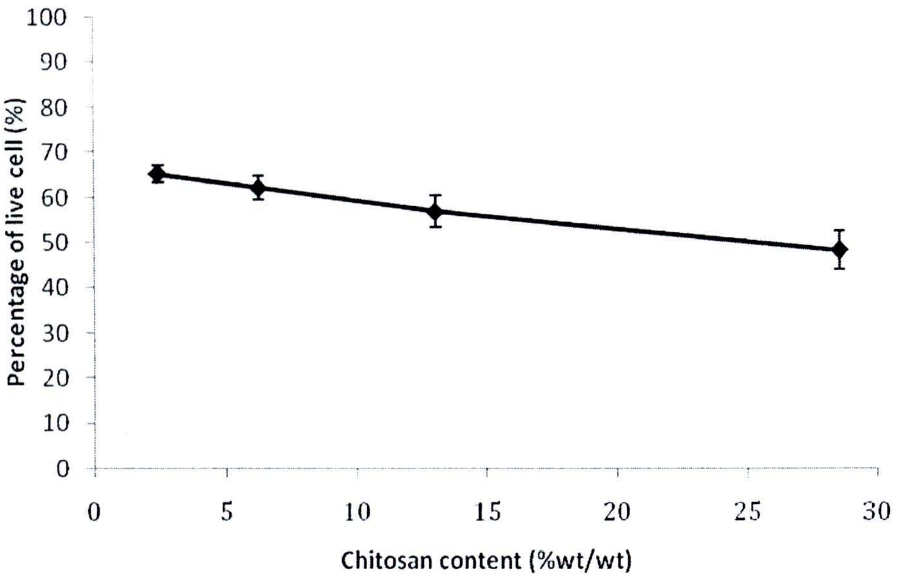


Figure 4.80 Percentage of live cells for Gram-positive *Brevibacillus agri* strain 13 attaching on chitosan/PVA nanofibers with various chitosan contents, after the incubation time of 24 h.

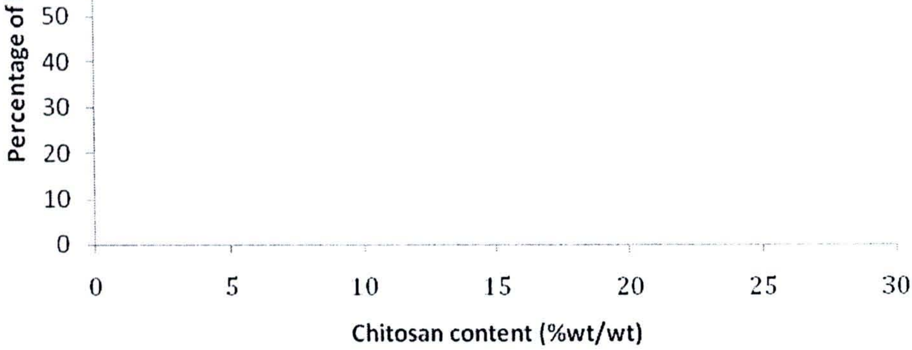


Figure 4.81 Percentage of live cells for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers with various chitosan contents, after the incubation time of 12 h.

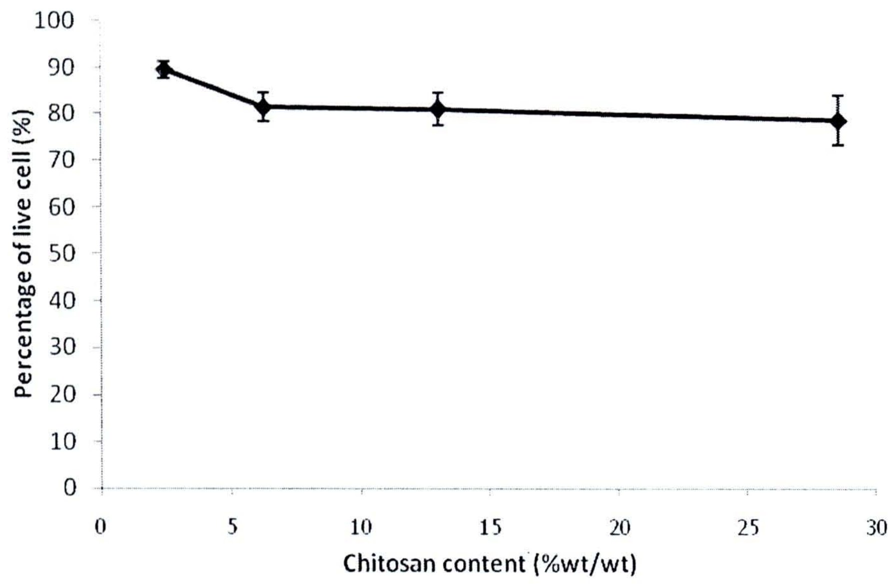
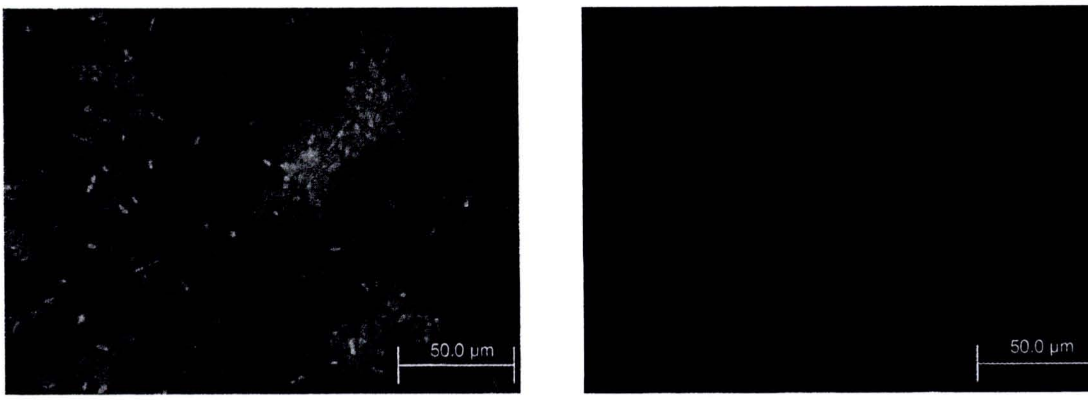
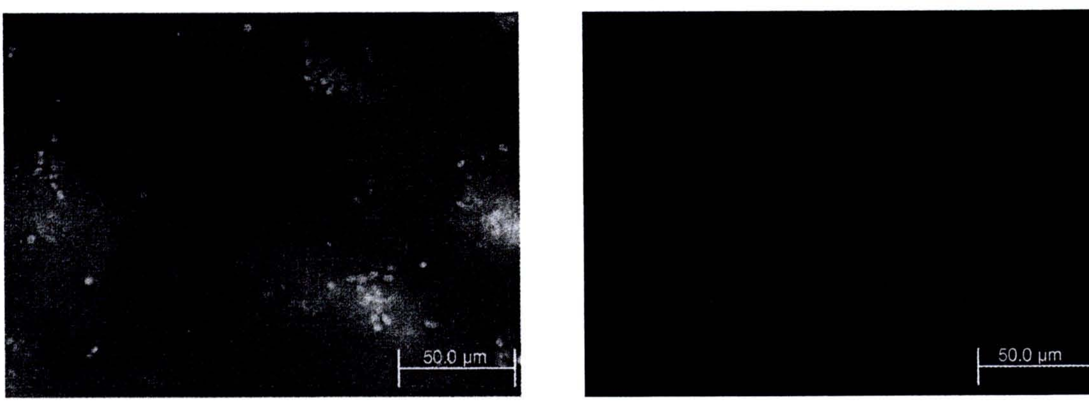


Figure 4.82 Percentage of live cells for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers with various chitosan contents, after the incubation time of 24 h.

At the lowest of chitosan content investigated (i.e., 2.44 %wt), all experiments for both types of bacterial cells indicated that the percentage of live cells attaching on surface was the highest. When the chitosan content was increased, it resulted in the decrease in the viability of cells on the surface. The results could be explained in the similar manner as what have been described in previous sections. The positive charges chitosan interacts with negative charges on the surface of the cells. The amino groups (NH_3^+) as the active functional group was found to be effective in obstructing the bacterial cells attachment [33]. The increase of chitosan content would increase the number of NH_3^+ groups leading to the free amino group that could be alter cell permeability. Due to the damage of the outer cell membrane, the hydrophilicity and charge density of the cell surface may be changed. The fluorescence micrographs of live cells and dead cells on chitosan/PVA fibers fabricated with various chitosan contents are shown in Figure 4.83-4.86.

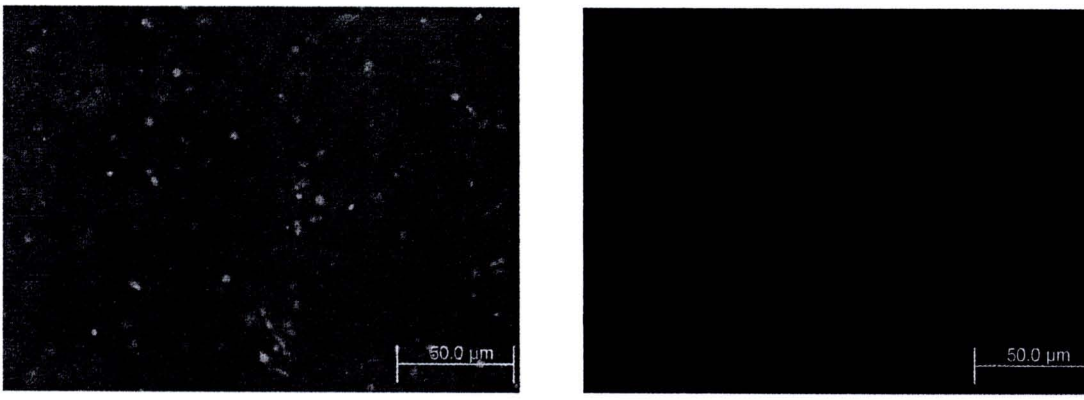


2.44 %wt

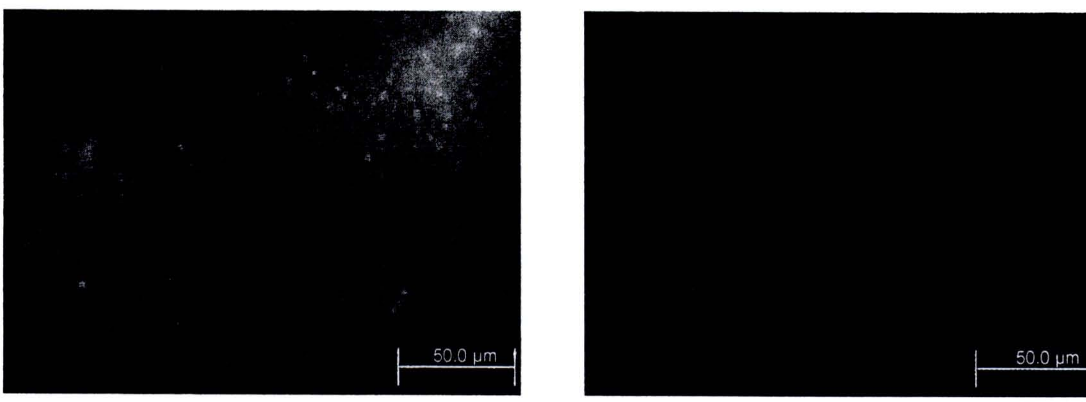


6.25 %wt

Figure 4.83 Florescence micrograph of Gram-positive *B. agri* strain 13 attaching on electrospun chitosan/PVA fibers that were prepared with various chitosan contents, after 12 h of incubation time.

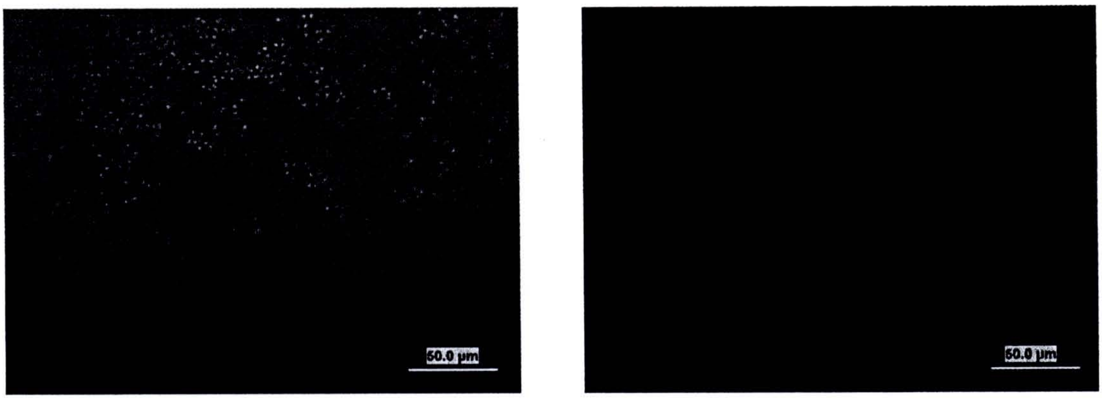


13.04 %wt



28.57 %wt

Figure 4.83 (continued).

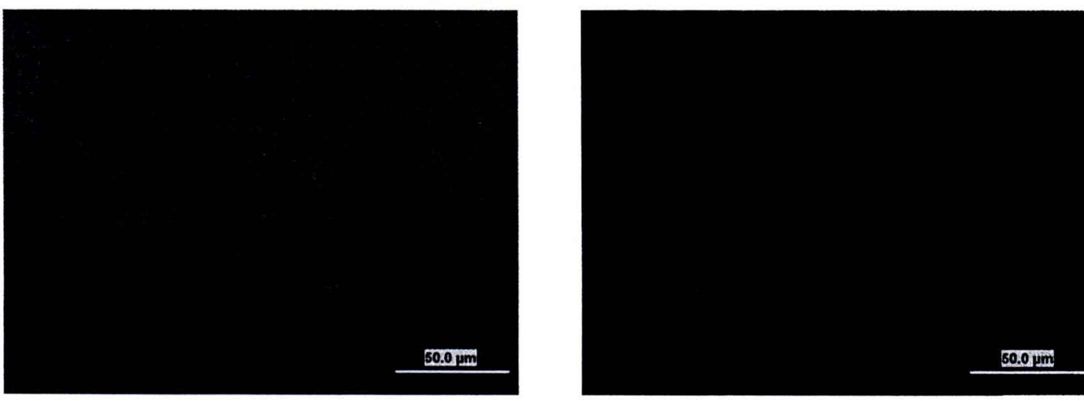


2.44 %wt

6.25 %wt

Figure 4.84 Florescence micrograph of Gram-negative *A. Baylyi* strain GFJ2 attaching on electrospun chitosan/PVA fibers that were prepared with various chitosan contents, after 12 h of incubation time.

13.04 %wt



28.57 %wt

Figure 4.84 (continued).

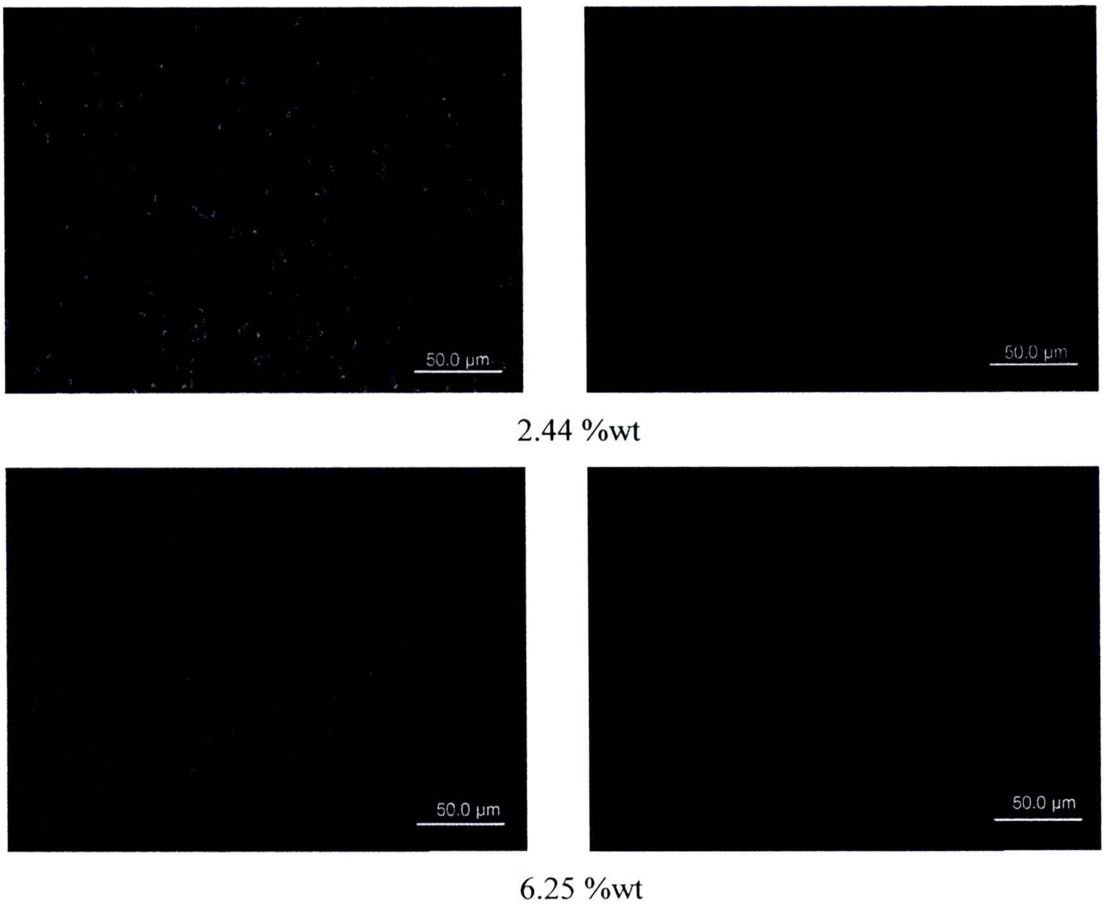
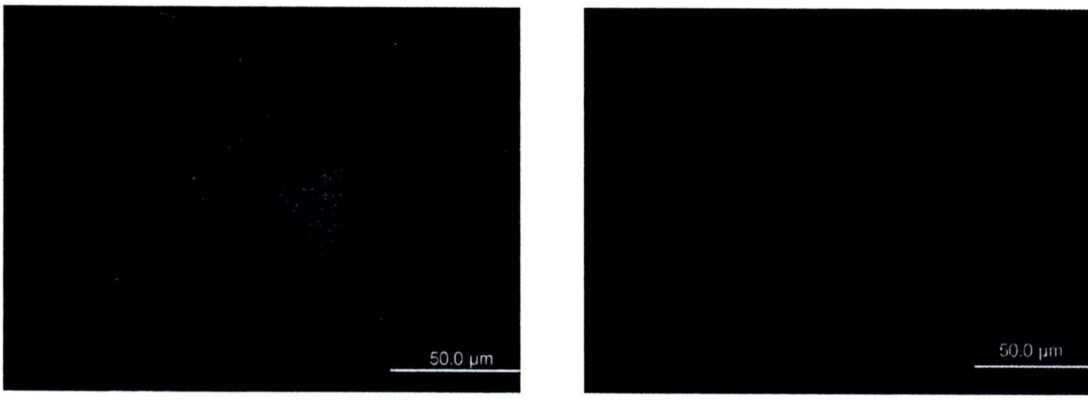
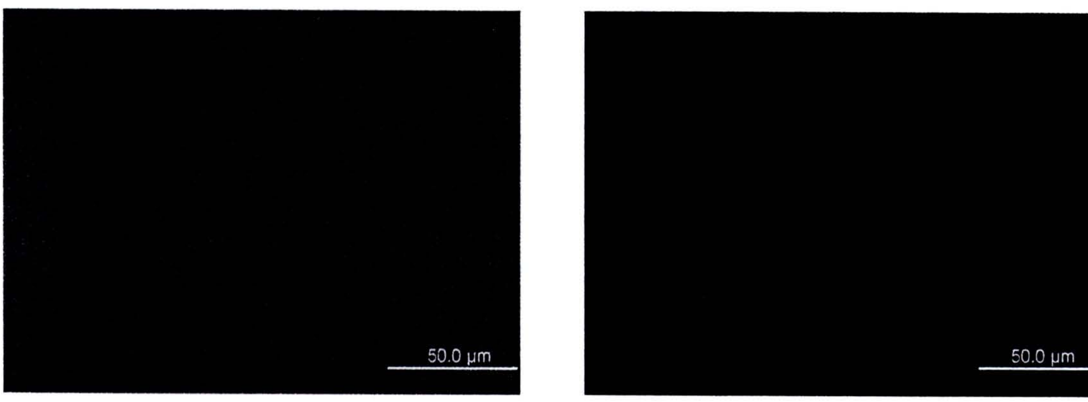


Figure 4.85 Florescence micrograph of Gram-positive *B. agri* strain 13 attaching on electrospun chitosan/PVA fibers that were prepared with various chitosan contents, after 24 h of incubation time.

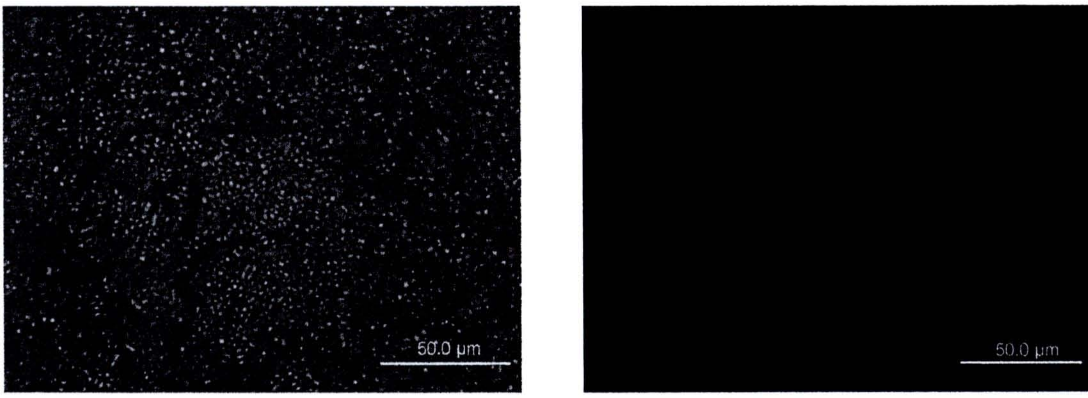


13.04 %wt

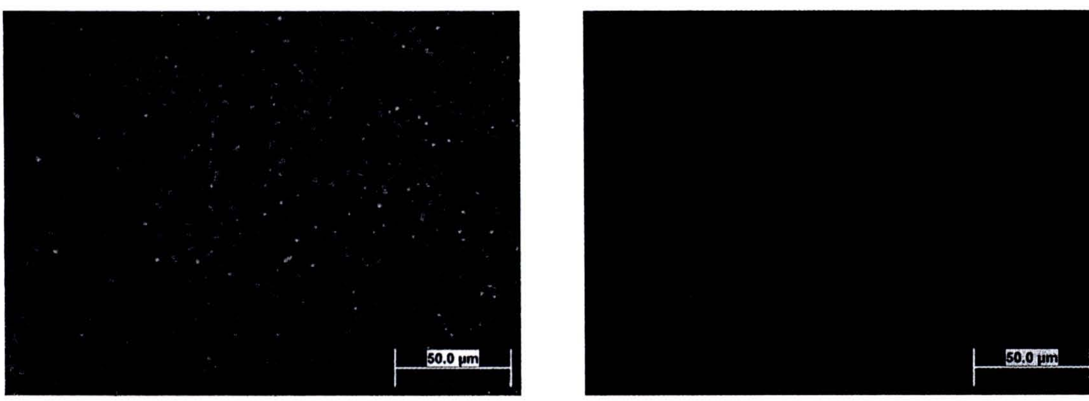


28.57 %wt

Figure 4.85 (continued).

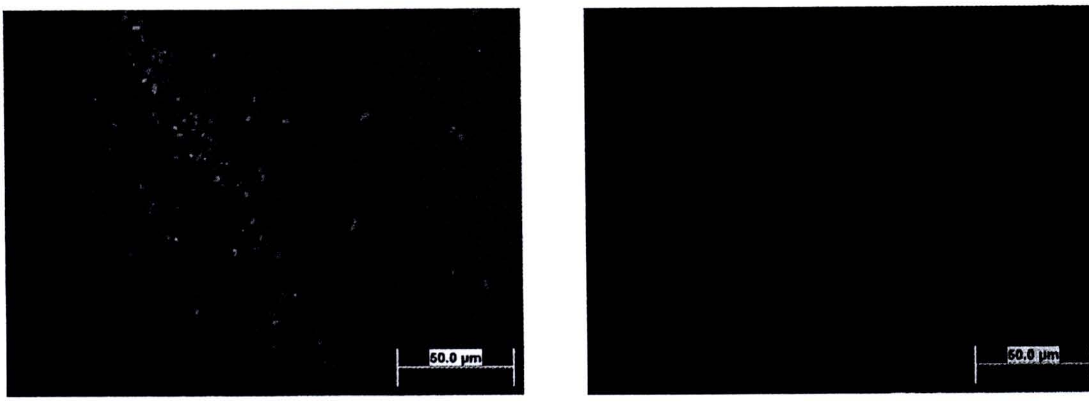


2.44 %wt

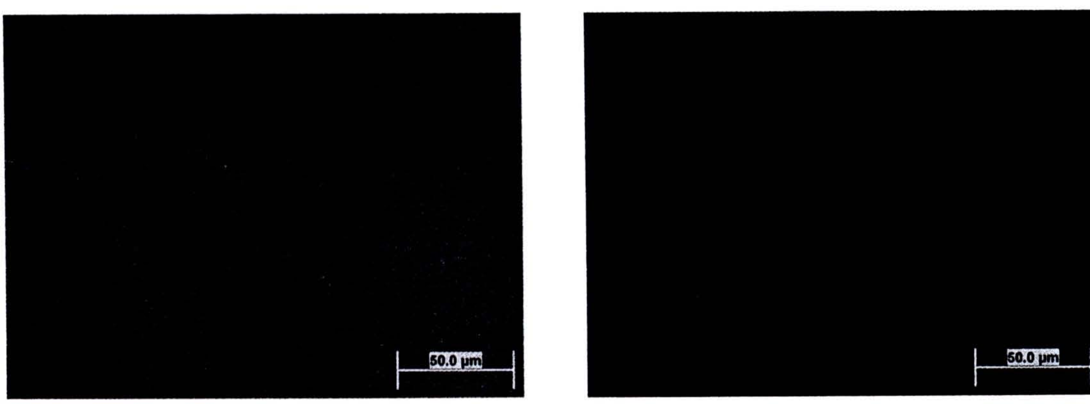


6.25 %wt

Figure 4.86 Florescence micrograph of Gram-negative *A. Baylyi* strain GFJ2 attaching on electrospun chitosan/PVA fibers that were prepared with various chitosan contents, after 24 h of incubation time.



13.04 %wt



28.57 %wt

Figure 4.86 (continued).

Furthermore, to investigate the effect of molecular weight on chitosan/PVA nanofibers fabricated by electrospinning, the chitosan with molecular weight 100 kDa, 400 kDa and 760 kDa were used. The nanofibers were tested with Gram-negative *Acinetobacter baylyi* strain GFJ2 bacteria using the incubation time of 24 h. The results are shown in Figure 4.87

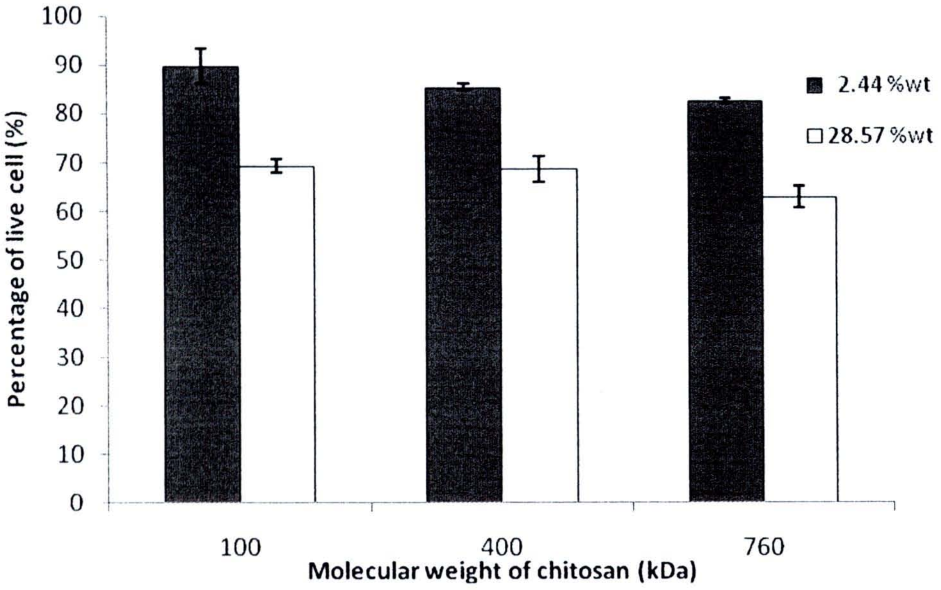
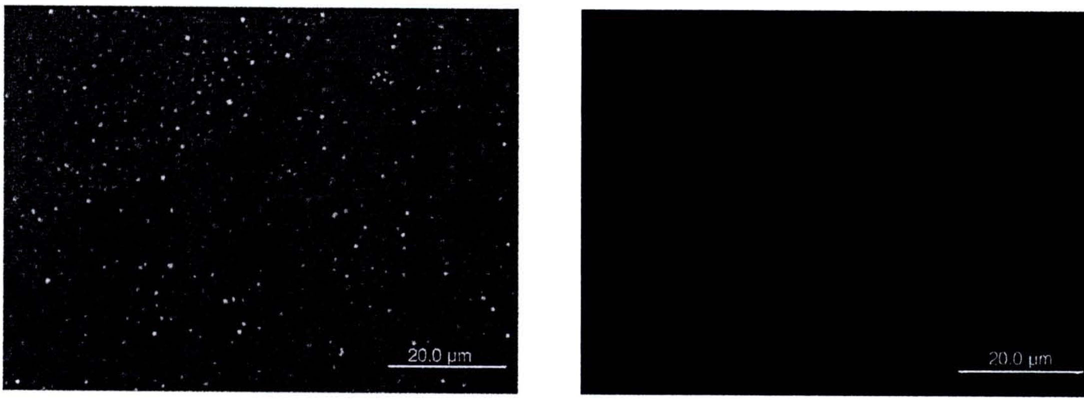
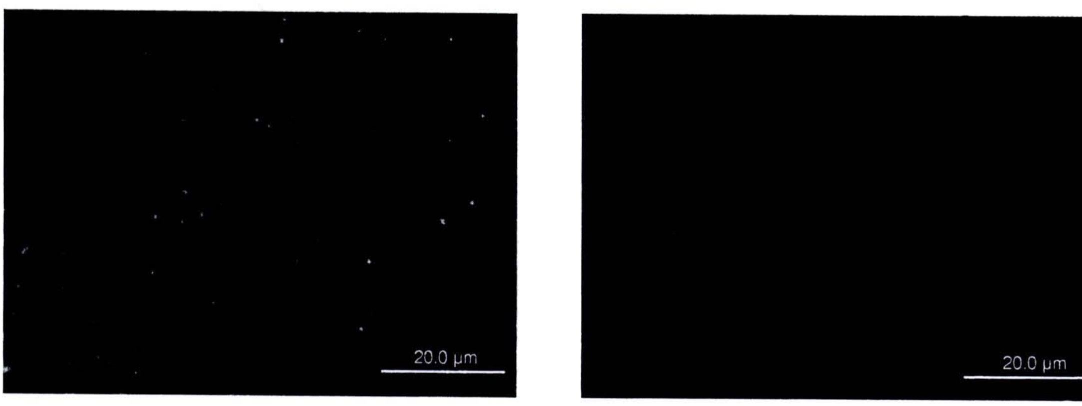


Figure 4.87 Percentage of live cells for Gram-negative *Acinetobacter baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers formed with various chitosan contents (%wt) and various chitosan molecular weights, after the incubation time of 24 h.

These results confirmed that molecular weight of chitosan affected viability of cells. Molecular weight of chitosan directly related to cationic the behavior of chitosan such as chain conformation, solubility and degree of substitution. High molecular weight chitosan could have block arrangement of acetylated and deacetylated units and would increase the number of amino groups (NH_3^+) per molecule. However, the maximum soluble amount of chitosan decreased with the molecular weight of chitosan, leading to the reduced number of free amino groups that are available for bacterial attachment [33]. The results regarding the micrograph of live cells and dead cells attaching on chitosan/PVA fibers are shown in Figure 4.88 -4.90.

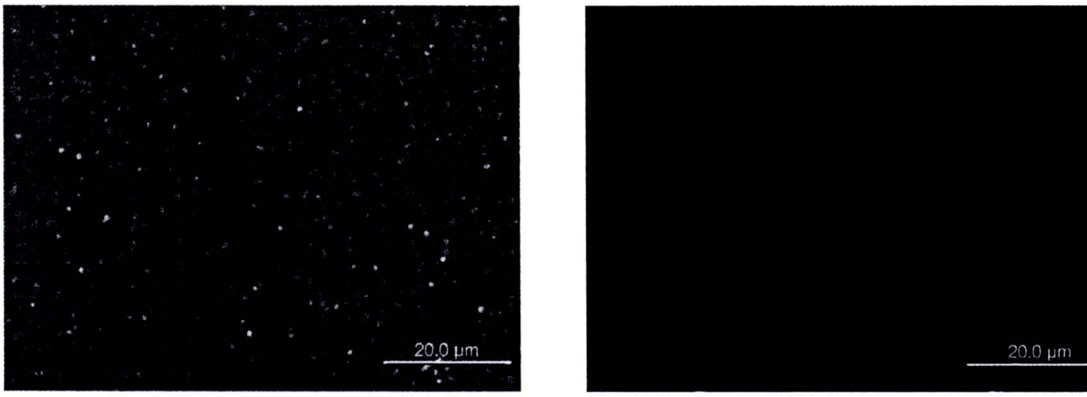


2.44 %wt

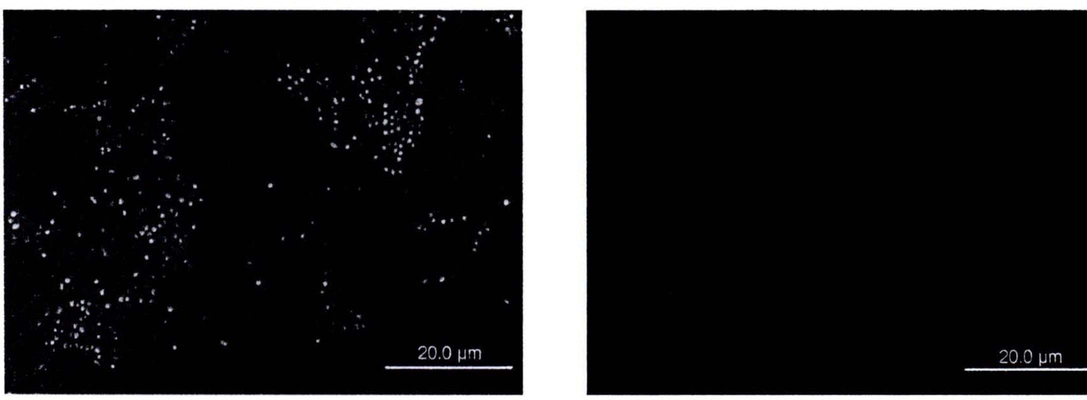


28.57 %wt

Figure 4.88 Fluorescence micrograph of Gram-negative *A. baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers that were formed from chitosan with molecular weight of 100 kDa in various chitosan contents (%wt), after 24 h of incubation.



2.44 %wt



28.57 %wt

Figure 4.89 Fluorescence micrograph of Gram-negative *A. baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers that were formed from chitosan with molecular weight of 400 kDa in various chitosan contents (%wt), after 24 h of incubation.

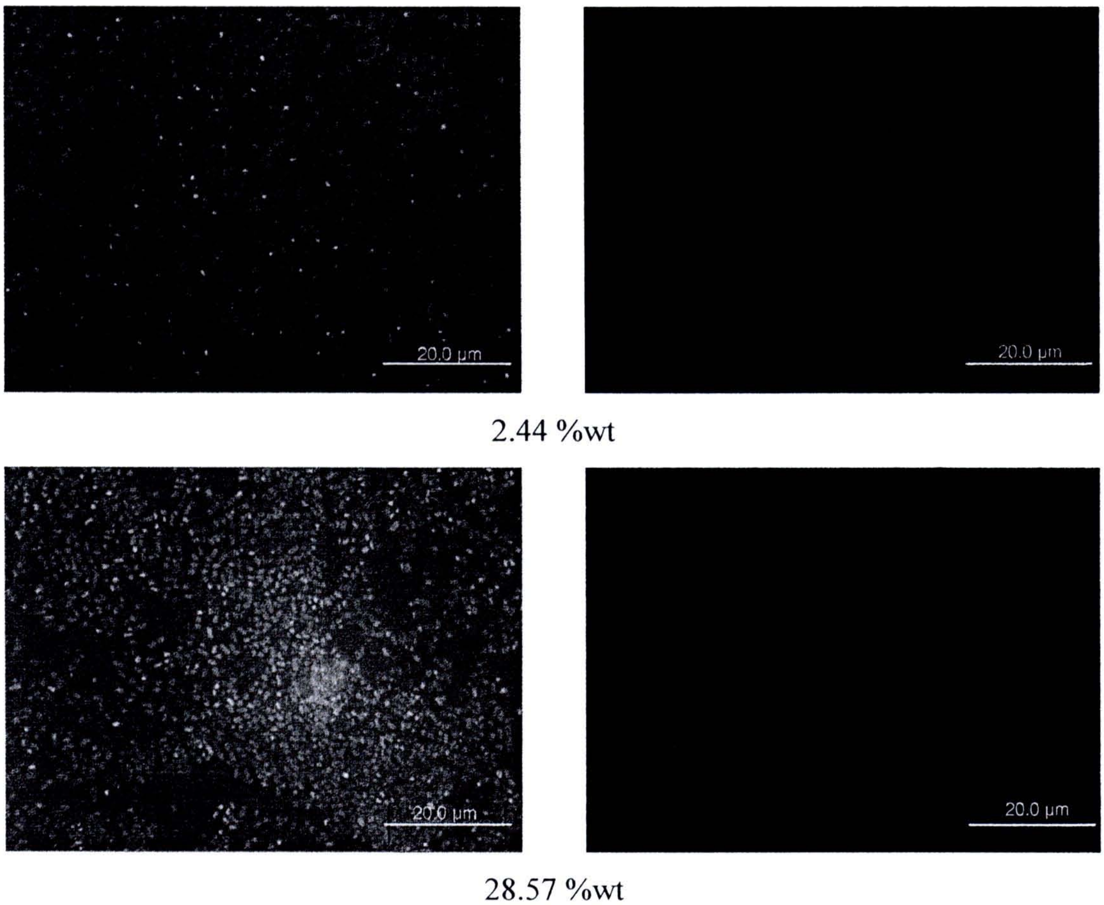


Figure 4.90 Fluorescence micrograph of Gram-negative *A. baylyi* strain GFJ2 attaching on chitosan/PVA nanofibers that were formed from chitosan with molecular weight of 760 kDa in various chitosan contents (%wt), after 24 h of incubation.