B.U.L.M. technique for increase of the bearing capacity in the pavement layers subjected to biological treatment

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ABSTRACT

In this paper the Authors present the results of an experiment which is part of a wider study, started some years ago in the framework of a project financed by the Italian Ministry of University and Scientific and Technological Research, together with the Environmental Microbiology Centre "Locorotondo" in Palermo.

The aim of the project is the development and application of an interesting biological technique, named B.U.L.M. (Bacteria Method for Unbound Layers), to verify the advantages of using metabolic wastes of a particular estremophilous bacterium, the Bacillus Pasteurii ATCC 6453, when wastes are active in the unbound layers of road construction realized with aggregates.

The B.U.L.M. technique can be related to the definition of an empirical model of a particular dynamic system based on the conjunction of two fundamental components: active base (bacteria) and passive base (stone granulates). The advantages of the B.U.L.M. technique consist of an increase of internal cohesive strength in the unbound road pavement layers, leading to an increase of the bearing capacity in the pavement layers subjected to biological treatment.

For this reason, the advantages can be related to the deterioration of functional characteristics of the pavement. For the analysis of results obtained with the B.U.L.M. technique, the AASHO modified test and CBR test were carried out.

Keywords: plugging, bacillus, biotechnology, CBR index, road pavements.

1. INTRODUCTION

Some years have now passed since the Authors of this document embarked on their first steps along a reasoned, multidisciplinary path of research which lies very much on the border between the world of biotechnological applications and that of road construction engineering.

In order to reach today's results, various problems of different types had to be overcome: first and foremost, that of organisation, when supplies were needed from the United States of America of the primary material for research – the bacterial stock Bacillus Pasteurii ATCC 6453 – whilst having to deal with an international scenario which, in the very first days following the events of 11 September 2001, was still imbued with understandable diffidence towards the supply of bacterial material to anyone at all; secondly, several problems of a scientific nature needed to be resolved, since it was necessary to perfect the technique for "awakening" the bacterial stock, supplied in a lyophilised form, taking into consideration the right combination of optimal characteristics of temperature, pH of the growth culture, and composition of the nutrient broth.

Today, thanks to the scientific contribution of the microbiologists on the staff of the 'Locorotondo Laboratory' – the Environmental Microbiology Centre of Palermo, widely accredited in the scientific area of the sector - the obstacles of some time ago have been greatly reduced, and it is increasingly easy to rapidly obtain the replication of the bacterial culture necessary for the plugging of inert materials undergoing biological compaction treatment.

The aim of the experiment presented here is to investigate the bearing capacity rate (CBR) in unbound road pavement layers, particularly the foundational layer, following the application of a treatment that uses the capacity of bacillus pasteurii to produce calcium carbonate (CaCO3) and compact the stone granulate into which the inoculation takes place.

To this end, it was interesting to carry out a significant comparison between the mechanical characteristics seen in the non-treated material, and those obtained working with the bacterial additive, using the traditional investigative process that uses the modified AASHO compaction of a granular mix, and the carrying out of the CBR test.

Finally, it may be observed that the method used, unlike other stabilizing systems, does not produce risks of pollution of the soil or the water table.

2. EXPERIMENTAL LAY OUT

Our previous experiments in biocementation, using Bacillus Pasteurii ATCC 6453, were carried out on stone materials of calcareous, basaltic and siliceous origin. They showed that the three lithic qualities respond well to biotechnological treatment, since they allow highly satisfactory levels of bacterial cementation.

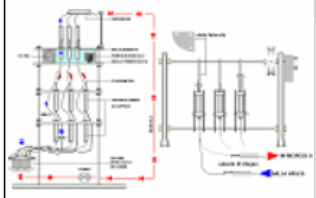
Bacterium growth and the level of microbial calcification in stone materials depend on some fundamental variables, the incidence of each of which was determined during previous experiments [3, 4, 5]. These variables are the following:

1. the granulometry of stone aggregates;

2. mineral qualities;
3. the typology of surface micro structure;
4. the initial and final pH of the host substrate;
5. the temperature of culture and inoculation medium;
6. the degree of bacterium concentration;
7. the chemical characteristics of the culture medium;
8. the duration of the inoculation.

The experiments carried out to date allow us to know the characteristics and the technical correctives of the biological treatment of road materials, in order to optimise their mechanical performances. This experiment is briefly described in table 1 and 2.

PLUGGING IN SYRINGES



Equipments realized at University Road department

AIMS

- *analysis of Ph-time*
- *analysis plugging-time curve*
- *Analysis of plugging level in sand materials*

MATERIALS

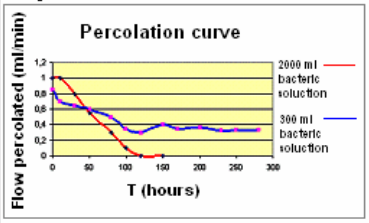
Calcareous, basaltic and siliceous sand passing in a sieve n.40 ASTM

METHOD

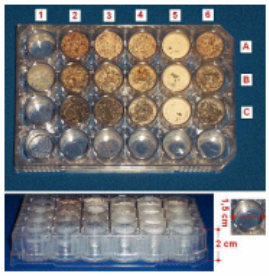
90 samples distinguished in n. 30 for material, which were biological treated with bacillus pasteurii and percolated with nutrient broth during 6 days.

EXPERIMENTAL RESULTS

The experimentation of first degree showed that the biotechnological method can be used to obtain a microbial cementation of calcareous, basaltic and siliceous sand. In particular we observed that to obtain the highest cementation, a week is necessary. During this time the bacterium must be treated with the percolation of an Urea nutrient broth. In the end of the development the formation of calcite CaCO3 was confirmed.



PLUGGING IN MULTIWELL



AIMS

-*Relation between microbial activity, Ph and stone aggregates*
- *analysis of the influence of the filler on the plugging*
- *analysis of the weight ratio between culture broth and nutrient broth*

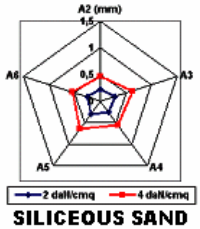
MATERIALS

calcareous, basaltic and siliceous sand

METHOD

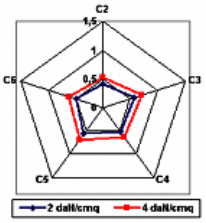
15 samples in multiwell, made and treated as shown in the near table. The essays were subjected to a load of 2 and 4 daN/cm² in order to evaluate the mechanical performances oj each material, according to the different conditions oj composition and biotechnological treatment. The results are shown in the near diagrams.

RESULTS



SILICEOUS SAND

CALCAREOUS SAND



BASALTIC SAND

Granulometric caracteristic of samples in multiwell

Ai (i=1..6): samples of basaltic sand
Ai (i=1..6): samples of siliceous sand
Ai (i=1..6): samples of calcareous sand

	Sand		Filler
$A_i \equiv B_i \equiv C_i$	Passing in a sieve 0,40 UNI	No Passing in a sieve 0,18 UNI	Passing in a sieve 0,075 UNI
	100%	100%	100%

BIOTHEC TREATMENT

	Sand	2 CICLI CULTURE BROTH+ NUTRIENT BROTH	Filler
$A_1 \equiv B_1 \equiv C_1$	4g+H_2O		
$A_2 \equiv B_2 \equiv C_2$	4g	1,8ml +1,8ml	
$A_3 \equiv B_3 \equiv C_3$	3,5g	1,0ml +1,0ml	0,5g
$A_4 \equiv B_4 \equiv C_4$	3,5g	1,25ml+1,25ml	0,5g
$A_5 \equiv B_5 \equiv C_5$	2,0g	1,0ml+1,0ml	2,0g
$A_6 \equiv B_6 \equiv C_6$	2,0g	1,25ml+1,25ml	2,0g

Table 1

PLUGGING IN PETRI DISHES

AIMS

First evaluations on the effects of the plugging in sand stone materials.

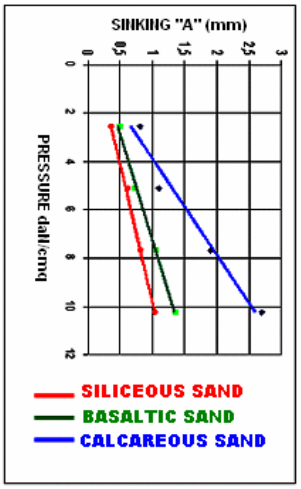
MATERIALS

Calcareous, basaltic and siliceous sand passing in a sieve n.40 ASTM

METHOD

The sands contained in the dishes were inoculated with the culture broth at the highest bacterial concentration equal to 9x108/ml. After the inoculation the ripening of the essays began at a 25 °C temperature, till the complete drying after two weeks.
The mechanical characteristics, reached by the sand materials after the bacterial treatment, were seen using the equipment requested by the B.U. CNR n.136 12.27.91, aimed to the punching of material surface.

EXPERIMENTAL RESULTS



PLUGGING IN MARSHALL

AIMS

Extension of the experimentation on basalt with Marshall punches and samples of material having a weight of 600gr belonging to two different granulometric curves

MATERIALS

Two granulometric compositions, which define the basalt groups G1 an G2, were utilized:

Granulometric caracteristic of samples G1				
I fraction		II fraction		III additive fraction
Passing 4 ASTM	Unpassing 8 ASTM	Passing 8 ASTM	Unpassing 12 ASTM	Passing 200 ASTM
300 g	300 g	200 g	200 g	100 g

Granulometric caracteristic of samples G2				
I fraction		II fraction		III additive fraction
Passing 12 ASTM	Unpassing 40 ASTM	Passing 40 ASTM	Unpassing 60 ASTM	Passing 200 ASTM
300 g	300 g	200 g	200 g	100 g

METHOD

The material, put in the punches without any compaction, was inoculated at the highest bacterial concentration equal to 9x108/ml . The ripening of the essays, at room temperature (about 25°C), lasted during four weeks. For the test loads, similar to those made on the essays in Petri dishes, we chose to sample making 4 measurements for each of the 4 load values examined: 2.56/5.11/7.67/10.23 daN/cm².

EXPERIMENTAL RESULTS

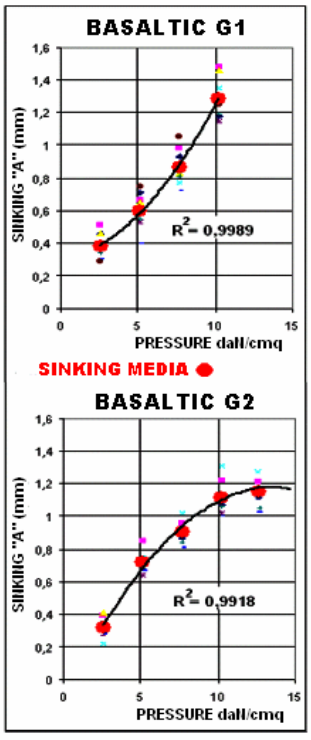


Table 2

The results of previous experiments indicate that siliceous material is the most congenial to bacterial plugging, although the bearing capacity of the biotreated basalt is equally satisfactory. This is because the microasperities that are typical of its texture provide an excellent grip for bacterial metabolites (CaCO3) which have the role of "cementing" the individual grains to each other (figure 1).

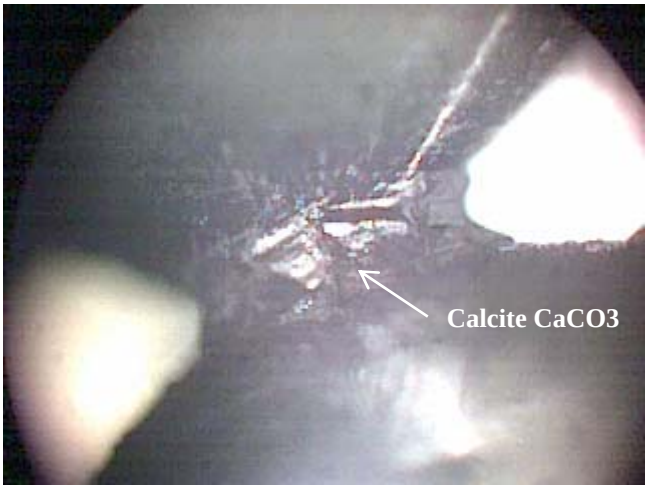


Figure 1 – Cemented basalt crystals (x1000)

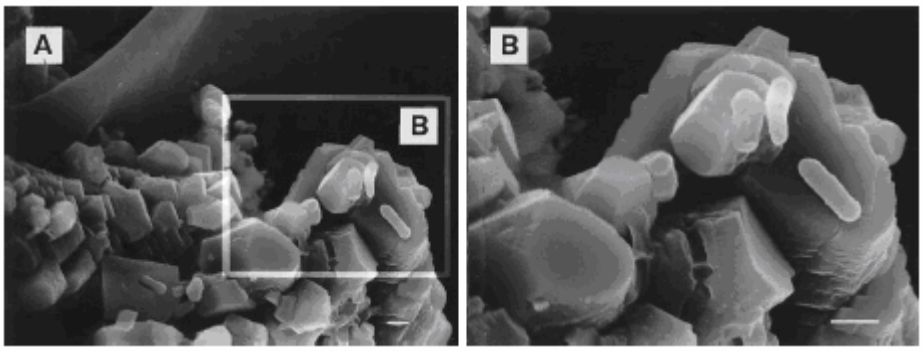


Figure 2 – The Bacillus Pasteurii during the CaCO3 precipitation phase

Thus, considering the fact that silica is a material chosen specifically for experimental comparison with the limestone and basalt and, obviously, is excluded *ab origine* from use for building purposes, throughout the rest of the paper, reference will be made exclusively to the bearing capacity characteristics acquired by the basaltic material treated using the bacterial cementation technique.

3. METHODS AND MATERIALS

3.1 Culture method

To develop the Bacillus Pasteurii ATCC 6453, we used a specific broth, the NH4-YE; for the replication we used a nutrient broth NB, which stimulates the cellular metabolism. The compositions of both media, as suggested in the protocol provided for the bacteria bank, are shown in table 3.

Table 3 Compositions of culture and nutrient broth

NH4-YE medium	20gr Yeast extract; 10 gr (NH4)2SO4; 0.13 M Tris buffer (pH 9); 2% Agar (culture gel) for the plates (Petri)
NB medium	3gr nutrient broth; 20gr Urea; 10gr NH4C1; 2.12gr (equal to 25.2mM) NaHCO3

In order to induce the germination of the bacterium, the bacterial spores were suspended again in 1 ml of NH4-YE broth; then 0.5 ml was diluted in about 5ml of a new broth, 1ml of which was diluted in a ratio of 1:5, while the remaining 0.5 ml were plated on Petri dishes with a NH4-YE broth enriched with 2% of Bacto-Agar.

Finally, the bacterium was maintained in culture by using the same nutrient broth, and the remaining part was frozen at -80°C.

At this point a part of the bacteria was plated on Petri dishes with NH4-YE broth + 2% of Bacto-Agar for determining the cellular concentration.

3.2 Bacterial growth curve

The growth curve of the bacillus pasteurii, as with any other cellular species, may be subdivided into phases that are distinguished as the start phase, the exponential phase, the plateau phase and the death phase.

Start phase

When a bacterial population is inoculated into a fresh medium, growth does not usually begin immediately, but only after a period of time known as the latency, or 'lag' phase; this period may be brief or extended, depending on conditions. If a culture in its exponential phase is inoculated into the same medium whilst keeping all the culture conditions unaltered, there is no latency phase, and the exponential growth continues at the same rate.

Conversely, if the inoculation is taken from a culture in its stationary phase and inoculated into the same medium, a latency phase may usually be observed, even if all the cells in the inoculation are live. This is due to the fact that usually, old cells do not contain a suitable quantity of coenzymes or of other cell constituents essential for growth, and therefore require a certain time to synthesize them again. Similarly, such a phase occurs when the inoculation is made up of cells that have been damaged (but not killed) by treatments with heat, radiation or toxic chemical compounds; in this case, the cells need to the time necessary to repair the damage undergone.

In the case in hand, an initial lag phase was observed in the bacillus pasteurii in relation to its transfer from the original culture medium NH4- YE (nutrient rich) to a poorer one made up mostly of a stony matrix subjected to biological plugging.

This is due to the fact that, in order to grow in a certain culture medium, the cells need to possess all the enzymes necessary for the synthesis of essential metabolites that are not present in the medium itself; consequently, the cells transferred to the poorer medium than that on which they grew, require a certain period of time to "adapt" with the synthesis of new enzymes.

For this reason, in the phase of inoculation of bacillus pasteurii in stone material, a "load" of nutrient broth NB is then added in order to balance the physiological metabolic slowdown.

Exponential phase

The exponential growth phase has already been discussed. As was said, the exponential phase is a consequence of the fact that each cell divides to produce daughter cells, which in their turn duplicate to produce four cells, and so on. Most unicellular micro-organisms grow exponentially, but the rate of exponential growth may vary greatly. For example, the pathogenic agent of typhoid fever, Salmonella typhi, grows very rapidly in culture, with a generation time of 20÷30 minutes, while the tuberculosis agent, Mycobacterium tuberculosis, grows slowly, duplicating just once or twice a day. The speed of exponential growth is influenced both by environmental conditions (temperature, composition of culture terrain), and by the genetic characteristics of the micro-organism itself. In general, prokaryotic micro-organisms grow more rapidly than eukaryotes, and small eukaryotes grow more rapidly than those of a larger dimension.

Plateau phase

In a closed system, exponential growth can not continue indefinitely. A single bacterial cell able to replicate within 20 minutes would produce, if it continued to grow exponentially for 48 hours, a population weighing about 4000 times the weight of the earth, a particularly impressive fact if we consider that the weight of a single bacterial cell is about 10^{-12} g. Obviously, mechanisms have to exist that limit the population growth well before arriving at this point. Generally, the end of the exponential phase is determined either by the depletion of an essential nutrient in the culture medium, or by the accumulation of waste products excreted by the organism, reaching inhibitory levels. When one of the aforementioned conditions occurs, the bacterial population reaches the phase known as stationary (plateau).

During the plateau phase, there is neither an increase nor a clear decrease in the number of cells. However, although there is not clear growth, many cellular functions, such as the metabolism of energy and some biosynthetic processes continue to occur. Some metabolites, known as secondary metabolites - amongst which, calcium carbonate $CaCO_3$ in the case of bacillus pasteurii - are produced particularly during the plateau phase, especially during the transition from the late exponential phase to the stationary phase.

In some micro-organisms, there may be a certain rate of growth during the plateau phase; in this case, some cells of the population grow, while others die, and the two processes are balanced so that there is neither an increase nor a decrease in the overall number of cells (this phenomenon is known as cryptic growth).

In spore-bearing bacteria, which amongst others include bacillus pasteurii, the endospore (the product of metabolism) is produced only when the culture enters the plateau phase.

Death phase

If incubation continues after the population has reached the stationary phase, the cells may survive and continue their metabolism or die. When the cells start to die, it is said that they have entered the death phase. During the death phase, the total count (measured as a direct count under the microscope) may remain constant, but the live count goes down slowly. In some cases, death is accompanied by cellular lysis, determining a decrease in number of cells counted directly under the microscope, and in their turbidity, together with the decrease in live numbers.

It is important to underline that the different phases on the growth curve of a bacterial population are the result of events that concern the whole population and not a single cell. The terms start phase, exponential phase, plateau phase or death phase are not applicable to single cells, but only to a population of cells.

We analysed the growth curve of the bacillus pasteurii with a spectrophotometric technique, which was used almost exclusively for quantitative analysis.

Once the germination of the bacillus pasteurii was obtained, we prepared two 250 ml Erlenmeyer flasks, one with 100 ml of NH4-YE broth and the other one with 100 ml of NB broth. Both the flasks were inoculated with 3 ml of growing bacteria with a concentration of about $1x10^7$/ml, corresponding to spectrophotometric reading (to 580nm) of 0.28 O.D. We took 1ml of broth with growing bacteria and made the reading by means of the spectrophotometer every two hours. Afterwards, we used the same sample to determine the pH. After about 10 hours from the inoculation, an exponential bacterial growth (0.3 O.D.) was found; after about 21 hours the top of the exponential growth period and the beginning of the stationary one (1.5 ÷1.7 O.D.) were recorded (figure 3)

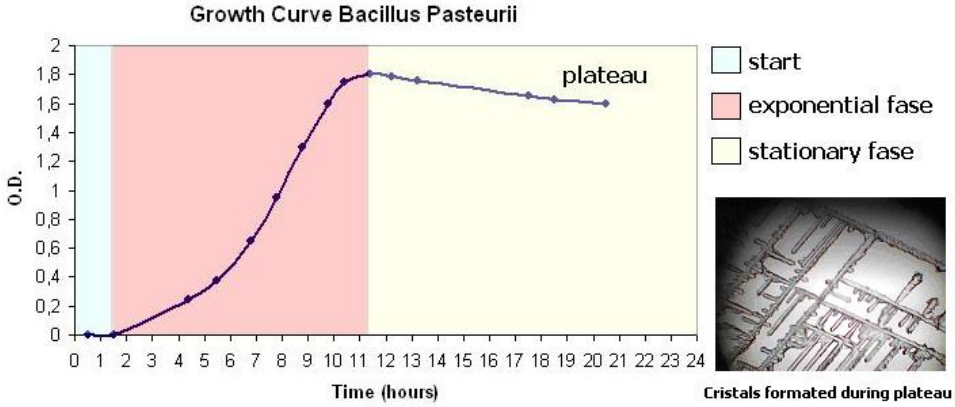


Figure 3 - Growth curve of the bacillus pasteurii

3.3 Calculation of cellular generation time

The phases of the life cycle of Bacillus pasteurii, as with all bacteria, are easily divided as shown in figure 3, so that the start phase, which coincides with the awakening of the bacterium when it is in a spore-bearing form, is followed by a second exponential growth phase and a third phase of slowing down or "sleepiness" known as the plateau phase.

Previously, it had been possible to explore the close relationship that exists between the plateau phase of the bacteria, and the opportunity to proceed, in such conditions, to inoculation into stone materials.

It should be noted, however, that although on a small scale it is easily possible to carry out a cell count and thus establish the beginning of the plateau phase, observing the growth gradient, on a large scale where the bacteria is cultivated in large fermentors (figure 4), it may be best to refer to a calculation of the bacterial population generation time.

The period of time during which two cells are formed starting from a single individual cell is known as generation, and the time required for the whole process is the generation time. The generation time is thus the time necessary for a cell to duplicate. Many bacteria have generation times of between 1 and 3 hours, but some micro-organisms are also known, amongst which the bacillus pasteurii, which grow very quickly, dividing within ten minutes, and others that have generation times of several hours or even days.

The calculation of generation time allows the definition of the time that is needed to obtain maximum concentration in a solution of bacillus pasteurii ATCC 6453 and thus the best moment to begin the release of the bacterial calcite and, consequently, the inoculation of the stone materials.

Figure 4 – 100 litre bacterial fermentors

The increase in the number of cells during the exponential growth of a bacterial culture is nothing other than a geometric progression in base 2. The duplication of two cells into four may be expressed as $2^1 \rightarrow 2^2$; the duplication of four cells into eight would be $2^2 \rightarrow 2^3$, and so on.

Precisely because of this geometric progression, there is a direct relation between the number of cells present initially in a culture, and the number of cells present after a certain period of exponential growth:

$$N = N_0 \times 2^n \qquad \text{(Eq.1)}$$

where N = final number of cells, No = initial number of cells, and n = number of generations in the period of exponential growth.
The generation time, "g", of the cell population is calculated as t/n, where "t" is the time in hours or minutes of exponential growth. Thus, knowing the initial and final number of cells in a population undergoing exponential growth, it is possible to obtain "n", and when "t" is known, it is possible to obtain the generation time "g".

The equation (1) for obtaining "n", after the necessary transformations, is expressed as:

$$n = \frac{log\ N - log\ N_0}{log\ 2} \qquad \text{(Eq. 2)}$$

Having expressed "n" in the function of easily measurable quantities, "N e N_O", it is possible to calculate generation time:

$$g = \frac{t}{n} \qquad \text{(Eq. 3)}$$

Vice versa, where generation time "g" is known, and the value of bacterial concentration to be reached, with this calculation it is possible to determine the length of time required for the bacillus to remain in the bacterial fermentor.

This leads to the possibility of prolonging fermentation up to the desired degree of concentration which, in this case, corresponds to the moment in which the inoculation of the stony round proves to be more profitable due to the quantity of released endospores.

It was calculated that, considering the generational capacity of the bacillus pasteurii, equal to 13'8" minutes, and wishing to reach a more productive concentration of $9x10^8$/ml, starting with a base concentration of $5x10^7$/ml, it is necessary to keep the bacteria under industrial fermentation for about 10 hours.

This highest concentration $9x10^8$/ml was taken as reference for the experiments we carried out in the stone substrate.

4. THE B.U.L.M. TECHINQUE

In this paper we present an experimental research on the biotechnological treatment of basaltic material. The stone material used belongs to an effusive quality from the province of Catania, from the caves of Belpasso.

The choice of granulometry takes into account the expanding nature of the bacteria in the *porous media* that contains it; in fact, in order to expand, its only possibility being to make use of the chemio-tactile properties in the long flagellum it possesses, it is important for it to be in a "granulometrically suitable" environment, so that the natural micro-porous that form according to the chosen mix design, may biologically cement together.

This research compares the bearing capacity rate CBR of a mix design, used for the construction of the layer of road pavement (according to CNR-UNI 10009 rule), with that obtained by the biological treatment of the material.

The granulometric curve, chosen to make the essays, corresponds to the lower limit of the granulometric spindle of acceptability specified in the article 2, table 2.3 of the above mentioned rule.

4.1 Modified AASHO Test

For the CBR test determination the essays were previously subjected to the experimental research of compaction humidity in order to guarantee the highest accumulation (the best humidity rate) according to the B.U. n. 69/78.

For this reason, the compaction test was carried out following the modified AASHO procedure on four essays of basaltic material, of the same weight and granulometric composition, which were humidified with a progressively growing quantity of water at the rate of 4, 6, 8, and in the end 10% of the weight of the dry stone material (6.5 Kg.).

The obtained AASHO curve is shown in figure 5.

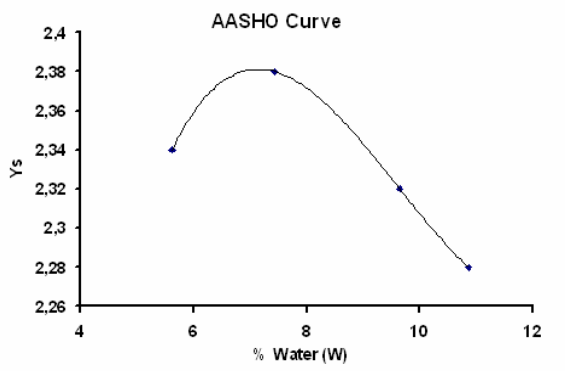


Figure 5 – Result of the AASHO test

The best humidity rate, gathered from the AASHO test carried out on material which was not subjected to biological treatment, was of about 7% and it was chosen as referring value for the compaction of the essays subjected to biological treatment. In this case the water was replaced with the broth inoculated with bacillus pasteurii at a $9x10^8$/ml concentration.

4.2 CBR test on not biological treated basalt

The CBR rate was found without making use of the saturation process, that is without humidifying the four essays of basaltic material which were subjected to the modified AASHO test. The referring CBR is the one of the material humidified with the 7% of water and with a dry specific weight (accumulation rate γs) equal to 2.38 g/cm^3.

The results of the CBR test are shown in figure 6.

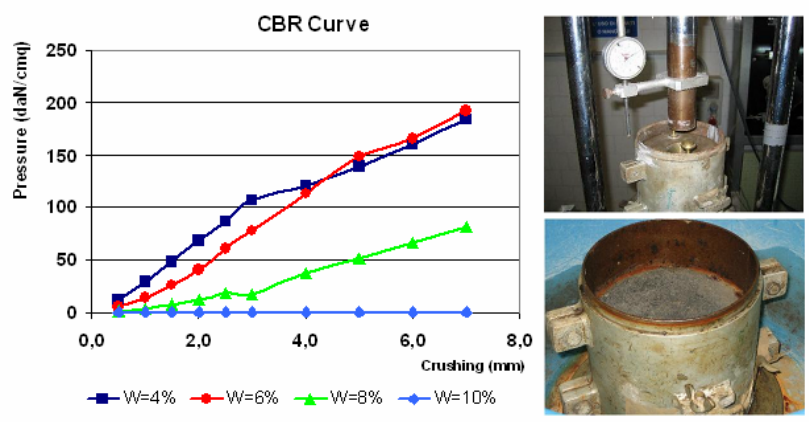


Figure 6 – CBR at different rate water

The results, reported in figure 7, show that, when we have the best humidity rate and the highest accumulation, the result of bearing capacity to be admitted, with a 5.0 mm sinking, is CBR 120.

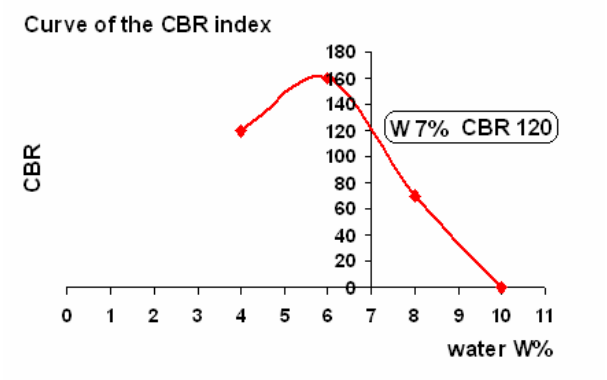


Figure 7 – CBR curve at 5,00 mm

4.3 CBR test on biologically treated basalt

After analysis of the non biologically treated material, we analysed the effects obtained on the granular mix by the application of the protocol of use of bacillus pasteurii.

For this purpose we prepared 12 punches, of 6.5 Kg each, consisting of basaltic material, which were made following the same granulometric composition of the neutral essays. The material in each punch was humidified using the broth of the bacterium which was inoculated with the highest concentration for volume unit ($9x10^8$/ml). It is interesting to point out that, from a physical point of view, the characteristics of the solution are not considerably different from those of the water. Each punch humidified with a quantity of biological solution equal to 7% of the weight (455gr) in comparison

with the weight of the inert material, settled during 12 hours and afterwards was subjected to the modified AASHO compaction procedure. The day after the compaction, the essays were treated with a second bacterial charge having a volume equal to 120 ml, allowing the natural perfusion of the solution to permeate the material. When the bacterial charge had completely filtered into the punches we provided further 200 ml nutrient broth (urea) in order to help the bacterial reproduction in the earthy substratum. After the compaction we let the essays ripen over periods of 10, 60 and 120 days, in a thermostatic room at a constant temperature of 27° C. After the ripening, the samples were subjected to the CBR test, in the following order:

- n° 3 punches after 10 days;
- n° 3 punches after 60 days;
- n° 3 punches after 120 days;

The results of the CBR tests on the all examined essays are shown in figures 8, 9 and 10 .

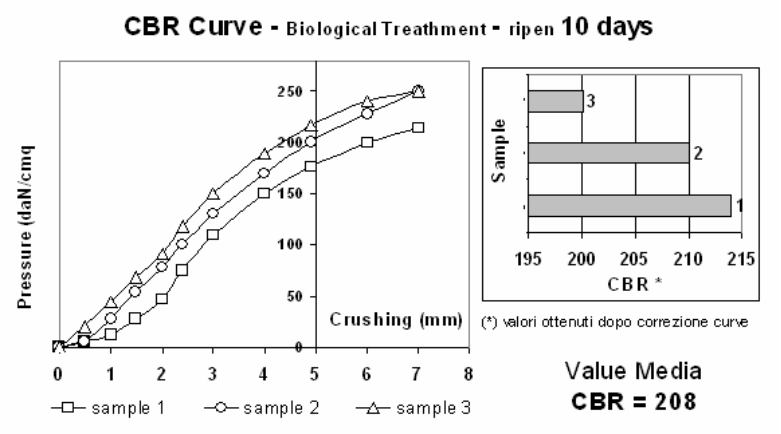


Figure 8 – CBR 10 days

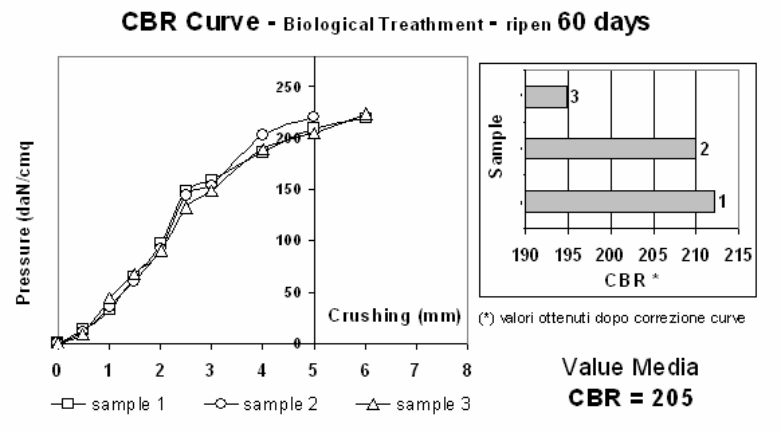


Figure 9 – CBR 60 days

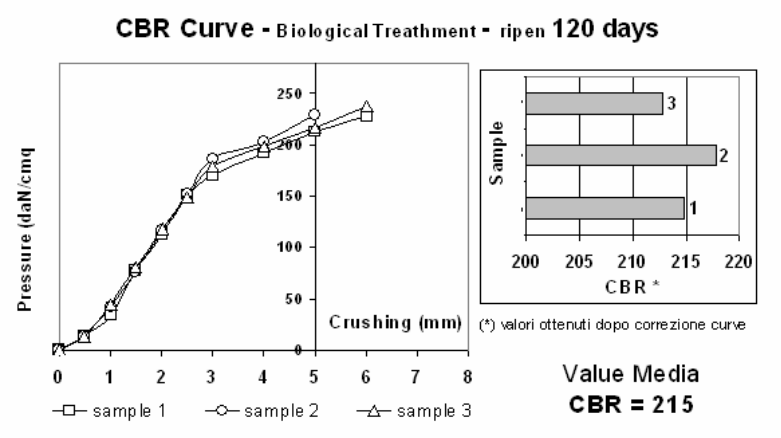


Figure 10 – CBR 120 days

ENDNOTES

We take the value CBR=215 as significant datum of bearing capacity for the granular biologically treated mix. It is interesting to observe that with the passing of time, there were no significant changes in the degree of bacterial compaction in the samples; the measured CBR values, in fact, differ from each other by barely one percent.

This is not at all surprising, and on the contrary, is a perfectly foreseeable result if one notes that bacillus pasteurii, as has been stated, reaches maximum concentration in ten hours, and in twelve hours completes the plateau phase. On completion of the said phase, the plugging process - that is, the cementation that leads to compacting within the material – ceases together with end of the life cycle of the bacterium, so that its effects are unaltered over time. This leads us to conclude that, in comparison with the neutral material (CBR 120), with bacterial plugging it is possible to increase the bearing capacity by 42%, after only 10 days.

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