ENCAPSULATION OF LIVE MARINE BACTERIA FOR USE IN AQUACULTURE FACILITIES AND PROCESS EVALUATION USING RESPONSE SURFACE METHODOLOGY.

Running title: Encapsulation of live marine bacteria for use in aquaculture

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Abstract

New strategies are being proposed in marine aquaculture to use marine bacteria as alternative to antibiotics, as nutritional additive or as immune-stimulant. These approaches are particularly promising for larval and juvenile cultures. In many cases, the bacteria are released in the seawater, where they have to be at appropriate concentrations. In addition, only low cost technologies are sustainable for this industry, without any complex requirements for use or storage. In this work we explore the possibilities of preservation of a potential marine probiotic bacterium (*Phaeobacter* PP-154) as a product suitable for use in marine aquaculture by addition to the seawater. A method which guaranteed the preservation of the viable marine bacteria in a saline medium and their rapid release in the seawater was searched for. In a previous step, classical procedures (freeze-drying and freezing) had been explored, but undesirable results of the interaction of the products obtained with natural seawater led to investigate alternatives. We report the results of the immobilization of the marine bacteria in alginate-calcium beads. The final product complies the salinity which allows the requirements of the bacteria without interference with alginate in the formation of beads, and a balanced hardness to retain the bacteria and to be easily released in the marine aquaculture environment. The process was evaluated using the Central Composite Rotatable Design (CCRD), a standard Response Surface Methodology (RSM).

Keywords

marine bacteria, *Phaeobacter*, aquaculture, encapsulation, RSM

INTRODUCTION

Aquaculture is probably the fastest growing food-producing sector. It is estimated that by 2030, the world will require the production of an additional 27 million tonnes (Tn) of fishery products to satisfy the growing demand for safe and quality aquatic food (FAO 2016a). With stagnating, and even declining in an immediate future, global capture fishery production and an increasing population, aquaculture has the greatest potential to increase production in the future to meet this demand (FAO 2011a). The production of aquatic animals from aquaculture has expanded rapidly (FAO 2011b), at an average annual growth rate of 5.8% from 2005, until 73.8 million Tn (estimated first-sale value of US$ 160.2 billion) in 2014. Production in marine waters accounted for 36% of this total by quantity (26.7 million Tn) and 44% by value (US$ 69.7 billion) (FAO 2016b), including many high-value finfish, crustaceans and mollusc species (FAO 2011b).

An adequate supply of quality juveniles is a major step towards establishing the foundation for sustainable global aquaculture production. In molluscs, the seed (juveniles) is obtained from both wild and hatchery sources (FAO 2011b), though hatchery-production is increasingly becoming the standard raw material for aquaculture, a trend that is likely to broaden in the future. The required development of this industry should be supported by technology and research. Recent advances in marine microbiology and biotechnology constitute an important tool in the management of marine aquaculture. As result, new strategies have been proposed to use marine bacteria as alternative to antibiotics, as nutritional additive or as immune-stimulant (Hoseinifar et al. 2018; Prado et al. 2010). These approaches are specially promising in larval and juvenile cultures, frequently affected by episodes of mortalities which compromise the economic viability of the industry and without effective treatment until now. For many of these new proposals, the bacteria have to be viable at appropriate concentrations in the marine environment. In addition, considering the narrow interaction between animal (mainly in initial stages of development) and its surrounding environment in aquaculture, many of the procedures are designed to release the bacteria in the seawater. Also, the production costs need to be low because expensive technologies are not sustainable for this industry. Further, because of similar reasons, it would be advisable that the product can be stored in the installations without special requirements and used by non-specialized personnel.

In this work, and taking into account the considerations above detailed, we explore the possibilities of preservation of a potential marine probiotic bacterium *Phaeobacter* PP-154 (Prado 2006) as a product suitable for use in marine aquaculture by addition to the seawater. This marine bacterium showed a wide spectrum of antibacterial activity, including against shellfish and fish pathogens, mainly *Vibrio* sp. The antibacterial compound is attached to the bacterial cells, without significant release of the substance. In seawater, PP-154 was able to inhibit vibrios when it was inoculated before or at the same time, but not after the pathogens were established (needed to be incorporated to the system previous or simultaneously to the income of the pathogen and the increase of its population in the environment). Micro-scale experiments showed that the strain PP-154 added to seawater reduced the mortality rates in flat oyster larvae caused by an inoculated vibrio, and it also enhanced the survival of natural infected larvae, with a drastic reduction of vibrios in culture seawater. Besides, the supply of PP-154 to phytoplancton (monospecific or mixture used as food) prevented the proliferation of vibrios and therefore avoided this route of entry of potential pathogens [Prado et al. 2009, 2010]. On the basis of those results, a method which guaranteed the preservation of the viable marine bacteria in a saline medium and their rapid release in the seawater was searched for. In a first step, classical procedures (freeze-drying and freezing) had been explored, but undesirable results of the interaction of the products obtained with natural seawater led to investigate alternatives.

Cell immobilization by encapsulation or entrapment in polymeric beads is a technique with applications in many fields (Callone et al. 2008; Duarte et al. 2013; Gillet et al. 2000). One of the most popular method is the external ionic gelation because of its simplicity, low cost and gentle formulation conditions that ensure high cell viability (Krasaekoopt et al. 2003). This procedure involves preparing a hydrocolloid solution, adding microorganisms, extruding the cell suspension through a syringe needle and dripping the droplets into a hardening solution (Martin et al. 2015). Alginate is an acid polysaccharide (polyanionic copolymers) mainly obtained from seaweed from brown algae (*Laminariales* and *Fucales*) (Goh et al. 2012; Kakita and Kamishima 2008). Alginates are composed by units of D-mannuronic acid and L-guluronic acid (Donati et al. 2005). Aqueous sodium alginate solution can undergo sol-to-gel transformation in the presence of cross-linking cations (divalent and trivalent ions) (Goh et al. 2012). Alginate is the most commonly used polyelectrolyte since it enables a simple encapsulation process that can be performed at neutral pH and at room temperature, and it does not require any harsh chemicals or drastic conditions (pH, temperature, ionic strength) for the formulation process (Westman et al. 2012). Divalent or trivalent cations are required for forming the alginate networks (insoluble alginate salts). Calcium (Ca^{2+}, as calcium chloride) is the most common cation used for the cross-linking (Mallón et al. 2007). However, other cations such as Fe^{3+} have been also utilized (Privman et al. 2016).

In this work, we report the results of the immobilization of the marine bacteria *Phaeobacter* PP-154 in alginate-calcium beads to be released in marine aquaculture environment and the evaluation of the process using Response Surface Methodology. In our study, the specific requirements of the final product should include: i) salinity requirements maintaining the bacteria alive and avoiding the precipitation of alginate, and ii) balanced hardness to retain the bacteria and to allow an easy release to the marine aquaculture environment.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The strain *Phaeobacter* PP-154 (deposited in the Spanish Type Culture Collection for patent purposes, CECT 5891) is marine bacterium isolated from flat oyster *Ostrea edulis* spat in a hatchery (Prado 2006). It grows well in Marine Agar (MA, Difco BD, Franklin Lakes, NJ, USA, Detroit), producing rounded cream-brown colonies with a brown diffusible pigment. This strain grows at salinity between 15 and 36‰, with more complex requirements than NaCl. Strain PP-154 displays a wide spectrum of antibacterial activity, which includes aquaculture pathogens, particularly *Vibrio*. It is able to develop the inhibitory activity in seawater, controlling the growth of vibrios. The antibacterial compound(s) is attached to the bacterial cells, without significant release of the substance to the medium. (Prado et al. 2009, 2010).

Bacterial cells of 4-day-old cultures in Marine Broth (MB, Difco, Sparks, MD, USA) at pH 7.0–7.7 were demonstrated to be the optimal to display inhibitory activity (Prado et al. 2009). In this work, strain PP-154 was grown under these conditions in shaking cultures at 23±1ºC for 4 days, yielding concentrations $\geq 10^9$ cfu/ml. For harvesting, the culture was centrifuged in sterile containers, using a high speed centrifuge Beckman J2-HS (Beckman Coulter, Indianapolis, IN, USA) at 9.000-10.000 rpm, for 12.5-15 min. The supernatant was discarded and the pellet suspended in sterile seawater (SSW or SSW15‰) in sterile containers and stored at 4ºC until use.

Preliminary assays for preserving marine bacteria by classical methods and interactions in seawater

Preliminary tests were performed using the classical methods for bacterial preservation: freeze-drying and freezing.

Freeze-drying

Strain PP-154 was freeze-dried using skim milk (SM) as cryoprotectant. Additives were prepared at concentrations of 5, 10 and 20% (w/v) (SM 5-10-20) and sterilized by autoclaving (110ºC – 10 min). PP-154 was cultured under the conditions above described. After 3-5 days, cells (400 ml) were harvested, suspended in the protectant and frozen. Total cfu was calculated on the basis of the bacterial concentration estimated by plating in MA. Cells were freeze-dried using Labconco

FreeZone® 6-L Freeze-dry System Model 79340-79480 (Labconco, Kansas City, MO, USA) at a collector temperature of −20/40°C for 48 h at 4 Pa. After the freeze-drying cycle was completed, the vials were sealed and stored at 4ºC. Freeze-dried bacteria were rehydrated with SSW. The recovery was determined by serial dilution in SSW and spread on MA plates. The plates were incubated at 25ºC for 48-96 h (to allow pigmentation). The loss of bacteria in the process was expressed as the difference between initial and final logarithms. With the purpose of determining if the strain PP-154 maintained the inhibitory activity, a sample of the rehydrated product (SM10) was tested in natural seawater (NSW). A simple assay was carried-out in a tank containing 5 l of NSW, inoculating 0.5 mL of rehydrated bacteria. Samples were taken at 0 and 48 hours and appropriate dilutions spread on MA and Tiosulphate-Citrate-Bile-Sucrose (TCBS, Oxoid, UK) plates to determine the presence of PP-154 and vibrios, respectively.

Freezing

Strain PP-154 was frozen at -20ºC using MB + glycerol (Panreac, Barcelona, Spain) 15% (MBg), inositol (Sigma) 10% (INO) or sucrose (Biolife Italiana Srl, Milano, Italy) 15% (SUC) as cryoprotectants. The recovery of thawed bacteria was determined on MA plates (see above). The different products obtained were added to NSW with vibrios to test the antibacterial activity of strain PP-154. Controls without bacterial addition and with only the protectant were included in each design. Experiments were carried-out in flasks with 100 mL of NSW and 10 µL of bacterial inoculum, in duplicate. Samples were taken at time 0 and after 24 hours and processed as previously detailed. The same design was reproduced in flasks with SSW.

Encapsulation as alternative for preservation of marine bacteria

Entrapment of bacteria in a gel matrix composed on alginates is the most popular system of encapsulation by the formation of a continuous coating around an inner aqueous gelled core. This technique of microencapsulation offers some advantages as adding a very low cost to production and the easiness of scale-up from laboratory to industrial scale. Ionic gelification of alginate with calcium ions was used in this work to fabricate living bacteria-containing beads to be used in a marine environment. With this application in mind, some parameters of the usual formulation process should be selected to obtain particles under these conditions, preserving viability of bacteria. Two factors, the cross-linking agent and salinity, were investigated in relation to the preparation of alginate beads, their effect on bacteria preservation and protection from the external environment.

Factors screening: cross-linking agent and salinity

In preliminary studies, in-house works, different cations naturally present in seawater (Mg^{2+}, Fe^{3+}, Ca^{2+}) were assayed to form the alginate networks (Millero et al. 2008; Tagliabue et al. 2017). Several salts were intended to use as cross-linking agents for alginate. Thus, $CaCl_2$, $Ca2SO_4$, $MgSO_4$ and $FeCl_3$ were added to alginate dispersion at different levels.

Particles preparation

According to the preliminary approaches, *Phaeobacter* PP-154 cells were immobilized by gel-entrapment in alginate matrix (ionic gelation), as described elsewhere (Kierstan and Bucke 1977) with some modifications to ensure the marine bacteria viability and the beads formation. Herein, low viscosity alginate (Alginate LV, Kelco Corp., Chicago, IL, USA) and Ca^{2+} (as $CaCl_2$) (Panreac, Barcelona, Spain) were used as polyelectrolyte and cross-linking agent respectively for the preparation of the beads. Alginate and calcium chloride solutions were prepared in SSW10‰. An experimental design was performed to evaluate the particulate formulation. Both, Ca^{2+} and alginate were used as variables at different concentrations (levels) according to a central composite rotational design (CCRD) (see Tables 1-2). The alginate LV was first dissolved in distilled water (DW) under shaking and appropriate volume of NSW (previously calculated depending on its salinity) was added to get the desired final volume of solution in SSW10‰. The calcium chloride was directly dissolved in the adequate volume of prepared SSW10‰ under stirring. Bacterial suspension in SSW15‰ (≥ 10^{10} cfu/ml) was prepared as previously described, Alginate solution (5 mL) and bacterial suspension (5 mL in 15% SSW) were mixed. The mixture alginate-bacteria was added drop-wise into the calcium chloride solution (250 mL) while stirring magnetically to prevent the droplets from sticking together. Beads were allowed to reticulate for 30 min. Once the beads were formed, they were harvested and separated by filtration using Whatman filter paper (No. 1). The beads were carefully collected and transferred to a sterile container (with SSW level enough for keeping the beads wet) and stored in a refrigerator (4±1ºC) until use.

Recovery: release of encapsulated cells [R]

Individual beads were weighted and homogenized in SSW (1 mL: 10 mg, v/w) using a mini-hand-blender (sterilized by flaming with alcohol). Appropriate dilutions were spread on MA and incubated as detailed above. Results were expressed as colony forming units per milligram of capsule (cfu/mg).

Viability of released bacteria [V]

These responses were estimated from the growth rate at different times. Experiments were carried out in 250 mL sterile flasks containing 200 mL SSW. One capsule corresponding to each formulation was homogenized (see above *Release of encapsulated cells*). One mL of the suspension obtained was inoculated. The cultures were incubated at 23±1 ºC on an orbital shaker (100 rpm). Samples were taken at days 5, 8 and 10 and processed as previously described. Results were calculated by the following equation:

$$GR = (\ln N_i - \ln N_0)/(t_i - t_0) \qquad (1)$$

where GR is the growth rate, N_i and N_0 are cfu/ml at time points t_i and t_0.

Experimental design: Response Surface Methodology (RSM)

In the present work, experimental design and response surface methodology (RSM) were used to study the effects of alginate and calcium concentration on bacterial entrapment. The experimental design and statistical analysis were done using Statgraphics Centurion XVI software package (StatPoint Technologies, Inc, VA, USA). The central composite rotatable design (CCRD), a standard RSM design, was used for the evaluation.

The number of tests required for CCRD includes the standard 2^n factorial with its origin at the center, $2n$ points fixed axially at a distance, say α ($\alpha = 2^{n/4}$), from the center to generate the quadratic terms, and replicate tests at the center (n_c); where n is the number of variables. The axial points are chosen such that they allow rotatability (Box and Hunter 1957), which ensures that the variance of the model prediction is constant at all points equidistant from the design center. Replicates of the test at the center provide an independent estimate of the experimental error. For two variables, the recommended number of tests at the center is five (Box and Hunter 1957). Hence the total number of tests required for the two independent variables, alginate and calcium concentration, is $N = 2^n + 2n + n_c = 13$.

Once the desired ranges of values of the variables are defined, they are coded to lie at five levels: ±1 for the factorial points, 0 for the center points and ±α for the axial points ($\alpha = 1.414$) (Mizumoto et al. 2007). The codes are calculated as functions of the range of interest of each factor as shown in Table 1. The values of independent variables are coded according to the equation $x_i=(X_i-X_0)/\Delta X_i$, where x_i is the coded value of the independent variable given by the experimental matrix, X_i is the real value of the independent variable, X_0 is the real value of the independent variable at the central point, and ΔX_i is the step of change (Zhang et al. 2013). The dependent variables (responses) were: recovery (*R*, cfu/mg), and viability at days 5 (*V5*), at day 8 (*V8*) and at day 10 (*V10*), measured as growth rate. The experimental sequence was randomized to minimize the effects of uncontrolled factors. The experimental results of the RSM were fitted via the response surface regression procedure using the following polynomial equation:

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^1 + b_{22} X_2^2 + b_{12} X_1X_2 \qquad (2)$$

where Y_i are the predicted responses, X_1 and X_2 are the independent variables, b_0 is the intercept term, b_1 and b_2 are the linear effects, b_{11} and b_{22} are the quadratic effects and b_{12} is the interaction term. This equation represents an empirical model, in which the response functions allow the estimation of responses due to changes in the dependent variables.

The adequacy of the fitted model equations was evaluated by variance analysis (ANOVA). The three-dimensional response surface, two-dimensional contour, main effect and Pareto plots were generated to visualize the effects of the process variables on the response variables, using Statgraphics Centurion XVI.

RESULTS

Preliminary assays for preserving marine bacteria by classical methods and interactions in seawater

Freeze-drying

The first approach was the freeze-drying using skim milk as protectant. The recovery was within an acceptable range, with losses between 1.3 and 3.5 logs in the rehydrated product respect to the initial concentration (Table 2). Skim milk 10% was selected, after the initial tests with SM 5-10-20. However, the recovery is not the only requirement for the product. The bacteria should be active when they were added to seawater, displaying the expected antagonistic ability, mainly against vibrios. Unfortunately, the first test carried-out in natural seawater showed that this was not the case. In addition, the appearance of the non-pigmented mutant was observed in the rehydrated product. Therefore, undesirable effects were recorded, summarized as follows. i) Non-pigmented mutant. Despite the good recovery after freeze-drying in skim milk, the rehydration of the product in SSW resulted in the appearance of the non-pigmented mutant. In 8 out of 10 tests, the non-pigmented variant was present, achieving a concentration only slightly lower than those of the pigmented type (differences between 0,2-1,6 logs). ii) Vibrios in natural seawater. The rehydrated bacteria added to NSW with initial presence of vibrios did not display the expected antagonistic activity, even though PP-154 increased from 9.4×10^3 to 1.2×10^5 cfu/ml (Figure S1).

Freezing

The protectants tested for freezing PP-154, MB+glycerol 15% (MBg), inositol 10% (INO) and sucrose 15% (SUC), resulted in acceptable values of recovery, with losses between 1.1 and 3.0 logs. Nevertheless, the addition of thawed bacteria to NSW resulted in the proliferation of vibrios, initially present in the seawater. The effect seemed to be related to the protectant, directly (MBg, SUC) or in combination with PP-154 (INO) (Figure 1). As expected, the controls in SSW, alone or with the substance, did not record any growth (results not plotted on graphics).

The processes and the substances assayed, alone or in combination, seemed to imply a risk of undesirable effects on the antibacterial ability of the marine strain in seawater. All these results led us to search a method for the preservation of marine bacteria minimizing the stress factors associated to the freezing / freeze-drying and the use of additives.

Encapsulation by ionic gelification-extrusion

Some parameters were preliminary considered to screen the adequate experimental factors to obtain stable beads compatible with live marine bacteria and salinity in seawater. Firstly, in preliminary in-house work, different cations naturally present in seawater were assayed to cross-link the alginate networks: Ca^{2+} ($CaCl_2$ and Ca_2SO_4, Feistel et al. 2008), Mg^{2+} (Mg_2SO_4, Millero et al. 2008), and Fe^{3+} ($FeCl_3$, Tagliabue et al. 2017). Only $CaCl_2$ and $FeCl_3$ allowed the obtaining of beads with adequate consistency and tuneable. Mg^{2+} (Mg_2SO_4) was discarded because it didn't allow the beads fabrication. On the other side, Ca_2SO_4 rendered beads with a low consistency and yield due to the low solubility of the salt that causes a slow cross-linking process. Thus, it was also discarded. Although the consistency of the beads using $FeCl_3$ was appropriated, the cross-linking agent altered the bacteria growth, thus this alternative was also quitted. In summary, none of the cations used provided an efficient cross-linking effect of alginate structure at any of the concentration and under the salinity conditions tested. Therefore, only calcium alginate was used as beads formation agent to encapsulate bacteria.

Furthermore, ions naturally present in seawater may displace the cross-linking agent along the time, and therefore affect the degradation of the gel (Sachan et al. 2009). The effect of the medium ionic strength in alginate viscosity led to determine the minimum salinity in seawater compatible with keeping bacterial viability, dissolving the alginate and forming stable beads. Because of the salinity requirement of marine bacteria, the formation of beads with sterile seawater (salinity ca. 33‰) instead sterile distilled water was assayed. As result, gelation did not occur and led to evaluate the salinity which allows the survival of marine bacteria join to the obtaining of consistent capsules. The minimum salinity required for the survival of the bacteria that does not interfere with the bead formation was 12.5% in the final alginate dispersion. Based on this set-up conditions, the bacteria were always suspended in SSW15‰, low viscosity sodium alginate in SSW10‰ (first dissolved in DW) and calcium chloride in SSW10‰.

Experimental design

Central composite rotatable design (CCRD) was used to evaluate the immobilization of a marine bacterium in alginate-calcium beads. The concentrations of alginate and calcium were selected as the two independent variables. This experiment was carried out to determine the effect of calcium and alginate concentration on bacteria encapsulation features. Thus, a total of 13 runs with two variables at five levels, including five replicates at the central point, were conducted according to CCRD in RSM. The lower and upper limits of the assay were stablished taking into consideration the lower and upper concentration of the components for the beads to be formed. Lowest concentrations of calcium and alginate did not render in cross-linking of polymer and the most concentrated led to a premature cross-linking.

Using factorial design and RSM, variations on recovery and viability of the marine bacterium at days 5, 8 and 10 were predicted as functions of the variations in alginate and calcium concentrations. The process variables used in the experimental design and response values obtained are shown in Table 3. The statistical package Statgraphics Centurion XVI was employed to perform the analysis and to generate polynomial models. Equations represent the obtained model for prediction of the response in the studied ranges of independent variables. The significance of each coefficient was determined using the F test and p-value. The corresponding variables were more significant if the absolute F-value became larger and the p-value smaller. The best equation for each response is suggested considering the significant parameters in the model ($p < 0.05$). Analysis of variance (ANOVA) of each response as a function of alginate and calcium are presented in Table 4. Complete data are reported as Supplementary Material (Tables S1-S2).

Response 1: *Recovery* [*R*]

The number of bacteria released from the beads formed under different alginate and calcium chloride concentrations was determined. Results were expressed as cfu/mg to minimize the differences in size/weight of the beads.

Equation (3) (Table 4) represents the obtained model for prediction of the recovery in the studied ranges of alginate and calcium concentrations. The equation shows the linear and negative effect and quadratic and positive effect of alginate, significant at a 5% probability level. The negative linear effect of calcium and the positive quadratic effect of calcium and of interaction between alginate and calcium were not significant, so they were omitted from Eq. (3).

$$Y_1=79439.6 - 404022\,X_1 + 277281\,X_1^2 \qquad (3)$$

A Pareto chart of standardised effects (Figure 2) showed significant effects of alginate (linear and quadratic) on recovery. Analysis of variance (ANOVA) supported that the resultant model adequately represented the experimental data (Table 4). The R^2 value (coefficient of correlation resulting from the regression of the model equation) indicated that 93.2% of the variability (R^2=0.932) in the recovery response can be explained by the model. The closer the R^2 value is to 1.0, the stronger the model and the better the response prediction. The adjusted R^2, derived from the samples size and the number of terms in the model equation, was 0.883 (88.3%). ANOVA was used to evaluate the significance of the coefficients of the quadratic polynomial models. A large *F*-value and a small *p*-value indicate a more significant effect on the response variables. Thus, the variable with the largest effect on recovery was the linear term of alginate ($p = 0.0001$), followed by the quadratic term of alginate ($p = 0.0014$). The effect of the other terms was not significant ($p > 0.05$).

The 3D-response surface (Figure 3) and 2D-contour (Figure 4, Figure S2) plots were used for finding the best combination of alginate and calcium that lead to an optimum maximum specific recovery. The maximum corresponded to the minimum alginate (and calcium). As the concentration of alginate increased, the recovery decreased. The minimum was in the area with alginate between 1.25-1.75.

Response 2: *Viability at day 5* [*V5*]

The response was given by a linear equation (Eq. (4)) with one significant coefficient, corresponding to alginate (Table 4).

$$Y_2=1.03516 + 0.348386\,X_1 \qquad (4)$$

The goodness of fit of the model checked by the coefficient of determination indicated that 87.8 % of the total variation was explained by the model. The Pareto chart (Figure 2) showed significant synergistic effect of alginate on viability at day 5. The response surface (Figure 3) and contour (Figure 4, Figure S2) plots estimated a growth rate corresponding to central points ≥ 1. The maximum value was expected with maximum of alginate (and calcium), while the minimum was estimated with minimum alginate and maximum calcium.

Responses 3 & 4: *Viability at day 8* [*V8*] & *10* [*V10*]

The results achieved in the analysis of data at days 8 and 10 were similar to those observed in viability at day 5, but with an attenuation of the synergistic effect of alginate (Figure 2, Figure 3 and Figure S2).

Y_3=0.68758 + 0.203754 X_1 (5)

Y_4=0.5354 + 0.172428 X_1 (6)

The overlapped main effects plots corresponding to *R*, *V5*, *V8* and *V10* (Figure 5), showed opposite effects of each independent variable on recovery and viability. Thus, the increase of alginate enhanced the viability but diminished the recovery, and to a lesser extent, similar effects were observed with calcium increase.

The overlapped contour plots of *R* and *V5* (Figure 4) illustrated that central point yielded $R > 7.0 \times 10^4$ cfu/mg and $V5 > 1.0$, a balanced result. The only chance to improve the results of central points would imply a slight decrease in alginate concentration (0.95) and increase in calcium (1.57), variations difficult to reach with accuracy, and that only offer a small enhancement of the results.

DISCUSSION

Preliminary assays for preserving marine bacteria by classical methods and interactions in seawater: Freeze-drying & freezing

The preservation of marine bacteria and the obtaining of products suitable for their use in marine environment of aquaculture facilities need specific research, considering the special conditions of both marine bacteria and marine environment.

Freeze-drying, or lyophilization, is a commonly used technique for preservation of bacteria (Broeck et al. 2016). In our experiments, the recovery of strain PP-154 using skim milk as lyoprotectant resulted in good values, but unexpected and undesirable effects were observed. The first was the appearance of the non-pigmented variant of the strain, lacking the dark extracellular pigment, after rehydration with SSW. The appearance of spontaneous non-pigmented mutants or variants has been observed in other *Phaeobacter*. The mutants are stable and lost the ability to produce the antibacterial compound(s). These variants were found “on rare occasions” (Bruhn et al. 2007) or only “several times” (Brinkhoff et al. 2004). However, in our experiments the rehydration after freeze-drying of strain PP-154 resulted in the appearance of the non-pigmented variant in 8 out of 10 tests, perhaps because the non-competitive conditions, under which the variant may be more frequent (Lutz et al. 2016). By the other hand, the rehydrated (and pigmented) PP-154 added to natural seawater with vibrios was not able to control them. Freeze drying implies some stress factors, as the cellular damage by ice crystals, osmotic damage by the unfrozen solutes or damages related to rehydration. It also has several draw-backs: it is an expensive and time-consuming process, additional processing could be needed to obtain powder particles, and the risk of cross contamination within a freeze-dryer has been reported (Broeck et al. 2016). All these considerations, joined to the results obtained, led to rule out this option.

Cryopreservation is other of the standard methods to preserve bacterial cultures. Freezing PP-154 with different protectants resulted in an acceptable preservation, but again undesirable effects came up when the thawed bacteria were added to natural seawater with vibrios. In the case of MBg and SUC, the protectant alone caused an increase of vibrios, which was not controlled (SUC) or only weakly (MBg) by thawed bacteria. In both cases the number of vibrios was above those recorded in controls without any addition. Respect to INO, though the substance itself did not enhance the growth of vibrios, the thawed bacteria with the protectant did it. From a commercial point of view, freezing has several disadvantages such as the need for subzero transportation and storage temperatures, and thus high energy costs. Moreover, cellular damage to the bacterial cells can be caused by freezing and thawing (Broeck et al. 2016). Again, these factors and the experimental data led to reject this option.

In summary, the results of the interactions of the substances, the seawater and the marine bacteria observed in the use of the products obtained by classical procedures, and the risks associated, made advisable the search of an alternative method for preservation, reducing the additives and minimizing the stress factors associated to the freezing and freeze-drying, and suitable for applying to heterotrophic marine bacteria.

Encapsulation of bacteria.

Extrusion technique is the most popular method for encapsulation of living cells. In this method, a polymeric solution is mixed with the cells and then extruded through an orifice as droplets into the solution of a cross-linking agent. Alginate is the most used polymer because of non-toxicity, its excellent biocompatibility properties, low cost, and mild and simple gelling conditions that ensure high cell viability (Martin et al. 2015; Rathore et al. 2013). Some drawbacks attributed to alginate microparticles, as their susceptibility to acidic environments or chelating agents, and their reduced barrier properties against unfavourable environmental factors (Martin et al. 2015; Rathore et al. 2013), are not relevant for the use as water additives in aquaculture facilities.

Only a few works addressed the specific application of the immobilization of live microorganisms for use in marine aquaculture environment. Marine microalgae have been successfully encapsulated in alginate-calcium (Chen 2003; Pales Espinosa et al. 2007). Live bacteria, probiotic lactic acid bacteria (*Bacillus* spp.), were successfully microencapsulated by emulsification/internal gelation in alginate-calcium beads (with maize-oil and Tween 80) and supplied as water additive to larval and post-larval cultures of Pacific white shrimp (*Litopenaeus vannamei*) (Nimrat et al. 2011). Regarding to marine bacteria, *Shewanella putrefaciens* Pdp11 was encapsulated in alginate-calcium beads for feeding juvenile fishes (*Solea senegalensis*) with the final aim of releasing the probiotic in the intestinal tract, without causing damage due to gastrointestinal conditions through the passage (Rosas-Ledesma et al. 2012). With the same strain, assays were also performed encapsulating crushed pellet diet with the probiotic for feeding juvenile sea breams (*Sparus aurata*) (Cordero et al. 2015).

In this work, a marine bacterium, *Phaeobacter* PP-154, was encapsulated in alginate-calcium beads by external ionic gelation by extrusion. The optimization of the process led to the design of a general and easy protocol to immobilise marine bacteria for quick release in seawater of aquaculture systems. The methodology was adapted to the general requirement of marine bacteria (salinity > 10‰), with further modifications to guarantee the formation of beads under these conditions.

Ions naturally present in seawater may displace the cross-linking agent on alginate structure, affecting mainly the degradation of the gel (Sachan et al. 2009). Therefore, the effect of the medium ionic strength in alginate viscosity led us to determine the minimum salinity in seawater compatible with bacterial viability, dissolving the alginate and forming stable beads. We focused on the cations normally found in coastal seawater, though different divalent and trivalent cations have been used (Sachan et al. 2009). Beads with adequate consistency were obtained with Ca^{2+} or Fe^{3+}. However, in our case, though beads were formed with Fe^{3+} as gelating cation, as reported by Goh et al. (2012), the growth of the bacteria was altered. Thus, Ca^{2+} was chosen as cross-linking agent for the rest of the experiments. It is also worthwhile to mention that different results on beads forming capsules may be obtained depending on the alginate provider. Different physic-chemical properties of alginate dispersion (molecular weight, viscosity, etc.) or gel formation can be shown, so the conditions reported here for beads formulations may not been completely comparable for all of alginate, depending on its origin. For example, Kakita and Kamishima (2008) has shown as the resistance of alginate fibers obtained with different molecular weight, and subsequently, different viscosities and different monomers ratio in their composition, resulted in different stability when cross-linked with calcium in the presence of sea water.

The encapsulation of living material in alginate beads cross-linked with Ca^{2+} using desalted water has been used for the encapsulation of bacteria for the treatment of residual waters (de-Bashan et al. 2002), yeast (Becerra et al. 2001), vegetal materials (Mallón et al. 2007), vaccines (Romalde et al. 2004) and microalgae (Pales Espinosa et al. 2007), etc. However, to keep alive the sea bacteria it is required the use of seawater to form the beads. The use of seawater (33-36‰) interfered with the beads formation. In previous studies, the formation of the gel under saline conditions was checked, as well as the degradation of the gel by other ions presented in the medium by displacing the cross-linking agent (Sachan et al. 2009). For example, high sodium contents may compete with the gelating calcium cations in the alginate carboxyl groups, promoting weak gel formation (Martinsen et al. 1989). In addition, the degradation of a Ca^{2+} cross-linked alginate gel can occur by removal of the Ca^{2+} ions caused by different factors, among them a high concentration of ions such as Na^+ or Mg^{2+}. As Ca^{2+} ions are removed, the cross-linking in the gel decreases and the gels are destabilized, with leakage of entrapped material and solubilization of the high molecular weight alginate polymers. Alginate gels also degrade and precipitate in a 0.1 M phosphate buffer solution (Sachan et al. 2009). Thus, the minimum concentration required for keeping alive the bacteria without interfering with the bead formation keeping the beads stability was adjusted under our experimental conditions.

Once the experimental conditions were adapted to marine environment, the process was evaluated according to CCRD in RSM (Becerra et al. 2001). Two different measurements, recovery and viability, were considered as dependent variables to adjust the encapsulation of the marine bacteria PP-154 in alginate-calcium beads, represented in four responses related to the independent variables, alginate and calcium. Alginate was the only significant variable for any of the responses, but with opposite effect on recovery and viability. The results showed a negative influence of the alginate concentration on the direct recovery of the bacteria from the bead, with a decrease in the bacteria released, related with an increase in the alginate used. However, when we analysed the effect of the variables on the viability, measured as growth rate at different times, the alginate concentration was shown to be a synergistic factor. In our experiments, the effect of calcium was not considered significant in the model. However, in the surface and contour plots a negative effect of the calcium on the recovery *R* was estimated for the lowest alginate concentration, probably associated to a partial collapse of the beads due to the low alginate concentration in presence of high Ca^{2+} concentrations, which reduced the water content and the bacteria entrapment. For the highest alginate concentration, the calcium exerted a slightly positive effect, under these conditions the high alginate in the matrix reduces the beads collapse, and subsequently, does not affect the water content and bacteria concentration in beads. Similarly, calcium showed a positive estimated effect on the viability *V5* (and to a lesser extent on *V8* and *V10*) for the highest alginate concentration. This fact may be related with higher permanency times of the intact microgels, caused by the high cross-linked of the beads by Ca^{2+} that reduced the disintegration rates of the microgels.

The combined results confirmed that the central formulation offered the most balanced result, with an acceptable preservation estimated as recovery > 7.0×10^4 cfu/mg, combined with a growth rate > 1.0 at day 5. From a practical point of view, it should be considered that the product is designed to be easily homogenized before supplying to the culture, not to be ingested by the animals, and then the size of particle was not considered as a relevant factor. This reason also supports the use of central formulation, avoiding those with a more densely cross-linked envelope (highest concentrations) which would difficult the bacterial release and need a more aggressive homogenization. By the other hand, the soft formulations (lowest alginate concentrations) would imply a high risk of leaks during the storage of the product. Therefore, the results of the model suggested that the central formulation (alginate 1%, Ca^{2+} 1.5%) was adequate and it was selected as definitive. A slight deviation of the composition from the central point (Figure 4) would improve the result, until guarantee 1.0×10^5 cfu/mg (and growth rate 1.0). However, from a practical point of view, the range of alginate

(0.92-1.0%) is too narrow to achieve only a small enhancement respect the central formulation. These results are not completely in accordance with previous works using the same components for preparing the beads. Pales Espinosa et al. (2007) and Chen (2003) encapsulated live microalgae using media with salinity ≥20‰, but the differences in the methods, formulations and aims are so pronounced that they impede the comparison. In assays with bacteria in marine aquaculture environments, Nimrat et al. (2011) encapsulated *Bacillus* spp., to be directly supply as water additives to the culture tanks. Again, there were substantial differences, considering that *Bacillus* has not salt requirements, the high alginate concentration (3%) used implies porosity and high risk of leaks in storage (Choi et al. 1999), and finally the use of additional substances (maize oil and Tween 80) in the formation of beads, undesirable in our case. All these discrepancies reveal the need of studies for each bacteria and use, considering the specific requirements and the application.

Encapsulation has demonstrated to be useful for the storage, preserving viability and function, of many products (Martin et al. 2015, Vemmer et al. 2013). The adequacy of the method for storage of live marine microorganisms has been also reported in the few studies on this field. Algal alginate-beads of the marine microalgae *Isochrysis galbana* kept in the dark at 4ºC for more than one year were capable of growth and initiated new cultures (Chen 2003). Regarding to bacteria, the viability of *Shewanella* Pdp 11 in calcium-alginate capsules was maintained after refrigeration (4ºC) for up to one month (Rosas-Ledesma et al. 2012). The viability of the strain PP-154 in beads under long-term storage should be the subject of specific studies, though we have observed that it remained viable in capsules kept at 4ºC for two months (data not shown), suitable for the storage in aquaculture installations without special requirements.In summary, the encapsulation of marine bacteria in alginate-calcium by extrusion, with appropriate modifications, is a viable method for the preservation and supply to marine aquaculture systems. The formulation of central points, alginate 1% and Ca^{2+} 1.5%, showed to be adequate according to the CCRD-RSM, considering not only the preservation and subsequent recovery of the bacteria, but also their viability once released in seawater, and the ease of use.

NOTES

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Compliance with ethical standards.

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1. Levels of real and codified values of independent variables utilized in the 2^2 CCRD.

Independent variables		Symbol		Level[a]				
				- α	- 1	0	+1	+ α
Factor		Coded	Uncoded	-1.414	-1.000	0.000	+1.000	+1.414
Alginate	% (w/v)	$x1$	$X1$	0.293	0.5	1.0	1.5	1.707
Calcium	% (w/v)	$x2$	$X2$	0.793	2.0	1.5	1.0	2.207

Table 2. Recovery of the strain PP-154 after freeze-drying using skim milk as protectant and loss in the process (difference between initial and final total numbers, expressed in logs). In the rehydrated product, the pigmented and nonpigmented spontaneous variant was differenced.

	Initial (cfu)	**Rehydrated**			**Loss** (log)
		Pigmented (cfu)	**Non-pigmented (cfu)**	Difference (log)	
1[a]	9.96 x 10^{12}	2.70 x 10^{9}	1.50 x 10^{8}	1.3	3.5
2	9.96 x 10^{12}	3.70 x 10^{9}	2.00 x 10^{8}	1.3	3.4
3[a]	9.96 x 10^{12}	2.10 x 10^{10}	1.50 x 10^{9}	1.1	2.6
4	5.20 x 10^{11}	6.40 x 10^{9}	2.00 x 10^{8}	1.5	1.9
5	8.84 x 10^{12}	2.20 x 10^{10}	1.36 x 10^{10}	0.2	2.4
6	8.84 x 10^{12}	1.62 x 10^{10}	4.00 x 10^{8}	1.6	2.7
7	6.20 x 10^{9}	2.80 x 10^{8}	-	-	1.3
8	1.04 x 10^{13}	3.50 x 10^{9}	1.60 x 10^{8}	1.3	3.5
9	1.52 x 10^{11}	5.60 x 10^{8}	4.00 x 10^{7}	1.1	2.4

[a] In experiments 1 and 3 skimmed milk were used at 5 and 20 % respectively.

Table 3. Central composite design matrix of two variables and experimental response values.

	INDEPENDENT VARIABLES				**RESPONSES**			
	Real values		Coded values		Observed			
Formulation	X_1 % **Alg**	X_2 % **Ca**	x_1 % **Alg**	x_2 % **Ca**	Y_1 cfu/mg ***R***	Y_2 GR ***V5***	Y_3 GR ***V8***	Y_4 GR ***V10***
F 1	0.5	1	-1	-1	1.150.000	0.4667	0.3666	0.3103
F 2	0.5	2	-1	1	640.000	0.4251	0.4085	0.2015
F 3	1.5	1	1	-1	60.000	0.9694	0.6444	0.4641
F 4	1.5	2	1	1	7.500	1.4135	0.9303	0.6749
F 5	1	1.5	0	0	258.000	0.7233	0.4880	0.4310
F 6	1	1.5	0	0	45.000	1.0401	0.7729	0.5922
F 7	1	1.5	0	0	33.100	1.1576	0.7345	0.5176
F 8	1	1.5	0	0	23.100	1.2059	0.8377	0.6868
F 9	1	1.5	0	0	38.000	1.0489	0.6048	0.4494
F 10	0.293	1.5	-1.414	0	1.080.000	0.4284	0.2831	0.1048
F 11	1.707	1.5	1.414	0	12.500	1.3448	0.8703	0.6367
F 12	1	0.793	0	-1.414	43.000	0.8173	0.5448	0.4147
F 13	1	2.207	0	1.414	20.000	1.0823	0.6719	0.3497

Table 4. Statistical ANOVA of the responses (Y_1-Y_4) for response surface model of marine bacteria encapsulated in alginate-calcium beads and equations describing the influence of independent variables.

	Y_1		Y_2		Y_3		Y_4	
Factor	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value
X_1	64.16	**0.0001***	40.88	**0.0004***	28.49	**0.0011***	30.81	**0.0009***
X_2	2.17	0.1838	3.18	0.1178	2.76	0.1405	0.00	0.9688
$X_1{}^2$	26.28	**0.0014***	2.88	0.1335	1.92	0.2085	4.84	0.0638
X_{12}	2.57	0.1529	2.48	0.1591	1.28	0.2958	3.31	0.1118
$X_2{}^2$	0.14	0.7238	1.34	0.2855	1.00	0.3512	4.11	0.0822
R^2	93.1555		87.7851		83.3827		85.7257	
Adj-R^2	88.2666		79.0602		71.5132		75.5297	
SEE[a]	142668		0.154123		0.107976		0.0878632	
MAE[b]	83168.3		0.078612		0.0539993		0.0496251	
Eq[c]	Y_1=79439.6 – 404022 X_1 + 277281 $X_1{}^2$		Y_2=1.03516 + 0.348386 X_1		Y_3=0.68758 + 0.203754 X_1		Y_4=0.5354 + 0.172428 X_1	

*Significant effect of factors on response. [a]SEE: standard error of estimate, [b]MAE: mean absolute error, [c]Eq: best-fit equation (positive sign of the coefficient: synergistic effects, negative sign: antagonistic effect.

FIGURE CAPTIONS

Figure 1. Bacterial counts of strain PP-154, marine heterotrophic bacteria other than PP-154 (MHB) and vibrios (V) in natural seawater (NSW, first frame), with addition of the protectant (NSW+protectant, second frame), and with thawed PP-154 (NSW+PP-154-protectant). Results are expressed in cfu/ml. Protectants: a) Marine Broth+glycerol 15% (MBg), b) Inositol 10% (INO) and c) Sucrose 15% (SUC).

Figure 2. Pareto charts in the central composite design for the standardized (α=0.05) effects of alginate [A] and calcium [B], and their interaction on (a) Recovery [R], and Viability (b) at day 5 [V5], (c) at day 8 [V8] and (d) at day 10 [V10]. The reference line (2.14) indicates that effects were significant with the α value of 0.05. Any term with a standardized effect (a measure of how far an observation lies from its mean, in units of standard deviation) $\geq$ 2.14 is considered significant at 95% confidence level.

Figure 3. Three-dimensional response surface plots for: a) recovery (R), and viability at b) day 5 (V5), c) day 8 (V8) and d) day 10 (V10), showing the interactive effects of alginate and calcium.

Figure 4. Overlapped contour plots for the effect of alginate and calcium on recovery (R, lines) and viability at day 5 (V5, regions). Area and white lines: best combined result (recovery $\geq 1.0 \times 10^5$ cfu/mg and growth rate $\geq$ 1.0). Blue lines: central formulation. Grey lines (recovery): values $< 1.0 \times 10^5$ cfu/mg.

Figure 5. Overlapped main effects plots showing effect of alginate and calcium on recovery (R, cfu/mg) and viability at day 5, 8 and 10 (V5-V8-V10, growth rate).