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Comparative study of the antibacterial activity of *Ulva lactuca* and *Pterocladia capillacea* extracts before and after encapsulation in Ca-alginate beads

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Abstract

The antibacterial activity of methanolic-L extracts of the marine algae *Ulva lactuca* (Linnaeus) and *Pterocladia capillacea* (Gmelin) against three fish pathogens; *Aeromonas hydrophila*, *Vibrio anguillarum* and *Pseudomonas fluorescens* was determined before and after their encapsulation into Ca-alginate beads. When the methanolic-L extract of *P. capillacea* was encapsulated, its antibacterial activity proved to be superior from that of free extract. The encapsulated extract of *U. lactuca* showed good antibacterial activity against *V. anguillarum* ($P<0.05$). Conversely, the free methanolic-L extract of this alga exhibited significant ($P<0.05$) reduction of *A. hydrophila* and *P. fluorescens* activities more than the encapsulated extract. The surface morphology of the formed beads was studied using scanning electron microscope (SEM). Changes of the characteristics of the beads were investigated by FTIR spectroscopy.

Keywords: Antibacterial activity, Ca-alginate beads, encapsulation, *Pterocladia capillacea, Ulva lactuca*

1. Introduction

Marine macroalgae are a good source for new antimicrobial agents and are able to produce a variety of secondary metabolites characterized by a broad spectrum of biological activities. There are numerous reports concerning the inhibitory activities of macroalgae against human pathogens, fungi and yeast but only a few contain data about their effects against fish pathogens (Gonzalez del Val *et al*., 2001 and Kubanek *et al*., 2003).

The treatment of microbial diseases in fish is still difficult, not particularly effective, and costly and might involve environmental hazards. A possible method to confront this problem might be the oral administration of antimicrobial materials to the fish larvae through the food chain, using the bioencapsulation technique (Verpraet *et al*., 1992). It is a physico-mechanical process in which the active components are covered by a layer of another material such as natural carrier polymer (Lakkis, 2008). The natural polymers such as the algal polysaccharides; alginate, carrageenan and agarose should have the advantage of being nontoxic, biocompatible and biodegradable (Murano, 1998).

Alginate is an anionic biopolymer, produced by marine brown algae, and possesses good biocompatibility, biodegradability, non-toxicity, gelation, and film formation properties (Lapasin and Pricl, 1995). Alginate consists of linear chains of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues joined by 1,4-glycosidic linkages (Johnson *et al*., 1997). Sodium alginate can be cross-linked using calcium chloride, with formation of insoluble calcium alginate beads (Rajaonarivony *et al*., 1993).

Alginate has been widely studied for particle formulation in the size range of 100 nm to 2 mm for drug delivery (Babu *et al*., 2007; Martins *et al*., 2007; Sarmento *et al*., 2007), but it has not been used for formulation of beads containing an antibacterial algal extract. Moreover, there have been several investigations for use of alginate gels as carriers for a variety of drugs.

Scanning electron microscope (SEM) has been a primary tool for characterizing the surface morphology and fundamental physical properties of the beads surface. It is used for determining the particle shape, porosity and average particle size (Arami *et al*., 2008).

In the present study, we have investigated the effect of encapsulation process on the antibacterial activity of the methanolic-L extracts of *U. lactuca* and *P. capillacea* against three fish pathogens; *A. hydrophila, V. anguillaum* and *P. fluorescens* using the shake-flask

method. The free algal extracts have previously been proved to be active by well-cut diffusion technique (Wefky *et al*., 2009). The physico-chemical properties of the plain and encapsulated beads with the bioactive algal extracts were determined by FTIR analysis and scanning electron microscope (SEM).

2. Materials and methods

2.1. Plant material and preparation of algal extracts

Marine algae *U. lactuca* (Linnaeus) and *P. capillacea* (Gmelin) were collected from sublittoral rocks from Abu Qir Bay in the Mediterranean Sea, Alexandria, Egypt during May 2008. The freshly collected algae were thoroughly washed with fresh water to remove the sea-salt, sand and epiphytes and then identified. The macroalgae were air dried under shade at room temperature. The dried algae (100 g) were powdered in a grinder and then soaked in ethyl acetate (1.50 L) followed by methanol (1.50 L) for two weeks. The organic layers were filtered through a Whatman No. 4 paper and then evaporated under vacuum at 45^{o}C. The methanolic extracts were obtained as a green residue (methanolic-R) mixed with a pale yellow liquid (methanolic-L) which were separated by decantation and stored at -20^{o}C.

2.2. Bacterial strains

The test Gram negative bacteria (*A. hydrophila*, *V. anguillarum* and *P. fluorescens*) were kindly obtained from Fish Diseases Department, Faculty of Veterinary Medicine, Alexandria University, Egypt.

2.3. Encapsulation of bioactive algal extracts

2.3.1. Beads formation

The encapsulating agent used was sodium alginate (2% w/v). Sodium alginate was purchased from Sisco Research Laboratories Pvt. Ltd., India. Beads were obtained by mixing the active extract with sodium alginate solution, then homogenized and dropped from a hydrodermic syringe to 100 ml of calcium chloride solution (2%) with constant stirring at room temperature. The formed beads were maintained in the gelling bath to harden for 1 h. Then, they were filtered through a Whatman paper No. 1 and washed with sterile distilled water.

2.3.2. Beads characterization

The morphology of the beads was analyzed using a Jeol JSM-6360LV (Japan) microscope. Alginate beads were dried before coating with gold for SEM analysis. Beads were attached to stubs using a two-sided adhesive tape, then coated with a layer of gold (15-20 nm) and examined using acceleration voltage of 20 kV. Analysis by Fourier transform infrared (FTIR) spectroscopy was performed on the plain and encapsulated Ca-alginate beads at Mubarak City for Science and Technology. A FTIR spectrophotometer (FTIR-8400S Shimadzu-Japan) was used. Samples were scanned from 500 to 4000 cm^{-1}.

2.4. Antibacterial activity of free and encapsulated extracts

The test microorganisms were grown at 30^{o}C for 24 h on nutrient broth. A cell suspension of each microorganism was used for the antibacterial test. The antibacterial activity of free and encapsulated algal extracts was evaluated by using the shake-flask method (Ye *et al*., 2005). In this test, 50 ml of each cell suspension and predetermined amounts of beads were placed in a sterilized flask and continuously shaken at 150 rpm on a rotary shaker. At prescribed time intervals, 1.0 ml of sample solution from the bead/microbial suspension system was removed by pipetting and optical density (O.D.) was measured at 550 nm (Kim *et al*., 2007). The optical density of the free extract samples was also measured as mentioned above.

2.5. Statistical analysis

Data analysis was performed with the software package Microsoft Excel, Version 2003. Statistically significant difference was determined using paired Student's t-test and $P<0.05$ was used as a limit to indicate statistical significance.

3. Results

3.1. Beads characterization

Figure 1 displays SEM observations of the external surface of plain and encapsulated Ca-alginate beads. The surface is rough, irregular and contains some pores (Figure 1a & 1b). Cracks and severe wrinkles are observed on the external surface of Ca-alginate bead encapsulated with the bioactive extract of *U. lactuca* (Figure 1c).

SEM micrographs of the internal surface of the formed beads are presented in Figure 2., It shows the presence of considerable number of pores (Figure 2a, 2b). The internal surface of Ca-alginate beads encapsulated with bioactive extract of *U. lactuca* contains some crystals (Figure 2c).

To gain information regarding the molecular structure of plain and encapsulated calcium alginate beads, the FTIR spectra for these beads were obtained (Figure 3). FTIR demonstrates the characteristic peaks of plain calcium alginate beads at 1614 and 1419 cm^{-1} assigned to the asymmetric and symmetric carboxylate (COO^{-}) vibrations, respectively (Figure 3a). The spectrum shows also some characteristic peaks at 804,

1276 (C-O), 2219, 2312 and 2925 cm^{-1} (CH_2 stretching).

In case of the spectrum obtained from beads encapsulated with *P. capillacea* extract (Figure 3b), two new peaks at 3776 and 2860 cm^{-1} (CH_2 stretching) are observed and the lack of peaks at 2312 and 2219 cm^{-1}. The peak at 804 cm^{-1} is decreased and appeared at 806 cm^{-1}. The spectrum of beads encapsulated with *U. lactuca* extract was characterized by the presence of the typical bands found in plain calcium alginate beads in addition to the presence of new absorption peaks at 675, 894, 1336, 2254, 2858 (CH_2 stretching) and 3774 cm^{-1} (Figure 3c). The peak at 804 cm^{-1} is clearly decreased and shifted to 813 cm^{-1}.

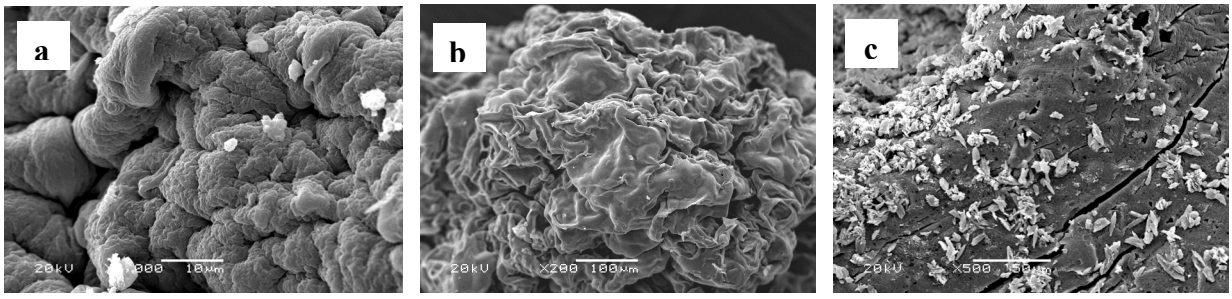

Figure 1. SEM micrographs of the external surface of (a) plain Ca-alginate bead, (b) Ca-alginate bead encapsulated with *P. capillacea* extract and (c) Ca-alginate bead encapsulated with *U. lactuca* extract.

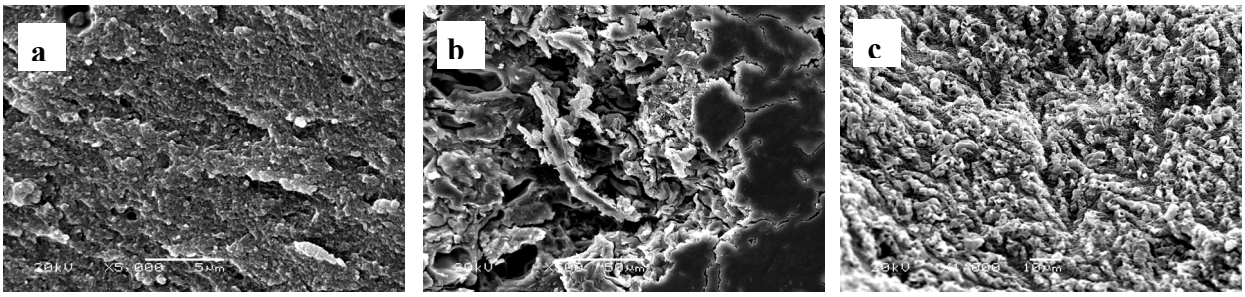

Figure 2. SEM micrographs of the internal surface of (a) plain Ca-alginate bead, (b) Ca-alginate bead encapsulated with *P. capillacea* extract and (c) Ca-alginate bead encapsulated with *U. lactuca* extract.

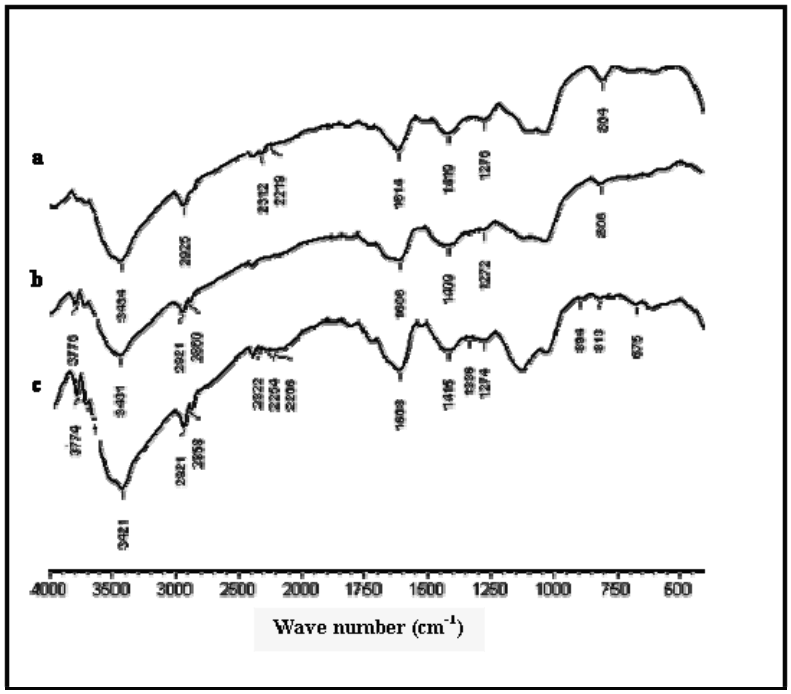

Figure 3. FTIR spectra of (a) plain Ca-alginate beads, (b) Ca-alginate beads encapsulated with *P. capillacea* extract and (c) Ca-alginate beads encapsulated with *U. lactuca* extract.

3.2. Antibacterial activity of free and encapsulated algal extracts

The antibacterial activity of the methanolic-L extract of *P. capillacea* increased noticeably after encapsulation in Ca-alginate beads where the exhibited antibacterial activities appeared stronger (significant at $P<0.05$), against *A. hydrophila*, *P. fluorescens* and *V. anguillarum* (Table 1, Figure 4). This extract reduced the growth of *A. hydrophila* by about 6 fold after 5 min while, the growth reduction of *P. fluorescens* was 1.8 fold after 10 min.

Furthermore, the encapsulated methanolic-L extract of *U. lactuca* showed better activity (significant at $P<0.05$) against *V. anguillarum* only compared to the free extract (Table 2 & Figure 5). It started its effect 5 min post addition of the beads and the effect increased with time up to 20 min (Figure 5b). Conversely, the free methanolic-L extract of *U. lactuca* possessed higher antibacterial activity ($P<0.05$) towards *A. hydrophila* and *P. fluorescens* than the encapsulated extract (Figure 5a & c). It exhibited complete elimination of *A. hydrophila* after 5 min only.

Table 1. Antibacterial activity of free and encapsulated extracts of *P. capillacea* against fish pathogens

	A. hydrophila			*V. anguillaum*			*P. fluorescens*		
	Optical Density (O.D*) x 10^{-3}								
Time (min)	Free bacteria	Free extract	Encapsulated extract	Free bacteria	Free extract	Encapsulated extract	Free bacteria	Free extract	Encapsulated extract
0	48	48	48	48	48	48	48	48	48
5	64	63	8	72	71	32	96	96	60
10	65	56	14	75	46	22	100	66	33
15	77	55	13	92	47	30	102	66	50
20	119	55	11	120	57	28	121	70	52
25	169	46	14	149	58	30	132	72	55
30	197	40	12	250	60	38	230	77	56
35	229	62	40	351	80	48	437	78	57
40	339	89	61	472	100	69	630	78	184

*O.D was measured at 550 nm

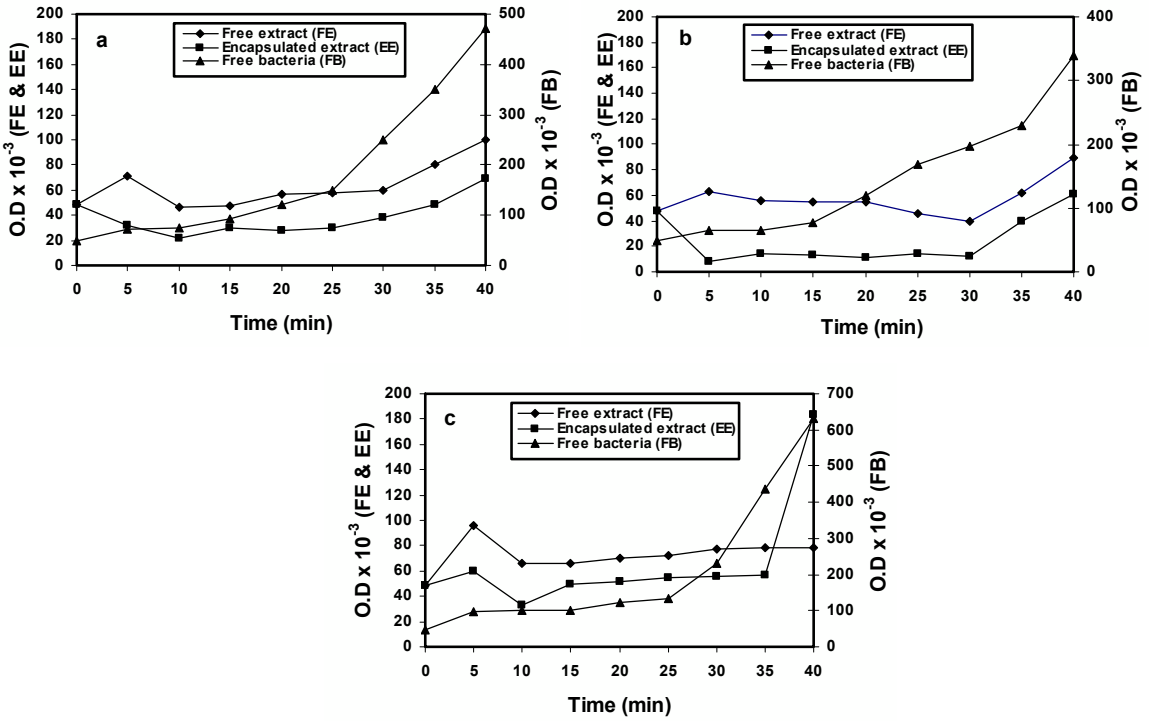

Figure 4. Antibacterial activity of free and encapsulated extracts of *P. capillacea* against (a) *A. hydrophila*, (b) *V. anguillarum* and (c) *P. fluorescens*.

Table 2: Antibacterial activity of free and encapsulated extracts of *U. lactuca* against fish pathogens.

	A. hydrophila			*V. anguillaum*			*P. fluorescens*		
	Optical Density (O.D*) x 10^{-3}								
Time (min)	Free bacteria	Free extract	Encapsulated extract	Free bacteria	Free extract	Encapsulated extract	Free bacteria	Free extract	Encapsulated extract
0	48	48	48	48	48	48	48	48	48
5	64	0	27	72	12	8	96	1	22
10	65	0	8	75	17	5	100	4	32
15	77	0	3	92	30	4	102	5	36
20	119	0	3	120	54	4	121	18	36
25	169	0	5	149	140	17	132	32	46
30	197	0	20	250	230	58	230	96	99
35	229	0	50	351	254	138	437	348	118
40	339	0	162	472	460	296	630	554	243

*O.D was measured at 550 nm

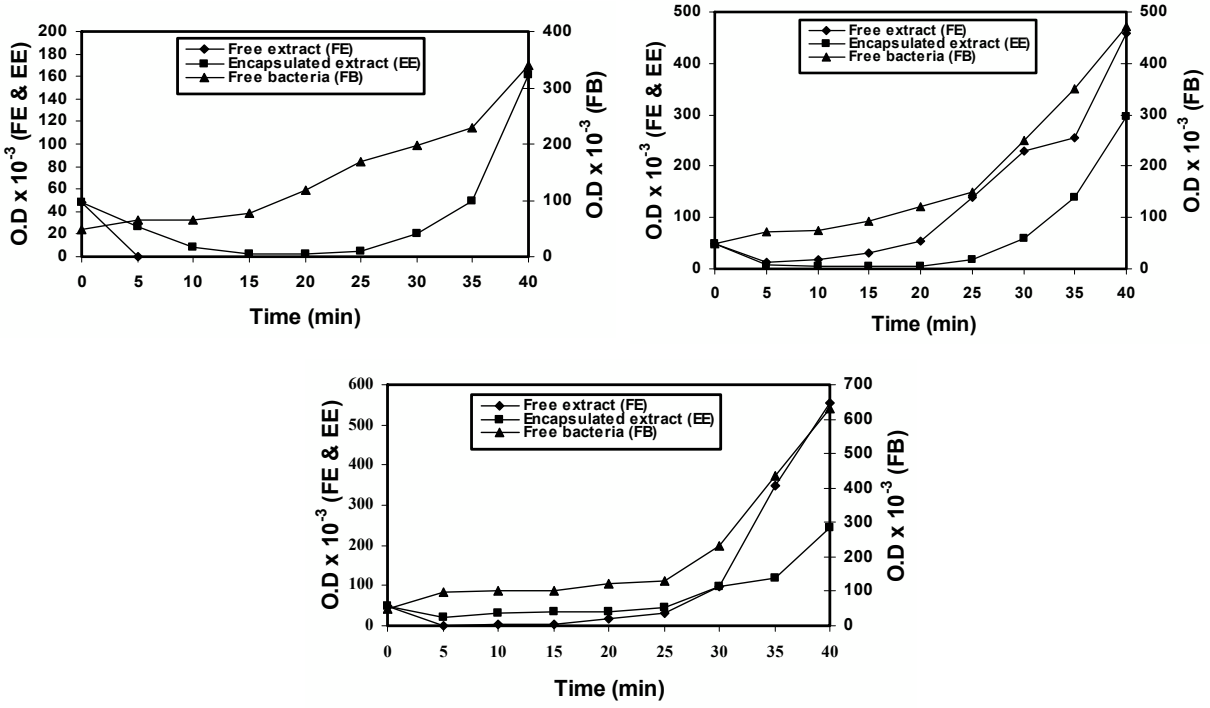

Figure 5. Antibacterial activity of free and encapsulated extracts of *U. lactuca* against (a) *A. hydrophila*, (b) *V. anguillarum* and (c) *P. fluorescens*.

4. Discussion

Scanning electron micrograph of the external surface of beads, encapsulated with the bioactive extract of *U. lactuca*, showed the presence of cracks which might be due to the partial collapsing of the polymer network during drying as reported by Pasparakis and Bouropoulos (2006). The SEM pictures of the internal surface of the beads possess very distinguished dark spots which can be taken as a sign for effective entrapment of the active extract in the cavities and pores of these beads. Moreover, the inner pores seem to be large to allow the release of the bioactive extract from the inner surface to the outer surface, resulting in the release of the bioactive extract from beads into the medium (Liu *et al*., 2008). The presence of crystals on the internal surface of Ca-alginate beads, encapsulated with the bioactive extracts of *U. lactuca*, could be attributed to sodium chloride. During gel formation, calcium ions interact with alginate to form the matrix, thus sodium ions are free to interact with chloride ions from the gelling bath. After drying of beads, sodium chloride crystals could have remained over the surface as suggested by Deladino *et al*. (2008).

FTIR, spectra of plain and encapsulated Ca-alginate beads showed the appearance of new peaks and the lack of others and these spectral changes further confirm the trapping of extracts inside alginate beads.

Immobilization either by covalent linkage to an insoluble matrix or by entrapment into gel of film support (Dumitriu, 2005) could provide stability to antimicrobial agents as reported for enzymes and bacteriocins (Le-Tien *et al*., 2004). Because alginate beads have unique physico-chemical properties, they have been used as an alternative cell immobilization matrix for several biotechnological applications.

In our previous work, the methanolic-L extracts of *P. capillacea* and *U. lactuca* have been proved to show significant antibacterial activity against some fish and human pathogenic bacteria (Wefky *et al*., 2009). In the present study, the methanolic-L extracts of *P. capillacea* and *U. lactuca* were immobilized by encapsulation into Ca-alginate beads in order to evaluate the effect of encapsulation process on their antibacterial activity. Consequently, a comparative study of the antibacterial effect of free and encapsulated extracts of *P. capillacea* and *U. lactuca* was investigated on the fish pathogens; *A. hydrophila*, *P. fluorescens*, and *V. anguillarum* using the shake-flask method (Ye *et al*., 2005) aiming for better activity and using them in the aquaculture.

Gortzi *et al*. (2008) reported that nanoencapsulation generally improves the antimicrobial activity of compounds and maintains the stability of antimicrobials over prolonged periods of time. In the present investigation, both encapsulated algal extracts of *U. lactuca* and *P. capillacea* exhibited different mode of action.

A. hydrophila was the most susceptible fish pathogen to the action of the encapsulated methanolic-L extract of *P. capillacea* followed by *V. anguillarum* and *P. fluorescens* but without complete elimination of the bacterial growth. This effect is attributed to the following processes: adsorption of extract onto the bacterial cell surface, diffusion through the cell wall, binding and disruption of the cytoplasmic membrane, and release of cytoplasmic constituents resulting in cell death (Massi *et al*., 2003). In addition to the presence of rigid lipopolysaccharide layer in the cell wall of the three Gram negative test pathogens which hinders the complete penetration of the bioactive substances and thus reduces the antibacterial action of the bioactive components. The inhibitory effect was proportional to time of exposure as mentioned by Millette *et al*. (2007).

Another observation was increasing the bacterial growth inhibition by using the encapsulated extract rather than the free extract, which could be explained by the protection of bioactive components into calcium alginate beads in addition to controlled release of them (Millette *et al*., 2007). Another explanation is the hydrophobicity of calcium alginate beads entrapping the extract, which retains the integrity of the beads and prevents their degradation over the course of experiment and thus allows more surface of contact between the extract and the bacterial surface (Kim *et al*., 2007). The increased antibacterial activity after encapsulation in Ca-alginate beads could promote the use of this extract as potent antibacterial agent in aquaculture.

The methanolic-L extract of *U. lactuca* showed reverse behavior of action where the bioactive components linked to alginate caused lower reduction rates than free extract when applied in the bacterial cultures of *A. hydrophila* and *P. fluorescens*. The same result was observed by Millette *et al*. (2007). Diffusion limitation of the bioactive components is an important factor affecting this process too (Bailey and Ollis, 1986).

5. Conclusions

The present study revealed that encapsulation of algal extracts into calcium alginate beads modified their antibacterial activities and could be used successfully to control the growth of fish pathogenic bacteria. The encapsulated extract of *P. capillacea* exhibited significant antibacterial activity against the tested pathogens. The free extract of *U. lactuca* showed good activity towards *A. hydrophila* and *P. fluorescens* whereas, the encapsulated extract of this alga possessed remarkable activity against *V. anguillarum*. Fourier transforms infrared (FTIR) and scanning electron microscopy (SEM) were useful tools to confirm the encapsulation of tested algal extracts into Ca-alginate beads.

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دراسة مقارنة للنشاط المضاد البكتيرى لمستخلصات طحلبى *Ulva lactuca* و *Pterocladia capillacea* قبل و بعد التقييد داخل كريات ألجينات الكالسيوم

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تمت دراسة تأثير مستخلصات الميثانول-L التي تم استخلاصها من بعض الطحالب البحرية مثل *Ulva lactuca* (Linnaeus) و*Pterocladia capillacea* (Gmelin) على النشاط البكتيري لأنواع من البكتيريا الممرضة للأسماك مثل *Aeromonas hydrophila*, *Vibrio anguillarum*, و*Pseudomonas fluorescens* ذلك قبل وبعد تقييد هذه المستخلصات داخل كريات الألجينات. وقد لوحظ تفوق مستخلص الميثانول-L الاتى من طحلب *P. capillacea* بعد تقييده وذلك بالمقارنة بالمستخلص الحر. بينما اظهر مستخلص الميثانول-L المقيد الاتى من *U. lactuca* نشاط ضد *Vibrio anguillarum* ($P<0.05$) فقط. على العكس تفوق مستخلص الميثانول الحر لنفس الطحلب على المستخلص المقيد فى نشاطه ضد *Aeromonas hydrophila* و *Pseudomonas fluorescens* عند ($P<0.05$) . كما تم فحص شكل الأسطح الداخلية والخارجية لكريات ألجينات الكالسيوم وذلك باستخدام الميكروسكوب الاليكترونى الماسح (SEM). هذا بالاضافة إلى إستخدام طيف الأشعة تحت الحمراء للتعرف على التغيرات التى طرأت على التراكيب الكيميائية لهذه الكريات.