Journal of Applied Science and Advanced Technology
Journal Homepage : https://jurnal.umj.ac.id/index.php/JASAT

Characterization of Pigmented Bacillus Species Isolated from Aquatic Environment

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ARTICLE INFO

JASAT use only:
Received date : 25 August 2021
Revised date : 21 October 2021
Accepted date : 28 November 2021

Keywords:
Characterization
Bacillus
Isolated
Pigment

ABSTRACT

The genus of Bacillus includes several group of genetically and phenotypically different. Some species of spore-forming bacteria are pigmented, although the role of these pigments has not been fully elucidated. This study aimed to isolate a new species of Bacillus species and characterize both pigmented and pigment-producing bacteria product. For this purpose, samples were used water to isolate spore-forming bacteria and verify the presence of pigmentation. All bacteria found pegmatite were characterized at the species level and one of those selected for further studies. The physiological and biochemical analysis carried out revealed that: I) the product is carotenoid pigment, II) its synthesis takes place in stationary phase of growth. These features have allowed for us to assume that the carotenoid is produced under conditions of cellular stress and probably involved in cellular adaptation to some environmental conditions are not optimal for the cell.

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INTRODUCTION

Bacilli species are a spore forming, gram positive organisms, identified due to their characterization by the ability of differentiation with endospore, these bacteria found in soil which considered as a main environment of Bacillus species, bacilli spores identified at many different habitats also isolated from guts of many animals and various insects [1]. A massive distribution of environment spread due to spore ability for surviving for a long time of water drought and absence of nutrients, also withstand of harsh environments which may or can kill other types of cells [2].

The Ability of Spore forming bacteria to survive, depending on the peculiarity structure of their spores [3]. These spores were formed due to considered inactive chromosome in dehydrate cytoplasm, also by series of protective layers [1]. The most inner layer is a rich cortex peptideglycan which itself covered completely with an additional layers from material of protein, in some species of exosporium as a coat [4]. The compounds together which reported to protect spores from Ultra Violate (UV) radiation, extremes of pH or rising of temperature, also to exposure to Hydrogen peroxide and solvents, in addition to the exposure to lytic enzymes and toxic chemicals [5].

Spores of bacteria start germination when there is a presence of appropriate water or moisture in environment in addition with essential and appropriate nutrients, a fast process during which the protective cell growth is allowed [6]. Some of Bacilli types are reported as pigmented [7] producers, such as *Bacillus ceruse* can produce green pigment [8], and brown pigment from *Bacillus subtilis* [9].

Natural pigments are found and widesored in nature, identified in prokaryotes and eukaryotes organisms. Pigments function at day light and as well as they can work like photo productive factor in photosynthetic organisms [10]. The genus of Bacillus includes several groups of different genetically and phenotypically [6]. Although of the role of these pigments hasn’t been fully clarified has not been fully elucidated. This study aimed seeks for isolation of a new Bacillus species also to characterize both of pigmented and pigment-producing a product of bacteria, so samples were

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DOI: https://dx.doi.org/10.24853/JASAT.4.2.51-62

used seawater for isolation of spore-forming bacteria, as well as verify pigmentation presence. At the level of species, all bacteria found pegmatite were characterized with one of those which selected for further studies.

The physiological and biochemical analysis carried out revealed that: I) the product is a carotenoid pigment, II) its synthesis takes place in stationary phase of growth, III) the temperature at which it is produced is lower than optimal for growth. These features have allowed me to suppose that the carotenoids are produced under cellular stress conditions and is probably involved in cellular adaptation to some environmental conditions are not optimal for the cell.

EXPERIMENTAL METHOD

Culture media

One litter of Luria-Bertani (LB) medium was prepared for 1L of: (5 g Bacto-yeast extract, 10 g Bacto- Tryptone, 5g Sodium chloride NaCl at Ph 7). Induction medium of sporulation: Difco Sporulation medium (DSM): 1 liter of distilled water, 8g of Bacto-nutrient broth (Difco), 10 ml of 10% Potassium chloride KCl (w/v), 10 ml of Magnesium Sulphate Heptahydrate MgSO4 x $7H_2O$ 1.2 % (w/v), 0.5 ml of 1M of NaOH, the medium was autoclaved and cooled to 50 °C before adding the following sterile solutions: 1 ml of Calcium nitrate Ca $(NO_3)_2$ 1 M,1 ml of Manganese Chloride MnCl2 00:01 M and 1 ml of Ferrous sulfate Fe SO_4 1 mM as a modified method [10].

Screening of pigmented Bacillus

Samples of sea water (250 ml) taken at different distances from the coast (3m, 50m, 70m) were collected in Niskin bottles. The microbial population present in samples of sea water was concentrated using a filter of 0.22 micron (Millipore). Each filter was put on DSM agar plates were incubated at 25 °C and 30 °C for 2-3 days. All colonies were collected in liquid DSM, treated with heat (80 °C for 10 minutes), plated on DSM agar then incubated at 25 °C and 30 °C for 2-3 days. The resistant colonies were isolated by plating on DSM agar then were analyzed till presence of spores using light microscopy for recognizing [10].

The biochemical test

Depending on obtained results from Kit API CHB 50 (BioMerieux): of each bacterium, A single colony was inoculated into 5ml of LB medium then let to growing at 37 °C along 24 h later, a dilution in 5 ml of LB medium to 0.1 was prepared and read every hour at absorbance of 600 nm 1 ml for each culture. Where all the bacteria were reached in exponential phase then centrifuged at 7000 rpm for 10 minutes and the pellets were washed twice with PBS 1X. After washing all obtained pellets then they were resuspended in 10ml of medium and CHB 50 rate of each culture was added in 49 wells of the kit, results of diagnosis were carried out through following as described in instruction manual [11].

Physiological characterization

Growth at different temperatures and determining the optimum temperature for growth: a single colony of each culture was inoculated in a volume of 20 ml from LB medium and left to grow along 24 hours in following temperatures 4-15-25-30-37-42-50-55-60-65-70 -75-80 ° C. To determine an optimal temperature for the growth of bacteria, a single colony was selected and inoculated in 5ml of LB medium, then incubated at 37°C for 24 hours, as a continuous work, an incubated cultures were performed in a dilution up to 0.1 OD in 50 ml of LB medium. The cultures were incubated at 25-30-37-42-50 ° C, with determination of optical density for 1 ml of each culture at 600nm using a spectrophotometer [12].

Growth under anaerobic conditions

For the determination of anaerobic growth in the strain of interest was smeared on DSM agar containing 5mM of Potassium nitrate and 2.5 mM of Potassium as electron acceptors. At this step of work, plates arranged at the presence of a positive control bacteria (*Clostridium perfringens*) with a negative control (*Bacillus pumilus* SC2220) at same time, were they incubated in anaerobic conditions at chamber of Don Whitley at 30 °C, then after 3 days growth was observed [11].

Survival at different concentrations of NaCl

The single colony from the strain of interest was streaked on LB agar which was added at different NaCl concentrations (5%, 8%, 10% and 12%), for two days, cultured plates were incubated at 25 and 30 °C [10].

Determination of sporulation efficiency

As a control, the Sporulation of PY79 and SF214 where measured in parallel using medium of DSM for sporulation induction [13]. The colony of each strain were inoculated in a volume of 20 ml from LB medium, then incubated for 24 hour at 37 °C, was made the day after a dilution to 0.4 OD in 200 ml of DSM. Bacterial growth was followed until the entry into stationary phase (T0) with

spectrophotometric every hour; the reading was performed at 600 nm. The next step was performed after 24 hours, from 3.8 and T0 200 microliters were taken from both cultures: 100 microliters were treated via subjected to the heat treatment under 80 °C for 20 minutes, and 100 micro liter had not treated with heating. the serial dilution of DSM agar were prepared in order to culture them with treated and untreated samples from previous step. In order to determine efficiency of bacteria sporulation, the number of resistant cells to increase temperature for treating in relation to untreated cells. [14].

Analysis the production of biofilms in *B. pumilus* SF214

24-well plates were set up in wells which were added 2 ml of the MSgg minimal medium, and 200 µl of bacterial culture obtained from overnight growth in a volume of 200 ml from LB medium, then the plates were incubated along 5 days at 25 °C, at 37 °C for 1 day and 42 °C for 5 days. After days previously indicated the cells was removed gently with a 2ml syringe, was then added to each well 1 ml of PBS (1X) to remove the cells may be present. Was then added 1 ml of Crystal-violet 0.1% and left for 30 minutes, after these minutes, the crystal-violet was removed with a syringe, added 1 ml of deionized water was subsequently removed with syringe. The plates were then air drying for about 30 minutes, and then added 1 ml of ethanol and acetone in a ratio 80:20. At this point the spectrophotometer reading was performed at a wavelength of 570 nm using a Multy scan Spectrum (Thermo Electron Corporation) in order to quantify the biofilm. [11].

Chromosomal DNA Extraction of *B. pumilus* SF214

One fresh colony from culture of B. pumilus SF214 was inoculated in a volume of 7 ml from TY mixed with selected specific antibiotic, then incubated overnight at 37 °C, later cells were centrifuged at 7000 rpm for 5 min. and obtained pellet washed with 5 ml of Lysis Buffer consisting of: 50 mM EDTA with 0.1 M NaCl. Centrifuged again and resuspend cells with about 1 ml Lysis Buffer to which were added 0,250 ml of lysozyme, we then proceeded to an incubation at 37 °C for 10 min. After these minutes, were added 0,075 ml of 20% Sarkosyl, stirred by inversion and incubation continued for another 5 min. At this point 1 ml of phenol was added, stirred vigorously then centrifuged at 4 °C for 10,000 g for 1 min. Then samples were moved to precipitate DNA, by adding 0.1 volumes of naach and 2.5 from [Et-OH] 95%. Subsequently, precipitated DNA was washed with solution of [Et-OH] 70%, then centrifuge again, always in the same conditions. At this point, resuspend the pellets in 500µl of sterile deionized water. [15].

Polymerase Chain Reaction (PCR)

DNA fragments were amplified by Polymerase Chain Reaction (PCR) using chromosomal DNA or plasmid as a template. The oligonucleotides shown in table (2) (synthesized by GIBCO BRL Custom Primers) were used as a trigger. In the reaction mixture were: oligonucleotides (5 mM), dNTP deoxyribonucleotide triphosphate mix (0.2 mm), MgCl2 Magnessium dichloride (1.5 mM), PCR buffer (GIBCO) and Taq DNA polymerase (GIBCO, 5U), and template DNA (20ng).The program for the PCR run included a first cycle of denaturation (3 min at 94 °C), 60 cycles of denaturation (1 min at 94 °C), annealing for (1) minute at (50-56 °C), according to the percentage in C / G oligonucleotide), elongation for (1) minute at 72 °C), plus (10) minutes at (72 °C) for the termination of the chains. The reaction products were then visualized on agarose gel or sent to sequencing [16].

Purification of plasmid DNA on a large scale

For purification of plasmid DNA, a single bacterial colony was taken and inoculated in 50 ml of TY contained 50 mg/ml Ampicillin and was prepared to grow overnight at 37 °C. Cells were centrifuged at 6,000 rpm for 5 min. at 4 ° C then resuspended in 1 ml of a buffer prepared with adding: 25 mMTris / HCl, 10 mM EDTA pH 8.0, 50 mM glucose. 5 min later, incubated at room temperature, then 2 ml of lysis buffer (0.2 M NaOH, 1% SDS) were added, directly the mixture was incubated for 5 min. on ice. Then a volume of 1.5 ml of a solution of 3M sodium acetate, pH 5.2 added to specimens then left on ice for 5 minutes. All specimens were centrifuged at 7,000 rpm for 10 min at 4 °C, and then the supernatant was recovered, and extracted with saturated phenol with 0.1 M of Tris for precipitation via addition of 2.5 volumes of ethanol. The plasmid DNA was collected by centrifugation in 4°C at 7,000 rpm for 10 min, later washed with 5 ml from ethanol concentration of 70% in order to remove any of salts which could precipitated, then resuspended with 0.5 ml of TE (10 mMTris-HCl pH 8.0 and 0.1 mM EDTA with controlling pH to 8.0 modefied method according to [17].

Enzyme Digestion

The plasmid DNA, was obtained depending on mentioned above protocol, was digested with restriction enzymes to 20 µl as a final volume in the presence of buffer which recommended by the supplier. Then digested fragment was loaded on gel and migrated at 100 volts for 45 min. Besides the samples was made to migrate a molecular weight standard consisting of the bacteriophage λ DNA digested previously with the enzyme BstEII [18].

Agarose gel electrophoresis

Agarose gel: 1 g agarose (Molecular Biology Certified Agarose, BIO-RAD) in 100 ml 1X TAE supplemented with 1 mg / ml ethidium bromide. Samples, to which was added 1 / 10 volume of loading solution (0.5% bromophenol blue, 30% glycerol in water), were loaded on gel and migrated at 100 volts for 45 min. Besides the samples was made to migrate a molecular weight standard consisting of the bacteriophage λ DNA digested previously with the enzyme BstEII as a modified method [19].

Elution of DNA from agarose gel

The fragments of DNA were eluted from the agarose gel using the protocol for elution QIAquik Gel Extraction Kit (QIAGEN). DNA bands corresponding to the interest of the gel was cut into pieces minutes and 1.5ml tubes in these places. The agarose was dissolved by adding 3 volumes of buffer QG (QIAGEN) then incubated for 10 min. at 50 °C. The solution was loaded onto the resin capable of binding to DNA, while the aqueous solution was discarded after a centrifugation for 1 min at 13,000 rpm. Finally, the DNA was eluted by 30 µl from TE Modified method [20].

Ligase reaction

The DNA fragment (insert) was combined with the DNA vector in a molar ratio of 5:1 then incubated till the presence of 3 units of enzyme T4 DNA ligase (Gibco) and 5X reaction buffer (Gibco) to final volume 10-15 µl for 12-16 °C. [20].

Preparation of competent cells and transformation of *E. coli*

To make competent, entry of DNA, cells of *Escherichia coli*, a bacterial colony was inoculated in a volume of 5 ml from TY medium to grow at 37 ° C for 12 hours with shaking. The inoculum was diluted 1:100 in 30 ml of TY medium and grown to exponential phase (OD 590, 0.3-0.5). At 4000 rpm for 5 min. and temperature of 4°C cells were centrifuged, then re-suspended with 15 ml of precooled 50 mM from CaCl2 with incubation on ice for 30 min. then cells were resuspended with a volume of 2 ml of a solution containing 100% glycerol and 50 mM $CaCl_2$ after the step of centrifugation, and then aliquoted and stored at -80 °C. The transformation was done by adding to 200 µl of competent cells, the reaction mixture of ligase, and then all was incubated at 4 °C for 1 h, later at 42 °C for 1 min, in order to induce a heat shock necessary for passage DNA within the cell. Then were added 800 µl of TY and bacteria were incubated at 37 °C for 40 min and then plated on TY plates containing the appropriate antibiotic [21].

RESULTS AND DISCUSSION

Samples of sea water, taken at different distances from the coast, were analyzed in order to isolate spore-forming bacilli pigmented. Sea water was filtered and filters that can retain all microorganisms, incubated on solid culture media. All bacterial colonies capable of growing were collected, treated at 80 degrees centigrade for 10 minutes and then seeded onto solid ground to identify individual colonies. The heat treatment was intended to eliminate all the cells to heat labile and thus facilitating the isolation of heat-resistant cellular forms, including bacterial endospores. All bacteria obtained by this method were analyzed by light microscopy to detect and make sure of the spores presence. The obtained fourteen strains of spore-forming bacteria were all able to grow in aerobic conditions and have been used previously considered to belong to the genus Bacillus. Biochemical analysis (API tests) have allowed me to confirm all isolates were belonged to the genus Bacillus, while the analysis of the nucleotide sequence of the gene coding for 16S ribosomal RNA allowed for me to identify them to species level (**Table 1**). Chromosomal DNA extracted from each isolate used it as a template in amplification reactions (PCR) using synthetic oligonucleotides as primer Ribo- rev and Ribo- for (**Table 2**).

Journal of Applied Science and Advanced Technology 4 (1) pp 51-62 © 2021

Table 1. Species and pigments of the isolates

Isolate No.	Species[1]	Produced colour
1	*Bacillus pumilus*	Yellow-orange
2	*Bacillus firmus*	pale orange
3	*Bacillus acquimaris*	Orange
4	*Bacillus firmus*	pale orange
5	*Bacillus pumilus*	Orange-red
6	*Bacillus firmus*	pale orange
7	*Bacillus acquimaris*	Yellow
8	*Bacillus pumilus*	Yellow
9	*Bacillus pumilus*	Yellow
10	*Bacillus pumilus*	Yellow-orange
11	*Bacillus pumilus*	Yellow-orange
12	*Bacillus pumilus*	Yellow-orange
13	*Bacillus firmus*	Red
14	*Bacillus acquimaris*	Orange

Table 2. Used primer to amplify the gene encoding the 16S rRNA

Primer	Sequence	Amplified fragment
P1 (sense)	[5'-GCGGCGTGCCTAATACATG]	1482 bp
P2(antisense)	[5'CACCTTCCGATACGGCTACC]	
P3 (sense)	[5'-ACGCCGCGTCATGAAG-3']	689 bp
P4 (antisense)	[5'-CATCTCACGACACGAGC-3']	

Among the pigmented bacteria isolated decided to focus my attention on the SF214 strain of the species *Bacillus pumilus*, which produces an orange-red pigment. As can be seen in **Figure 1**, the pigment production takes place both on the ground, LB (favoring vegetative growth) and on DSM medium (which induces the formation of spores), although it appears to be greater in the first case. The purpose is to define an optimal temperature to the growth of SF214 determined before the temperature range within which the bacteria grew. As we watched the growth of SF214 between 15 and 50 °C (data not shown), decided to determine the growth curves of SF214 25, 30, 37, 42 and 50 °C as can be seen in **Figure 2**, the results showed an optimum growth temperature of SF214 in LB liquid medium was 37 °C.

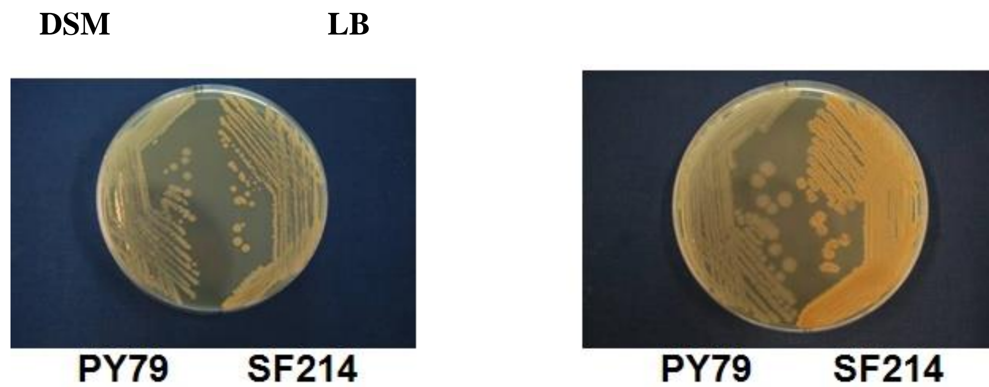

Figure 1. *B. pumilus* SF214 on DSM agar (left) and LB agar (right), PY79 (*B. subtilis*) is a non –pigmented *Bacillus* which used as a reference

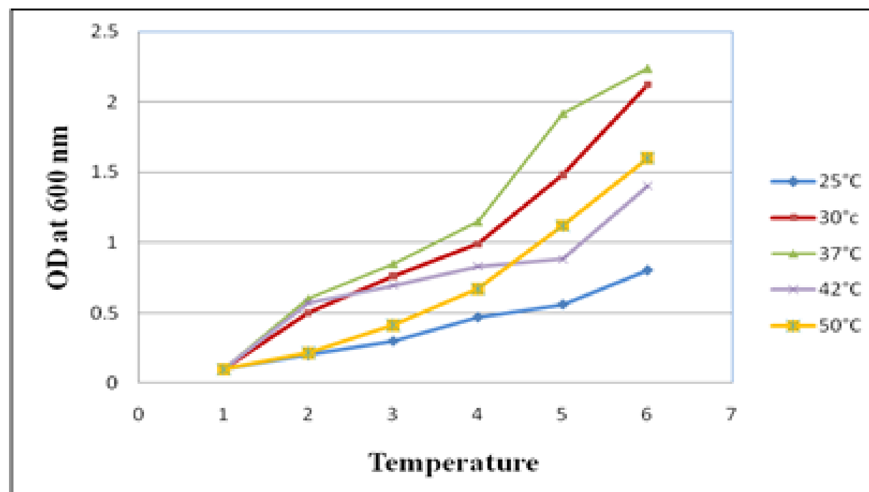

Figure 2. Curve of the growth of SF214 at a various temperature in LB medium

Some aerobic bacilli such as *Bacillus subtilis*, are able to grow in aerobic conditions by the respiration in the presence of nitrate as a terminal electron acceptor [22]. We decide to see if SF214 has ability to grow under another condition of anaerobic one. For this purpose solid medium incubated in SF214 DSM supplemented with potassium m nitrate (5 mM) and potassium nitrite (2.5 mM) as electron acceptors. The plates were incubated in an anaerobic chamber for 3 days at 30 °C during the presence of the negative control (the aerobic bacterium *B. pumilus* SC2200) and the positive control (anaerobic bacterium *Clostridium perfringens*). The lack of growth of SF214 allowed me to conclude that this strain is an aerobic required. The marine bacteria are osmotically sensitive to changes in salinity [23]. Many of them, known as halophiles, grow only in the high concentrations of Sodium Na^+. I decided to test the potential application of NaCl SF214 determining growth on LB solid medium supplemented with increasing amounts of NaCl. Strain used as reference was *B. subtilis* PY79 known to be capable of growing concentrations of NaCl up to 12%. As reported in **Table 3**, SF214 was able to grow up to 10% NaCl concentrations, although at this concentration growth is reduced.

Table 3. Growth in the presence of NaCl (%)

Isolate	5	7,5	10	12
B. subtilis PY79	+	+	+	±
B. pumilus SF214	+	+	±	-

Many pathogenic bacteria produce exotoxins such as Hemolysin. The Hemolysin has the ability to lyse the cells and can act in two ways: either by involving a protein capable of forming pores or through a phospholipase [24]. Hemolytic reactions can be easily spotted crawling the organism of interest on a blood agar, and they are classified generally as Alpha, Beta or gamma, depending on how taken from the area surrounding the isolated colonies grown on the ground with:- Alpha hemolysis if the colony was surrounded with a zone of intact red blood cells but the discolored and greenish, is usually caused by the action of peroxides which produced by bacteria; For Beta hemolysis, if the colony is surrounded with clear halo due to the complete rupture of red blood cells due to vicinity of the bacterial colony;- while Gamma hemolysis when the blood agar plate appears brownish in the absence of hemolysis around the colony. This is a normal reaction of blood due to the growth conditions which used 37 °C in the presence of carbon dioxide. In order to know whether SF214 produced hemolysin, this bacterium was smeared on blood agar, prepared as described above in "Methods." The plates were incubated at 37 °C for one day. After a day was not any visible halo typical of alpha or beta hemolysis SF214 is possible to conclude that gamma-hemolytic. Furthermore to characterize *B. pumilus* SF214 to determine efficiency of sporulation, its expressed as a percentage of resistant cells to the high temperature (80 °C for 20 minutes) which present in the DSM culture after 3, 8, and 24 hours after entry into the stationary face (T0) which compared to the total cells. Obtained results showed that after 24 h. from T0 to sporulation of *B. pumilus*SF214 efficiency was 10.1%. the low efficiency of sporulation observed for *B. pumilus* SF214 compared was compared to the reference which was *B. subtilis* PY79 at least partly due to the fact that the experimental conditions that used (Culture medium, pH. and temperature) were ideal for *B. subtilis* as shown in **Table 4**.

Table 4. Efficiency of sporulation

Strain	One hour after T_0	Number of live cells (ml-1)	Number of spores (ml-1)	Efficiency of sporulation (%)
SF214	3	$3.5x10^7$	0	0
	8	$8x10^7$	$0.12x10^7$	1,5
	24	$128x10^7$	$13x10^7$	10,1
PY79	3	$13x10^7$	0	0
	8	$35x10^7$	$2.3x10^7$	6,57
	24	$40x10^7$	$18x10^7$	76,6

The similar rules for the formation of Carotenoid and biofilm were assumed that processes could be coordinated. To test this hypothesis carried out a test of the production of biofilm at 25, 37 and 42 °C in soil msgs, specifically in considering the production of biofilm (Materials and Methods). This experiment was conducted in the presence of the positive control (a strain of *B. amyloliquefaciens* biofilm producer) while the negative control was (a strain of *B. firmus* that no biofilm). The quantification of the biofilm was performed after 5 days of incubation at temperatures as indicated by spectrophotometer reading at 570 nm using a Multiskan Spectrum (Thermo Electron Corporation). B. *pumilus* SF214 can produce biofilm at all experimented temperatures, while producing the Carotenoid at 25 °C and less than 37 °C while its produced at 42°C as shown in **Figure 3**. This result allowed to us to conclude that in spite of these two processes, they are regulated in a similar manner, but they were independent of one to another.

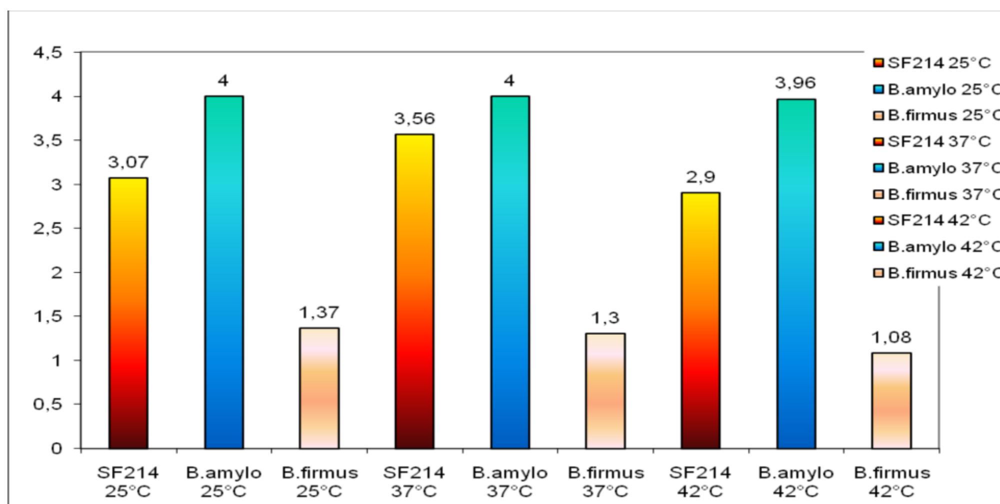

Figure 3. Production of biofilm in *B. pumilus* SF214 temperatures: 25 °C, 37 °C, 42°C. *B. amyloliquefaciens* which was used as positive; *B. firmus* as a negative control

Our study of the regulation of Carotenoid synthesis would be greatly facilitated if the bacteria were transformed. This would allow the construction of mutants, an essential step to understand the function of a gene. For this reason I decided to try to transform SF214. In the absence of plasmids capable of replication in *B. pumilus*, decided to try to transform SF214 with non-replicative carriers that could be integrated on the chromosome of SF214. This integration can only happen if there are regions of DNA between the plasmid and the homologous chromosome. It was therefore obtaining a preliminary non-replicated plasmid that contained a piece of SF214 chromosome. To this end, I decided to use a plasmid replication in *Escherichia coli, pER19*, unable to replicate in SF214, to enter a piece of chromosome of SF214.Chromosomal DNA was extracted as described above in Materials and Methods and the gene encoding of 16SrRNA amplified by PCR using oligonucleotides P1 and P2 (**Table 2**) as a trigger. The obtained DNA fragment of 1500 bp was cloned into the vector pGEM-Teasy (Promega). This carrier is specific for cloning PCR amplification products as it is linearized and has at both ends of the polylinker of the individual T that allow direct cloning of the fragments obtained from PCR. The mixture of ligase was used to transform the cells of *E. coli* (DH5α) made competent as described in Materials and Methods. The cells of transformed *E. coli* were cultured on LB agar mixed with Ampicillin, IPTG (Isopropyl β-D-1-tiogalattopiranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D galactopyranoside) as the vector contains the gene that confers resistance to ampicillin and the gene with the lacZpolilinker between promoter and coding part. Transformants were selected on the basis of blue-white screening and positive clones (white colonies because the insert has interrupted the lacZ gene) were inoculated in LB ampicillin, and most were grown at 37 °C. The volume of 5 ml from aliquot of this culture was used to extract plasmid DNA (Materials and

Methods). To check and determine the orientation of the insert in the vector, carried out an enzymatic digestion with the enzyme PstI, which recognizes a specific cleavage site is present within the insert in position within the polylinker. The plasmid obtained was digested with Saci and SmaI enzymes to extract the insert encoding the 16S rRNA of SF214. The resulting DNA fragment of 1500 bp then was cloned into a vector pER19 was digested with the same enzymes. The plasmid PER19 is also unable to replicate in SF214 but contains the gene conferring resistance to the antibiotic chloramphenicol, selectable in SF214. The ligase mixture was used one time more to transform cells of *E. coli* (DH5α) made competent also its described in Materials and Methods. Colonies resistant to the antibiotics ampicillin and chloramphenicol were grown in liquid medium and used to extract plasmid DNA. This was then digested with various restriction enzymes to verify the presence of the insert. Of the several positive clones obtained, a pBL1 was chosen for further analysis. The plasmid pBL1 obtained can be used to test the transformability of SF214, either through the preparation of protoplasts for electroporation (**Figure 4**).

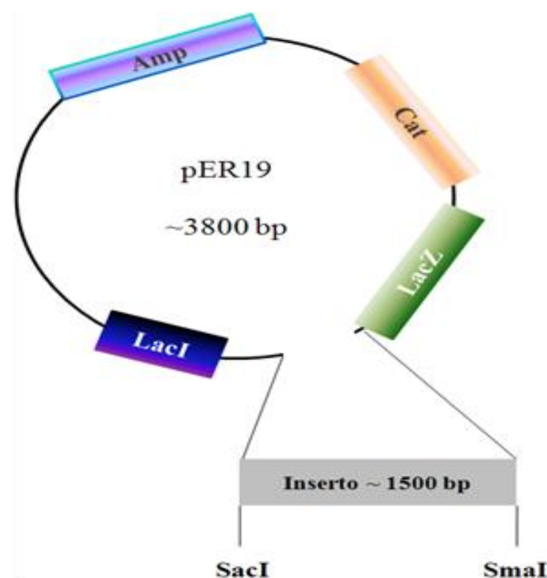

Figure 4. Diagram of the plasmid pBL1 obtained

CONCLUSION

Till now the pigmented spore-forming bacteria are still unknown well and poorly known. At this project, I have re-proposed for identification of species of pigmented *Bacillus* and characterize the pigments. Analysis of samples of sea water I got different strains pigmented and I decided to focus my attention on the island SF214, belonging to the *B. pumilus* species. Physiological haracterization of *B. pumilus* has allowed for us to understand the production or creation of the pigment is probably belonging to stress response because it is produced in poor conditions for the temperature to the availability of nutrients. The study of the regulation of Carotenoid synthesis would be greatly facilitated if the bacteria were transformed. This would allow the construction of mutants, an essential step to understand the function of a gene. For this reason I decided try to transform SF214. In the absence of plasmid capable of replication in *B. pumilus*, I decided try to transform SF214 with non-replicative carriers that could be integrated on the chromosome of SF214. This integration can only happen if there are regions of DNA between the plasmid and the homologous chromosome. It was therefore obtaining a preliminary non-replicated plasmid that contained a piece of SF214 to SF214 chromosome. To this end, I decided to use a plasmid replication in *Escherichia coli*, pER19, unable to replicate in SF214, which I inserted a piece of chromosome of SF214. Then I built a plasmid that had all the requirements for the transformation of SF214, which can be used to verify transformability, both through the preparation of protoplasts for electroporation.

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