

Effect of Storage Temperature, Duration and Types of Biofertilizer Carriers on Survival and Numbers of Bacterial Strains *Bacillus megaterium* var. *phosphaticum*, *Azotobacter chroococcum*, *Rhizobium leguminosarum* and Transformant, Transconjugant *B. megaterium* var. *phosphaticum*

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Abstract—this study was carried out in order to compare the different types of biofertilizers carriers, storage temperature and storage duration on the survival and numbers of bacterial strains in biofertilizers. Fifty soil samples and 25 nodules samples were collected from Erbil city, Kurdistan Region, Iraq. *Bacillus megaterium* var. *phosphaticum*, *Azotobacter chroococcum*, *Rhizobium leguminosarum* were isolated, purified and then identified by biochemical tests, isolated bacteria were capable of fixing atmospheric nitrogen and dissolving phosphate in the soil. Chromosomal DNA located *nif* genes responsible for nitrogen-fixing from *Azotobacter chroococcum* as a donor cell transferred to *Bacillus megaterium* var. *phosphaticum* as recipient cell by conjugation process. Plasmid DNA of *Rhizobium leguminosarum* with nod genes transferred to the conjugated cells by transformation process. The cells containing symbiotic nod genes and non-symbiotic *nif* genes were obtained and having the ability to dissolve phosphate and fixing atmospheric nitrogen. To ensure the successful of gene transfer the PCR technique was performed. Four types of bacteria were carried on the three carriers with different structures (carrier 1 = CaCo₃ 20% + Charcoal 20% + Compost 20% + Clay 20% + Sand 19% + Gum1% , carrier 2 = Clay 50% + Compost 20% + CaCo₃ 20% + Charcoal 20% + Gum1% and carrier 3 = Sodium Alginate Sodium alginate solution (6% w/v)), and incubated for 6 months at three temperatures (5°C, 20°C and 30°C) and the bacterial number was enumerated monthly. The results indicated that the number of bacterial cells increased gradually up to 60 days of incubation, then decreased and reached to 13.250×10^{10} Cell /gm of carrier at the end of incubation time from 8.333×10^{10} Cell/gm of starting time, while the number of bacterial Cell in sodium alginate carrier remain stable and survive up to the end of the incubation time.

Index Terms—Biofertilizers carriers, Storage temperature, Storage duration

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I. INTRODUCTION

Biofertilizers considers one of the most safety methods to supply the plants with nutrient elements that it need, whether they are provided or dissolve non-elements are in the soil and in the recent biofertilizers contributed to a significant role in increasing the quality of the crops, because it relies mainly on reducing recourse to chemical fertilizers that have an impact worse on the health and safety of consumers through using with cultivation. The biofertilizers improved with bacteria and fungi have confirmed to be of great importance in improving the yield and quality of different agricultural crops [1], Some free living bacteria have the ability to fixed atmospheric nitrogen, a heterotrophic bacteria found in several environments such as water, air and soil, such as genus *Azotobacter* sp. [2]. As well as a group of bacteria, Gram negative and living as symbiotic with family Fabaceae can fix nitrogen through the formation of nodules such as (species of, *Allorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*). Where it has the ability to establish a relationship with a specialization in very different host plants [3], Where that plants responding chemotactically to flavonoid molecules induce the bacterial genes (nod genes) that can form nodules, which in turn produce lipo-chitoooligosaccharide (LCO) signals that start mitosis cell division in roots, leading to nodule formation[4]. There for some biofertilizers that contain phosphorus solubilization for instance genes *Bacillus megaterium* var. *phosphaticum*, which has the ability to solve the Non-dissolved phosphorus in the soil and converted it to available elements for plant uptake and improve nutrition efficiency and seeds germination [5, 6]. Recently headed the scientific research towards genetic transformation in bacteriology, creating it easier for a particular type do several tasks at the same time and at the same point be more portability and more efficient, according to several research's the transfer of genes from one cell to another and used in bio-field such as the conjugation and transformation processes [7]. It is well known that using of different types of carriers especially organic materials in biofertilizers [3, 8-11].

Fit carrier should be cheap, easily used, mixable, package able, and available, also the carrier must allow gas exchange, particularly oxygen, and has high organic matter content and high water holding capacity as well [12]. Due to the results of [13] the materials used in the production of the biofertilizer should be non-toxic for used bacteria. Additionally, Stephens and Rask [14] and Ferreira and Castro [15] indicated that the carriers should have near neutral adjustable pH, be abundant locally at a reasonable cost and able to sterilize. Otherwise, different materials such as Plant waste, rice straw and composite or industrial waste such as coal have been tested in the inoculum production [14, 16]. Alginate the most suitable material for using as carrier to biofertilizer is considered due to dry, non-toxic nature, the bacteria can analyzed it and retain bacteria a long time without elimination [17]. It also isn't cause environmental pollution, is used widely in the industry and can be stored for a long time as well as also can stored as small beads maintain the vitality of bacteria[18]. The aim of this study is preservation of the environment and to avoid the use of chemical fertilizers, through using biofertilizer and the best carrier to storage the bacteria with the most stable and efficiency. Moreover, selection the best temperature and duration for surviving the bacteria incubated with different carriers.

II. MATERIAL AND METHOD

Four different isolates soil bacteria (*Bacillus megaterium* var. *phosphaticum*, *Azotobacter chroococcum*, *Rhizobium leguminosarum* and Transformant transconjugant *B. megaterium* var. *phosphaticum*) were used as inoculum, three types of carriers (1,2 and 3) used in the conservation of those bacteria were stored at three different temperatures (5°C, 20°C and 30°C). This experiment was carried in the period between December 2015 to May 2016 at Biology Department, Education Collage, Erbil City, Kurdistan region, Iraq.

A. Source of strains

The bacterial strains were *Azotobacter chroococcum*, *Bacillus megaterium* var. *phosphaticum* were isolated from the soil and *Rhizobium leguminosarum* isolated from *Vicia faba* roots nodules: *A. chroococcum* was isolated from the soil of Erbil city, Iraq. During the period of November 2013 to March 2014 using Ashby's media for *A. chroococcum* . and Yeast Extract Mannitol Ager (YMA) for *Rhizobium leguminosarum* . Morphological and biochemical tests (Gelatinase, Catalase, Indole test, Oxidase test and Carbohydrate assimilation tests were performed followed stranded model[19-21].

Bacillus megaterium var. *phosphaticum* was isolated from soil rhizosphere in Erbil city using Sperber's medium followed the enrichment culture techniques [22]. Identification performed through a number of microbiological and biochemical tests. Aerobic spore formers pasteurize a diluted soil sample at 80 degrees for 15 minutes, then plated onto nutrient agar and incubated at 37°C for 24 hr., then the plates were examined for typical colonies identified as catalase-positive, Gram-positive, endospore-forming rods[23] .

Transformant transconjugant *B. megaterium* var. *phosphaticum* it is underwent two transgenetic process the first

process was conjugation and donor cell was *Azotobacter chroococcum* it had (*nif*) genes which located at chromosomal DNA responsible for the non-symbiotic nitrogen fixation and the second process was transformation where the plasmids transferred containing (*nod*) genes and responsible for symbiotic nitrogen fixation ,It has been confirmed the success of the process of gene transfer by PCR technique where proved the existence of those genes in the recipient cells (data not shown).

B. Preservation of bacterial isolates

The isolated bacteria were preserved at -20°C after suspension in 20% (W/V) glycerol [24].

C. Preparation of carriers

TABLE I THE DIFFERENT FORMULA OF BIOFERTILIZERS CARRIERS

Carriers	Formula	Reference
Carrier 1	CaCO ₃ 20% + Charcoal 20%+ Compost 20% + Clay 20% + Sand 19% + Gum1%	[25]
Carrier 2	Clay 50% + Compost 20% + CaCO ₃ 20% + Charcoal 20% + Gum1%	[26]
Carrier 3	Sodium Alginate , Sodium alginate solution (6% w/v)	[17]

1. Preparation of Biofertilizer

1.1. Preparation of Materials Used in the Carrier Composition

1.1.1. Preparation of Compost

Three types of compost were prepared by composing process using animal manure (sheep manure), wheat residues and mixture of them, as a source of organic matter, and suspension of soil, as a source of microorganisms [27]. Animal manure, wheat residues, and mixture of them were dried in an oven at 65°C, added to a pail flagged with coarse gravel, and whenever a layer of organic matter source (1kg) was added with thickness of 10 cm, spread with amount of soil suspension (1L). According to the size of pail four layers of organic matter were layout. The soil suspension that used for inoculation was prepared by addition of 250 gm soil to 2.5 liters of distilled water, shacked for 24 hours. After inoculation, the top layer of each pail was covered completely with 5 cm of clay to keep the moisture content during composing process, and then left at room temperature for 70 days in order to decompose the organic matters. After incubation period the clay layer was removed and the organic matters were dried at room temperature, then turned and crumbled in middling and crushed with electrical grinder then sieved through a sieve of 0.05mm. After sterilization, all three types of compost were stored in plastic bags.

1.1.2. Preparation of Charcoal

Charcoal was prepared from local oak tree coal. It is crushed with electrical grinder, and sieved through an electrical sieve of 0.01mm. After sterilization process stored in sterilized glass bottles.

1.1.3. Preparation of Gum

Local gum was crushed with electrical grinder, and sieved through an electrical sieve of 0.01mm. After sterilization process stored in glass bottles.

1.1.4. Preparation of Clay

The clay used in the carrier's structure was prepared from Ankawa soil. For clay preparation, the soil was passed through

an electrical sieve of 0.002 mm, and then the clay was sterilized and stored in sterilized glass bottles.

1.1.5. Preparation of Sand

For sand preparation the soil was passed through an electrical sieve of 0.1 mm, and then the sand was sterilized and stored in sterilized glass bottle.

1.1.6. Alginate beads preparation

Alginate was prepared according to Bashan [17] and Draget, Skjåk-Bræk and Smidsrød [28]. Sodium alginate solution (6% w/v) were autoclaved at 121°C for 20 min, then cooled and mixed slowly with growth of each strain at the rate of (1:1v/v). The alginate-cells mixture was stirred gently for 30 minutes at 100 rpm to be homogenous. For preparing the beads, the mixture was added dropwise, with the aid of micropipette, into 0.1 M CaCl₂ solution with continuous stirring at rate of 100 rpm for 10 minutes under ambient temperature. The CaCl₂ was then drowning out and the formed beads were washed twice with water, after washing, the beads were dried at room temperature. Alginate was obtained from El Nasr Pharmaceutical Chemicals Co., Egypt.

D. Preparation of inoculums

The bacterial isolates were activated, *Azotobacter chroococcum* on Ashby's broth media [29], *Rhizobium leguminosarum* on Yeast Extract Mannitol broth media [3] and both of *Bacillus megaterium* var. *phosphaticum* and Transformant transconjugant *B. megaterium* var. *phosphaticum* on Sparer's broth [22]. After 7 days at 30°C, cell densities were adjusted to be 7.016, 10.360, 9.803 and 9.018 Log₁₀ of CFU respectively. Furthermore, each group was sub-divided into 3 parts to be stored at 5°C, 20°C and 30°C. The moisture of the carriers was adjusted to 50% of the water holding capacity (WHC). Alginate was prepared under non-aseptic condition according to the method described by Bashan, Hernandez, Leyva and Bacilio [30]. Preparation of different formula of solid state inoculants using various carrier materials Broth culture of previous bacterial isolates having a cell density as shown in table (II) were used to inoculate the prepared bags sterilized using sterile plastic syringe with hypodermic needle until reaching 50% of WHC.

TABLE II THE DIFFERENT CELL DENSITY THAT WE STARTED

Bacterial Isolates	Cell density, Log ₁₀ of CFU (Colony Forming Unit)
<i>Bacillus megaterium</i> var. <i>phosphaticum</i>	7.016
<i>Azotobacter chroococcum</i>	10.360
<i>Rhizobium leguminosarum</i>	9.803
Transformant transconjugant <i>B. megaterium</i> var. <i>phosphaticum</i>	9.018

The bags were thoroughly mixed to ensure similarity in distribution and absorption of the liquid culture into the carrier. Third of the inoculated polyethylene bags, containing the tested formulations were incubated at 5°C the other bags were incubated at 20°C and 30°C for 6 months. Survivals of bacterial isolate during the storage period periodical samples were taken from the prepared formulations every month during the storage period. Standard plate count technique was applied using Nutrient Agar media to determine both of *Bacillus megaterium*

var. *phosphaticum* and Transformant transconjugant *B. megaterium* var. *phosphaticum* [31] and using yeast extract Mannitol Ager (YMA) to determine *Rhizobium leguminosarum* [32], while most probable number method was applied using Modified Ashby's broth medium to determine the total count of *Azotobacter chroococcum* [29]. Samples of alginate beads were rehydrated with NaCl solution (0.8% W/V, pH 7) with shaking at 100 rpm for 30 min according to Ivanova, Teunou and Poncelet [33] before testing the survival of the strains. Evaluation the biological efficiency on carriers after 6 months of incubation, the prepared formulations which exerted survival cells equal to or more than 108 CFU/gm were assessed for their efficiency to fix nitrogen according to Hardy, Burns and Holsten [34].

III. RESULTS AND DISCUSSION

From 50 soil samples and 25 nodule samples collected in Erbil city Iraq, *Bacillus megaterium* var. *phosphaticum*, *Azotobacter chroococcum* and *Rhizobium leguminosarum* were isolated, purified and identified depending on cultural, morphological characteristics, biochemical tests and molecular assays (data not shown).

A. Survival of *Bacillus megaterium* var. *phosphaticum*

Data shown in Fig. 1 that the starter densities was 7.016 log₁₀ CFU/gm in all the carriers numbers began to increase in the first three months reached to 10.258 CFU/gm, then tended to decline gradually by the end of the storage period, which reached 6 months to almost arrived at the first carrier to 6.501, 6.000 and 6.000 log₁₀ CFU/gm when storage at 5, 20 and 30°C respectively. Although the results followed the same line with second carrier the numbers of bacteria reached 10.688 log₁₀ CFU/gm, while it arrived 6.355, 7.000 and 6.233 log₁₀ CFU/gm when incubated at 5, 20 and 30°C respectively at the end of the storage period. While the results headed in a different manner with the third carrier, where marked relatively stable from the beginning to the end of the storage duration with all temperatures where almost began from 7.016 log₁₀ CFU/gm, after 4 months the number become 7.190 log₁₀ CFU/gm, and after six month was 7.000, 7.019 and 7.018 log₁₀ CFU/gm during incubation temperatures 5, 20 and 30°C respectively. These results were convergent with the results of [35], that was perhaps due to the Appropriate conditions of the media used for growing *Bacillus megaterium* var. *phosphaticum* and there was adequate supply of nutrients and the incubation temperature was favorable.

Encapsulation using alginate exerted high stability densities up to the end of the storage period (6 months) at 5, 20 and 30°C, These results agreed with those of Bashan [17] and Bashan and González [18], who informed that bacteria can survive in alginate beads for long periods.

B. Survival of *Azotobacter chroococcum*,

Results illustrated in Fig. 2 that the number of bacteria started from was 10.630 log₁₀ CFU/gm to even reached 12.220 log₁₀ CFU/gm after fourth month, at the end of a period of storage rapporteur were in carrier (1) 8.125, 9.500 and 8.333 log₁₀ CFU/gm for all incubated temperatures respectively. Also in carrier (2) the Numbers reached to 11.500 log₁₀ CFU/gm after

three month of incubation at 30°C and taken tapering until it reached to 7.852, 9.966 and 9.254 log 10 CFU/gm when incubated at temperatures 5, 20 and 30°C respectively. It is shown in the results also kept the third carrier on the stability of the preparation of bacteria and vitality throughout the storage period, where bacterial numbers were 10.855, 10.550 and 10.500 log 10 CFU/gm at temperatures 5, 20 and 30°C respectively. These results are in agreement with those obtained by [36], [37] and [38]. The achieved results are in approval with those obtained by Bashan [17] and Bashan, Hernandez, Leyva and Bacilio [30], who informed that alginate beads can be stored for long periods in a comparatively small volume without any changes in the biological efficiency of the bacterial numbers. From these results, concluded that alginate is the best carrier to be used for protection and survival of bacteria, but it is expensive comparing to other carriers used in this study [39].

C. Survival of *Rhizobium Leguminosarum*

Results in Fig. 3 showed that the number of bacteria that started from was 9.803 log 10 CFU/gm of carrier and reached 11.477 log 10 CFU/gm after three months of incubation, at the end of storage period rapporteur were in carrier (1) 8.333, 7.355 and 8.000 log 10 CFU/gm for 5, 20 and 30°C respectively. Also in carrier (2) the Numbers reached to 11.477 log 10 CFU/gm after four months at 30°C, and taken tapering until it reached to 7.000, 9.200 and 8.254 log 10 CFU/gm at temperatures 5, 20 and 30°C respectively. The results indicated that the numbers of bacteria in third carrier remain stable during the storage period, and bacterial numbers were 9.910, 9.900 and 9.920 log 10 CFU/gm for incubation temperatures 5, 20 and 30°C respectively. It is note that the optimum temperature to maintain the vitality of the bacterial inoculum was 30°C, These results are consistent with [40], where they noted that the temperature and humidity play an important role in pH adjustment and thus has a direct impact on the development and vitality of bacteria inside biofertilizers carriers.

D. Survival of transformant, transconjugant *Bacillus megaterium* var. *phosphaticum*

Results Made clear in Fig. 4 that the number of bacteria began were 9.018 log 10 CFU/gm even reached 11.600 log 10 CFU/gm after three months of incubation, at the end of a period of storage were in carrier (1) 8.568, 7.200 and 8.254 log 10 CFU/gm when incubated at 5, 20 and 30°C respectively. Also in carrier (2) the number reached to 10.456 log 10 CFU/gm after four months at 30°C, and taken tapering until reached to 7.500, 9.255 and 7.254 log 10 CFU/gm at temperatures 5, 20 and 30°C respectively. The results also maintained on the same track with third carrier on the stability of bacterial number and vitality throughout the storage period, where bacterial number was 9.250, 10.250 and 9.350 log 10 CFU/gm at temperatures 5, 20 and 30°C respectively, these results agreed with the finding of [41], [35] and [42]. In this study our conclusion is transconjugant -transformant *Bacillus megaterium* var. *phosphaticum*

bacteria which received *nod* genes from *R. leguminosarum* and *nif* genes from *A. chroococcum*, has the ability to fixed atmospheric nitrogen symbiotically, non-symbiotically and solubilized phosphorus in the soil (data not shown), can be used

as a biofertilizer, and alginate is the best carrier to be used for protection and survival of bacteria to provide available nitrogen and phosphorus to the plants as a safe alternative to harmful chemical fertilizers to human health and polluting, moreover low-cost, and easily delivered to the farmers comparing with chemical fertilizers.

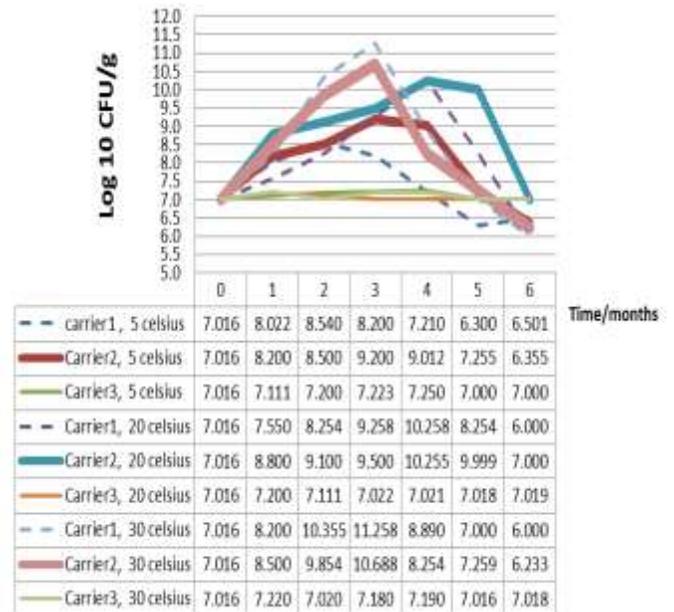


Fig 1. Survival of *Bacillus megaterium* var. *phosphaticum* different carrier (1, 2 and 3) stored at different temperature (5, 20, 30 °C) during storage period (6 months).

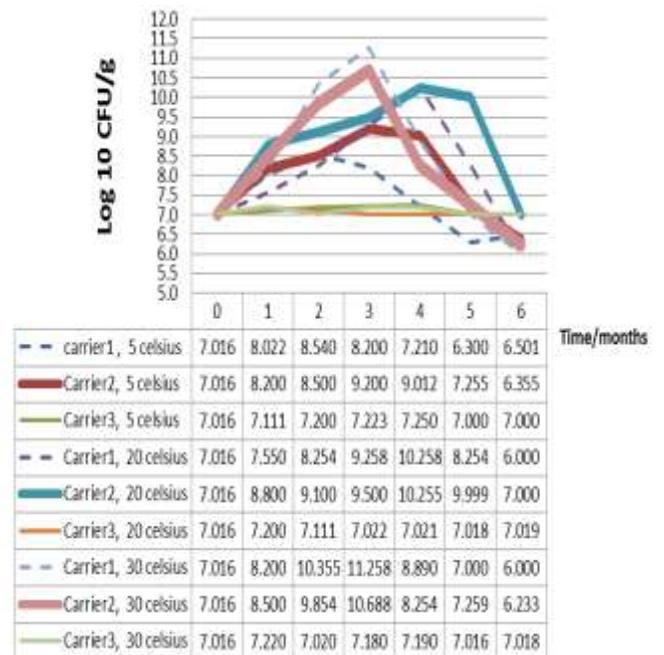


Fig 2. Survival of *Azotobacter chroococcum* on different carrier (1, 2 and 3) stored at different temperature (5, 20, 30 °C) during storage period (6 months).

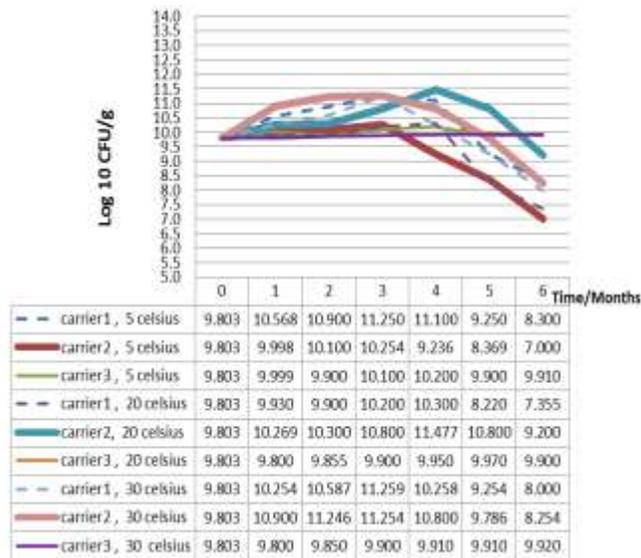


Fig 3. Survival of *Rhizobium leguminosarum* on different carrier (1, 2 and 3) stored at different temperature (5, 20, 30 °C) during storage period (6 months).

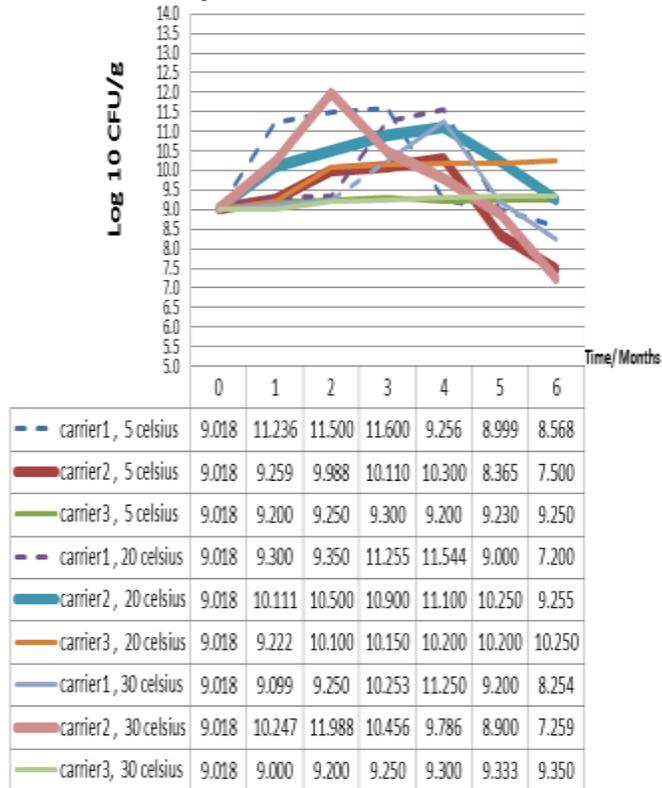


Fig.4. Survival of transconjugant-transformant *B. megaterium* var. *phosphaticum* on different carrier (1, 2 and 3) stored at different temperature (5, 20, 30 °C) during storage period (6 months).

IV. CONCLUSION

Recently thrust into the use of bio and organic fertilization to try to avoid the dangers of harmful chemical fertilizers which leads to risks the environment and the health of living creatures so it was the goal of research is to try to find a fertilizer vital, find a suitable carrier and also choose the temperature and duration storage. Our results show that the best carrier was sodium alginate beads because it gave the best ratio of the

stability of bacterial number. Moreover, it is note that the optimum temperature to maintain the vitality of the bacterial inoculum was 30°C.

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