



Culture conditions improvement of *Crassostrea gigas* using a potential probiotic *Bacillus* sp strain



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ABSTRACT

It is well demonstrated that some probiotics improve rearing water quality and thereby have beneficial effects on reared organisms. We conducted this study to determine the effect of *Bacillus* consortium on *Crassostrea gigas* reared in contaminated seawater with indigo dye priorly treated with *Bacillus* or no treated. This effect was studied by assessing hemocytes death using flow cytometry analysis. We found that the percentage of decolorization of indigo dye in polluted seawater in presence of *C. gigas* increased from 41% to 90% when using *Bacillus* consortium. In these conditions, the hemocytes mortality of reared *C. gigas* decreased from 87% to 56%. We have demonstrated also that seawater contaminated with priorly treated indigo with *Bacillus* consortium is less toxic than seawater contaminated with the no treated indigo. The percentage of hemocytes death is 81% for the contaminated seawater with indigo and 56% for no contaminated seawater. This consortium shows a protector effect of *C. gigas* against *Vibrio harveyi* contaminating reared seawater.

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1. Introduction

Most of pollutants are thrown out on soil and get washed off during monsoon months and are thereby conducted to lagoons and coastal seawater. They lead to ecological and sanitary problems such as the decrease of aquatic biodiversity and the intoxication of aquatic organisms particularly filter feeders like oysters. The world annual production of dyes is estimated at more than 80 000 tons used mainly in food industries, cosmetics, paper mills and especially in textile industries which absorb alone more than 70% of the produced total quantity [1]. The intense use of the synthetic dyes in these various industries generates sources of considerable pollution of the environment. Indeed, 10–15% of the quantity of dyes used is rejected into the natural environment [2]. These dyes, in addition to dangerous pollution that they can generate, constitute because of their toxicity a potential danger to both humans and environment [3]. Since the very early stages of the history of

mankind, several natural dyestuffs have been used. In fact, the art of dyeing with indigo dates back several thousand years. The Indigo is classified as strongly toxic dyes [4]. The indigo can cause a major problem for aquaculture in general and particularly in *Crassostrea gigas*.

The Pacific oyster *Crassostrea gigas* is one of the most important cultured species, with a high value and strong market demand [5]. Oyster farming practices usually include numerous transfers of oysters at all life stages, especially of spat and juveniles grown in hatchery and nursery systems before being transferred to oyster farming areas in open seawater [6]. Along with these farming practices, oysters must adapt to new abiotic and biotic environments. Biotic changes include interactions with new, potentially pathogenic or toxic microorganisms that infect oysters through the feeding and filtration process. The most important factor affecting aquaculture is the incidence of microbial pathologies, mainly bacterial in origin. *Vibrio harveyi*, and *Vibrio anguillarum* are most frequently isolated marine *Vibrio* species [7] [8], having been associated with large-scale losses of larval and juvenile penaeids [9] and also causing several opportunistic diseases to fishes [10] [11]. *Vibrios* are gram-negative, ubiquitous in marine and estuarine ecosystems as well as aquaculture farms, and comprise one of the

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major microbiota of these ecosystems. Many *vibrios* are serious pathogens for animals reared in aquaculture [12] [13]. *Vibriosis*, caused by infection by *Vibrio* spp, is one of the most prevalent diseases in fishes and other aquaculture-reared organisms and is widely responsible for mortality in cultured aquaculture systems worldwide [14] [15].

Disease outbreaks in shellfish aquaculture are managed using methods such as disease avoidance, frequent water changes, good husbandry, and the use of immune-stimulants and antibiotics [16]. Antibiotics have been used widely in aquaculture systems as a method for disease control. However, because of the emergence of antibiotic resistance and concerns about environmental pollution, alternatives to the use of antibiotics are needed [17], [18]. One of these alternative methods is the use of nonpathogenic microorganisms called probiotics. *Bacillus* sp. is widely used as probiotic in aquaculture but it is not autochthonous in gastrointestinal tract. However, it may be active during intestinal transit [19]. In a recent study, two marine bacteria, *Bacillus pumilus* RI0695 and *Phaeobacter gallaeciensis* were previously reported to provide significant protection of the Eastern oyster larvae *Crassostrea virginica* when challenged with the shellfish pathogen *Vibrio tubiashii* [20]. Additionally, *Bacillus* sp. had been shown to have health benefits for *Dicentrarchus labrax* larvae [21]. Probiotics have been tested successfully in shellfish culture. Chniti et al. (2003) [22] isolated two yeasts and one bacterial strain (designated SS1, AY1, and SY9, resp.) from the digestive tract of abalone (*Haliotis midae*). A diet was formulated with a mixture of the three putative probiotics. Each probiont was added to the feed to achieve a final concentration of approximately 107 cells g⁻¹ of dry feed. The growth rate of small (20 mm) and large (67 mm) abalone was improved by 8% and 34%, respectively, in eight months cultures. Furthermore, abalones supplemented with probiotics had a survival rate of 62% to the pathogenic bacterium *Vibrio anguillarum* compared to 25% survival of untreated animals. Another study describes that the daily application of *Phaeobacter inhibens* and *B. pumilus* RI06-95 mixed with algal feed to culture tanks in the hatchery increased survival of oyster larvae to experimental challenge with *V. coralliilyticus* RE22 [23]. Probiotic microorganisms have the ability to release chemical substances with bactericidal or bacteriostatic effect on pathogenic bacteria that are in the intestine of the host, thus constituting a barrier against the proliferation of opportunistic pathogens. In general, the antibacterial effect is due to one or more of the following factors: production of antibiotics, bacteriocins, siderophores, enzymes (lysozymes, proteases) and/or hydrogen peroxide, as well as alteration of the intestinal pH due to the generation of organic acids [18]. According to these data, the genus *Bacillus* is one of dominant probiotics, which was commonly used in aquaculture. More and more studies confirmed that endogenous or exogenous *Bacillus* strains could be effective in improving growth, immunity and disease resistance of shellfish [24] [25] [20].

This study thus investigated the possible protection effect of *Bacillus* sp for *Crassostrea gigas* reared in indigo polluted seawater and infected with *Vibrio Harveyi*.

2. Materials and methods

2.1. Samples collection and bacterial characterization

Three *Bacillus* strains, isolated from Tunisian hypersaline environments in a previous study in our laboratory [26], are used in this study as *Bacillus* consortium. Sequencing analyses showed that these bacteria corresponded to the genus *Bacillus* sp such as *Bacillus subtilis*, *B. cereus*, *B. coagulans* [26]. These strains were preserved in our laboratory and their purity was routinely checked. Stock cultures were stored at 80 °C in powdered skimmed milk suspension

with 25% glycerol. *Bacillus* strains were isolated, in this study, on nutrient plates and inoculated in nutrient broth prepared with seawater. It was incubated for 24 h at 20 °C [27].

Vibrio harveyi ATCC 14 126 was routinely cultured in marine broth. It is used for control as positive pathogen.

2.2. Screening and identification of micro-organisms with bioremediation activity

Indigo was submitted to decimal dilution in a mineral liquid medium (MLM) prepared as described previously by Ref. [28] up to a concentration of 1.75 mg/ml.

Isolated bacteria from sludge and wastewater of industrial textile plant were inoculated in flasks containing MIM medium with 1.75 mg/ml indigo dye concentration. Then flasks were incubated at 37 °C under shaking conditions (120 rpm) in a rotary shaker. Samples were collected at different time to determine decolorization. Optical densities (OD) were determined using a spectrophotometer, Shimadzu UV-240I PC model Kyoto, Japan) at the max λ max 664 nm. The percentage of decolorization was calculated as following:

$$\text{decolorization (\%)} = \frac{[\text{absorbance at } t_0 - \text{absorbance at } t_1] * 100}{\text{absorbance at } t_0}$$

2.3. Oysters challenge

Crassostrea gigas were purchased from a rearing plant in a lagoon after an acclimatization period of one week at 25 °C in aquariums containing 10 L of seawater. Experiments were carried out in six aquariums, each containing six specimens.

Aquarium 1: containing *C. gigas* and seawater; Aquarium 2: containing *C. gigas*, seawater and indigo (1.74 mg/ml); Aquarium 3: containing *C. gigas*, seawater and filtrate from a culture of *Bacillus* consortium where indigo was degraded; Aquarium 4: Same content as the second aquarium but reared seawater is inoculated with *Bacillus* consortium (10 ml of *Bacillus* suspension 10⁶ CFU/ml); Aquarium 5: seawater inoculated with *Bacillus* consortium (10 ml of *Bacillus* suspension 10⁶ CFU/ml); Aquarium 6: the same content as the fifth aquarium but oysters are infected with *V. harveyi* ATCC 14 126; Aquarium 7: the same content as the sixth aquarium but oysters are infected with *V. harveyi* ATCC 14126 and added with *Bacillus* consortium (10 ml of *Bacillus* suspension 10⁶ CFU/ml).

2.4. Hemolymph collection

C. gigas was collected from the lagoon of Bizerte (Tunisia). It is polluted with industrial and domestic wastewater.

Hemolymph was collected from the adductor muscle following Chen method (1996). A notch was filed on the dorsal side of the shell valve, adjacent to the adductor muscle. 2 ml of hemolymph were collected aseptically from each animal.

A total of 10 000 hemocytes were analyzed using a flow cytometer BD FAC Caliber coupled with a Power Mac G3PC equipped with a Cell Quest Pro software.

2.5. Hemocytes viability assay

150 μ l of hemolymph was mixed with 150 μ l of an antiaggregant solution (AASH) and 5 ml of propidium iodide 1 mg/l (PI) (Sigma) at a final concentration of 20 μ g/ml. The Propidium iodide binds to DNA double stranded and absorbs at wavelength above 630 nm. It enters and stains the cells but can't cross the membrane of viable cells. Tubes were incubated with PI and AASH for 10 min at 20 °C

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