Indian J. Applied & Pure Bio. Vol. 29(1), 67-76 (2014).

Screening the temperature tolerant strains of *Rhizobium japonicum* for high productivity yield of *Glycine max* using gum as carrier in tropical and subtropical regions of India

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ABSTRACT

Soybean is cultivated as subsistence crop in central India, this crop could also adapt as well to different environmental conditions and soil types. The most important agent of symbiotic nitrogen fixation is bacteria of genus *Rhizobium japonicum* for soybean.

Temperature seems to be an important factor affecting the *Rhizobium japonicum* population and their distribution. The survival of *Rhizobium japonicum* at high temperature is a challenging problem especially for tropical and subtropical regions with extreme temperature ranges 35-50 C.

Presently lignite is one of the most commonly used carriers in production of Bio-fertilizer. It has been observed that lignite shows low moisture holding capacity and proper maintenance of moisture is essential for growth of *Rhizobium japonicum*. In the present investigation a new approach has been made of using different types of gums. It is an important parameter to know effective symbiosis. It is a need to isolate and identify temperature tolerant strains of *Rhizobium japonicum.*

In the present study the effects of high temperatures 35-50 C was studied on different *Rhizobium japonicum* strain mixed with the lignite, which is taken as control. The strains is mixed with two different nutritive gums, they are Arabic and Guar followed by incubation to achieve the temperature in water bath for four hours daily for 10 days at 35-50 C. The growth and viability of strains was observed daily before and after heat treatment with the help of viable plate count method.

The outcome of this study suggests that screening of high temperature tolerant strain of *Rhizobium japonicum* in gums as carrier in compared to lignite could be key to sustain legume productivity in tropical regions of our country.

Liquid bio-fertilizer formulation could be considered as one potential strategy for improving the shelf-life of bio-fertilizer. Further, solid carrier based bio-fertilizers are less thermo-tolerant where as liquid formulations can tolerate the temperature as high as 55°C. Hence, improved shelf-life could be achieved by the application of a liquid bio-fertilizer formulation. Process cost of liquid bio-fertilizer is significantly higher than a solid formulation. Thus, successful commercialization of less expensive liquid bio-fertilizer is a challenge and shelf-life of such products is still a concern.

Key words : *Rhizobium japonicum*, screening, viability, symbiosis, lignite, Arabic, Guar.

All living things require nitrogen to make proteins needed for life. As soils are often low in nitrogen content, good plant growth often means supplementing soil nitrogen with fertilizer nitrogen which is expensive and is therefore too costly for many small farmers to buy. Fortunately some plants can be benefited (symbiosis) with micro-organism which convert atmospheric nitrogen to ammonia. This fixed form of nitrogen is then used by the plants to make proteins. Tolerance test are usually performed *in vitro* exposing the strains to adverse condition that mimic those that naturally occur in the soil[4,9].

Rhizobium japonicum is one of the most important agents of symbiotic nitrogen fixation. Growth and survival is effected by many factors such as plant stress and soil acidity. Temperaturs is the prime environmental factor affecting the survival of the *Rhizobium* population. Survival of *Rhizobium* at high temperature is a challenging problem especially in tropical region of extreme temperature in tropical country like India. To establish an effective symbiosis, there is a need for isolation and screening of high temperature tolerant strains. Presently lignite is the most commonly used carrier in production of Bio-fertilizer. Lignite show slow moisture holding capacity and comparatively considerable amount of moisture is essential for the growth of *Rhizobium*.

In the present investigation a new approach has been made of using different types of gums. The two different types of gums are used as carrier to know their efficiency in holding capacity of moisture even at high temperature, gums are nourishing and also having the important characteristics of adhesion, because it was observed that bio-fertilizer require such carrier which could adhere with the seed properly but lignite don’t have the characteristics to adhere with the seeds in that condition jaggery syrup is used for adhesion.

Chemically the gums are complex compounds, derived from carbohydrates. Specifically they are salts of either potassium, magnesium or calcium of acidic polysaccharides, the acidity of which is due to uronic acid.

Laboratory experiments demonstrate the feasibility of this approach. In the present study the effect of relatively high temperature (35-50^{0}C) was studied on 18 strains of *Rhizobium* isolated from various regions of Madhya Pradesh. However one strain (*Rj*(S)TAL102) is a *Bradyrhizobium japonicum* which was collected from Bio-fertilizer plant of M.P. Agro Industry, Bhopal, which is a slow growing bacteria where as *Rhizobium japonicam* is a fast growing bacteria. Fast growing Rhizobia are known to acidify the medium and slow growing Rhizobia make the medium alkaline. From this 18 isolates, 3 strains and *Rj*(S)TAL102 strain found to flourish well at this extended temperature both in broth and gums. From the viable count it was found that there was no serious mortality.

These strains were given heat treatment in water bath for 4 hours for 10 days at 35-50^{o}C and accordingly broth of these strains were also given heat treatment in water bath for 4 hours for 10 days at 35-50^{o}C.

The growth and viability of strains was observed daily before and after the heat treatment with the help of viable plate count method and observed OD by spectrophotometer at 600 nm.

The outcome of this study suggest that screening high temperature tolerant strains of *Rhizobium* could be key to sustain legume productivity in temperate and tropical regions of our country and using this strain for nitrogen fixation in liquid gum inoculums medium is more better than in lignite carrier. This gummy inoculums are available and serve not only as glue for the bacteria to stick on to the seed but also serve as food for the bacteria until the infection process is complete.

Qualities of a good carrier material are:-

Highly absorptive
Easy to process
Non toxic
Easy to sterilize
Inexpensive
Provide good adhesion to seeds.
Have pH buffering capacity.

Comparative studies indicate that viable counts are more in liquid medium than in lignite carrier. Survival of *Rhizobium & Bradyrhizobium japonicum* at high temperature is a challenging problem especially in tropical regions of extreme temperature range. Some attention had given to the effect of high temperature on *Rhizobium*[3,13]. Temperature is the prime environmental factor, which can limit the survival, density and capacity of inoculums[11]. Survival is only possible in narrow range of temperature called Bio-kinetic temperature. Jones and Tisdale[7] studied the effect of different temperature on nodulation and found that 24^{o}C was the optimum temperature for maximum nodulation. Temperature has been shown a marked influence on nodulation, nitrogen fixate on ability and longevity of *Rhizobium*.

Critical temperature for nitrogen fixation is ranges between 35 and 40^0C for soybean. Nodulation of soybean was markedly inhibited at 42 and 45^oC (Zahran[14]). The optimum temperature for its growth varies from 28 to 35^oC. Kretovich *et al.*,[8] and Jain and Rewari[6] observe no serious mortality upto 40^oC. High soil temperature have been found to have a detrimental effect on the growth and survival of *Rhizobium*[1].

Effect of temperature was studied according to the method as described by Vincent[12]. 18 isolates of *Rhizobium japonicum* were isolated including one strain of *Bradyrhizobium japonicum* Rj(S)TAL102 was taken from Bio-fertilizer plant, Bhopal. 100 ml of yeast extract mannitol broth in 250 ml conical flask were sterilized and is inoculated with 10ml of 8 to 10 days old broth. This inoculated broth was incubated for 3 to 4 days in shaker followed by gram staining and other biochemical test to confirm and check the growth of nitrogen fixing bacteria. All samples were tested in triplicates by serial dilution method by plating 1ml suspension from each flask. Then the inoculums was divided in to three parts. First part of the inoculums was maintained as control at room temperature, second part was mixed with lignite and third part was mixed in gum modified media in 1:1 ratio (at pH 6.8). This modified media have Arabinose, Trehalose, Fe-EDTA, PVP etc.

Temperature effect was studied at 35-55^oC. After incubation flask containing yeast extract mannitol broth with inoculants are kept in various temperature in seriological water bath for 4 hours. Before and after heat treatment is done to check the viable count. This test was done by drawing 1ml suspension from each flask and plating was done for upto 10 days and by spectrophotometer. Heat treatment was given after every 24 hours upto 10 days. For carrier 3 to 4 days old inoculants are mixed with lignite and gum. Viability is checked by plate count method of yeast extract mannitol media or congo red yeast extract mannitol media for both broth, lignite and gum. The lignite taken is sterilized in which charcoal and calcium carbonate is mixed in order to maintain the pH of the carrier. Mixing should be done in such a way so that the moisture % should be 40-50%. This mixture of 10gms is taken in flask and then they are kept in water bath in various temperatures for the treatment of 4 hours for 10 days. In this study also before and after heat treatment, testing is done and count was taken in by plate count method. Testing was done in triplicates. In mixture of 10gms of inoculums in lignite and 10 ml of gum liquid inoculum, 100 ml of distill water is dispensed to do the testing by serial dilution method.

Control set was maintained at room temperature, which received no heat treatment.

Temperature plays an important role in morphological and biochemical process. External temperature can have profound influence on the endogenous metabolism and has been shown to affect the nutritional requirement of many microorganisms[5].

Data on survival of different test isolates of *Rhizobium japonicum* is presented for *guar* gum whose shelf life was more than the Arabic gum shown in Table-1, 2, 3 and 4.

After giving heat treatment it was found that cells multiply rapidly when temperature was above 30^0C and this significant increase in multiplication was observed upto 50°C for 10 days and then growth declines in lignite but in gum liquid inoculums nitrogen fixing bacteria survive in 35-50°C for more than 25 days. In isolate *Rj*(S)04, *Rj*(S)05, *Rj*(S)08 and *Rj*(S)TAL102 it was observed, that survival was seen at 45°C for more than 25 days and at 50°C upto 19 days in gum liquid inoculums whereas in lignite survival was not observed in 50°C. But the interesting thing was that the strain *Rj*(S)TAL102 taken from bio-fertilizer plant survive for more than 24 days at 50°C temperature in gum liquid inoculums but in lignite it shows no growth at 50°C. bThis result is an interesting part of this study. *Rj*(S)01, *Rj*(S)02, *Rj*(S)03, *Rj*(S)06 and *Rj*(S)07 grew well upto 35°C for 10 days in both lignite and gum liquid inoculum, it was observed that nitrogen fixing bacteria grow slowly in lignite, on third day strain starts declining rapidly at 35-50°C.

Heat resistance in *Rhizobium* has been a subject of great interest. Temperature is one of the most important environment factors which can limit the survival of the inoculums[11]. In general it has been reported that majority of bacteria grow well at 37°C temperature. The temperature tolerance studies indicated survival of test isolates of *Rhizobium japonicum* at various temperatures. There was an increase in viable cell when temperature was raised from room temperature to 45°C. There was successive declining in the count of the cell when the temperature was further raised from 35-50°C.

In India, count of *Rhizobium japonicum* at temperature ranging from 28-35°C were appreciated while at 40°C mortality rate was high. Bajpai *et al.*,[2] also reported a gradual fall in viable count of *Rhizobium* above 30°C.

Raghuvanshi and Gangwar[10] reported that the favorable temperature for multiplication of most species of *Rhizobium* is upto 40°C above which there is a sharp decline in the viable count.

Kretovich *et. al.*,[8] have reported that bacterial growth is optimum at 37-42°C after which it declines sharply. They also reported that at 40°C *Rhizobium japonicum* multiplied to a greater extent.

In general it was observed that above 35°C temperature sharp decline in the Rhizobia population take place. Field survey reports of Rhizobial population under natural condition reveal that the Rhizobial population in general is poor throughout the country. Increase in exposure time at temperature range of 28-35°C resulted in progressive rise in the viable count. A reverse trend was found at 45-50°C in case of all the test isolates except, similar results were reported by Bajpai *et.al.*,[2]. It was observed that inoculums in gum survive in 50°C but in lignite it does not showed any count. So, as per present investigation efficiency of strains were checked in gum liquid inoculums in three modified media in comparison with the carrier lignite taking in yeast extract mannitol broth and in control also yeast extract mannitol broth was used.

Variation was quite wide between liquid inoculum, lignite and yeast extract mannitol broth.

Table-1. Showing viable count of *Rhizobium japonicum* (*Rj*(S)TAL102) in carrier exposed to heat treatment in (4 hours/day).

Heat Treatment	Control	In *Guar* gum (50°C)		In lignite (50°C)	
		BHT	AHT	BHT	AHT
Day 1	239 ± 3.61	302 ± 1.73	296 ± 2.52	283 ± 1.53	286 ± 2.65
Day 2	234 ± 2.31	312 ± 1.15	308 ± 2.08	293 ± 2.08	303 ± 1.15
Day 3	309 ± 2.08	329 ± 0.58	321 ± 2.08	312 ± 1.15	309 ± 2.08
Day 4	328 ± 1.53	344 ± 1.15	337 ± 3.79	300 ± 1.00	293 ± 2.08
Day 5	348 ± 1.16	331 ± 1.15	348 ± 1.15	281 ± 0.58	273 ± 1.73
Day 6	382 ± 1.16	307 ± 1.15	323 ± 1.15	257 ± 1.73	235 ± 1.73
Day 7	378 ± 1.73	261 ± 1.53	289 ± 1.73	206 ± 3.06	181 ± 1.15
Day 8	340 ± 1.16	193 ± 1.15	225 ± 2.08	161 ± 2.52	119 ± 0.58
Day 9	319 ± 1.53	149 ± 1.53	155 ± 2.08	103 ± 2.00	88 ± 1.15
Day 10	293 ± 2.08	129 ± 2.08	105 ± 1.73	72 ± 1.15	58 ± 1.15

BHT=Before heat treatment

AHT=After heat treatment

Data are mean of three replicates.

GRAPH 1:

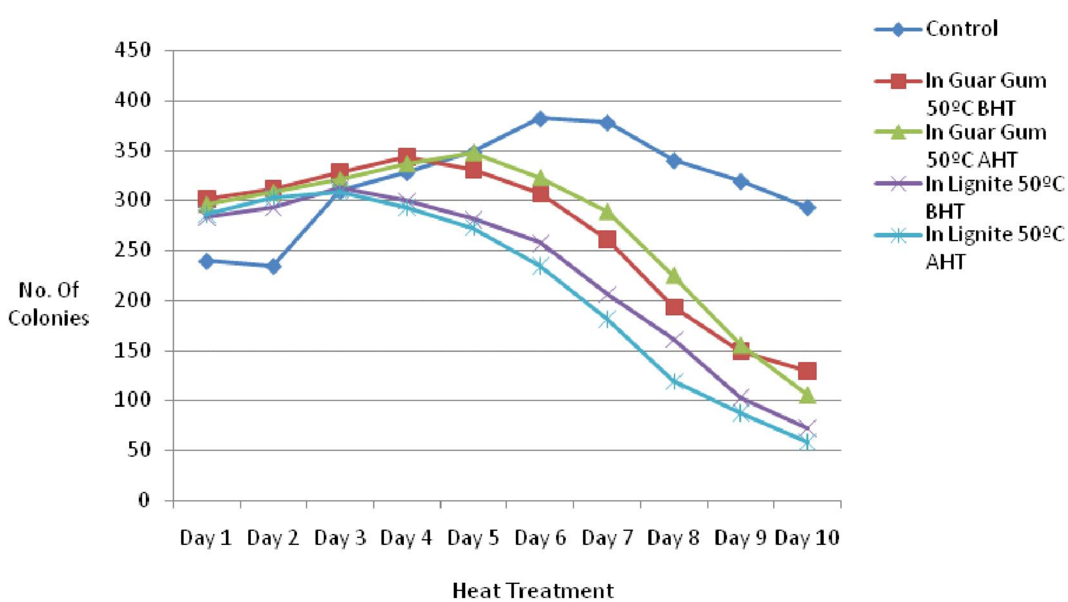


Table-2: Showing viable count of *Rhizobium japonicum* (*Rj*(S)05) in carrier exposed to heat treatment in in (4 hours/day).

Heat Treatment	Control	In *Guar* gum (50°C)		In lignite (50°C)	
		BHT	AHT	BHT	AHT
Day 1	260 ± 1.15	170 ± 2.31	150 ± 2.31	161 ± 1.53	120 ± 1.15
Day 2	271 ± 1.73	152 ± 2.65	141 ± 1.53	122 ± 2.52	100 ± 2.52
Day 3	278 ± 1.53	144 ± 2.31	130 ± 1.73	101 ± 1.15	90 ± 1.15
Day 4	281 ± 1.15	132 ± 1.73	120 ± 1.15	72 ± 2.08	59 ± 1.15
Day 5	290 ± 2.08	123 ± 2.52	119 ± 0.58	40 ± 1.15	20 ± 1.15
Day 6	300 ± 1.00	114 ± 1.73	98 ± 2.08	19 ± 1.53	0 ± 0.00
Day 7	313 ± 2.08	76 ± 1.00	65 ± 2.52	0 ± 0.00	0 ± 0.00
Day 8	320 ± 1.53	22 ± 1.15	10 ± 1.53	0 ± 0.00	0 ± 0.00
Day 9	300 ± 1.00	11 ± 1.00	6 ± 1.15	0 ± 0.00	0 ± 0.00
Day 10	262 ± 1.53	6 ± 1.15	0 ± 0.00	0 ± 0.00	0 ± 0.00

BHT=Before heat treatment

AHT=After heat treatment

Data are mean of three replicates.

GRAPH 2:

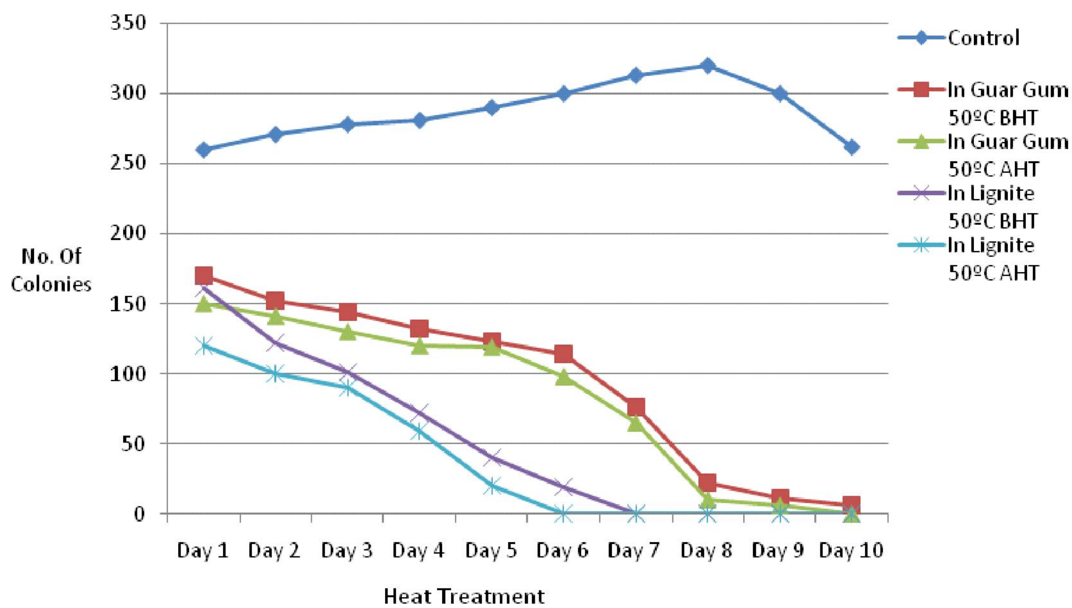


Table-3: Showing viable count of *Rhizobium japonicum* (*Rj*(S)08) in carrier exposed to heat treatment in (4 hours/day).

Heat Treatment	Control	In Guar gum (50°C)		In lignite (50°C)	
		BHT	AHT	BHT	AHT
Day 1	261 ± 1.73	264 ± 2.65	222 ± 2.31	261 ± 1.73	202 ± 2.65
Day 2	272 ± 1.53	271 ± 2.08	211 ± 2.08	193 ± 2.08	170 ± 2.31
Day 3	279 ± 2.08	193 ± 2.08	196 ± 1.00	164 ± 2.52	101 ± 1.15
Day 4	282 ± 1.16	164 ± 2.52	173 ± 2.65	133 ± 1.00	51 ± 1.53
Day 5	293 ± 2.08	133 ± 1.00	155 ± 1.73	120 ± 1.15	20 ± 1.15
Day 6	304 ± 1.53	114 ± 1.73	139 ± 1.73	104 ± 2.89	4 ± 0.58
Day 7	312 ± 1.15	96 ± 2.08	125 ± 2.08	96 ± 2.08	0 ± 0.00
Day 8	328 ± 1.53	66 ± 2.52	105 ± 3.21	72 ± 2.08	0 ± 0.00
Day 9	341 ± 1.15	44 ± 2.65	81 ± 0.58	64 ± 2.52	0 ± 0.00
Day 10	362 ± 1.53	26 ± 1.53	59 ± 2.65	56 ± 2.08	0 ± 0.00

BHT=Before heat treatment

AHT=After heat treatment

Data are mean of three replicates.

GRAPH 3:

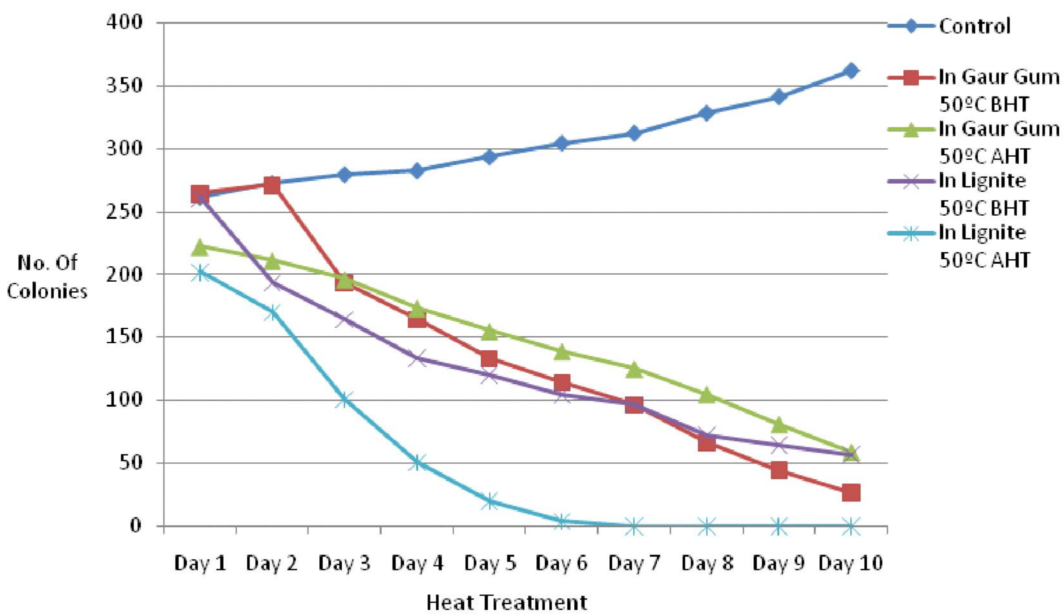


Table-4: Showing viable count of *Rhizobium japonicum* (*Rj*(S)04) in carrier exposed to heat treatment in (4 hours/day).

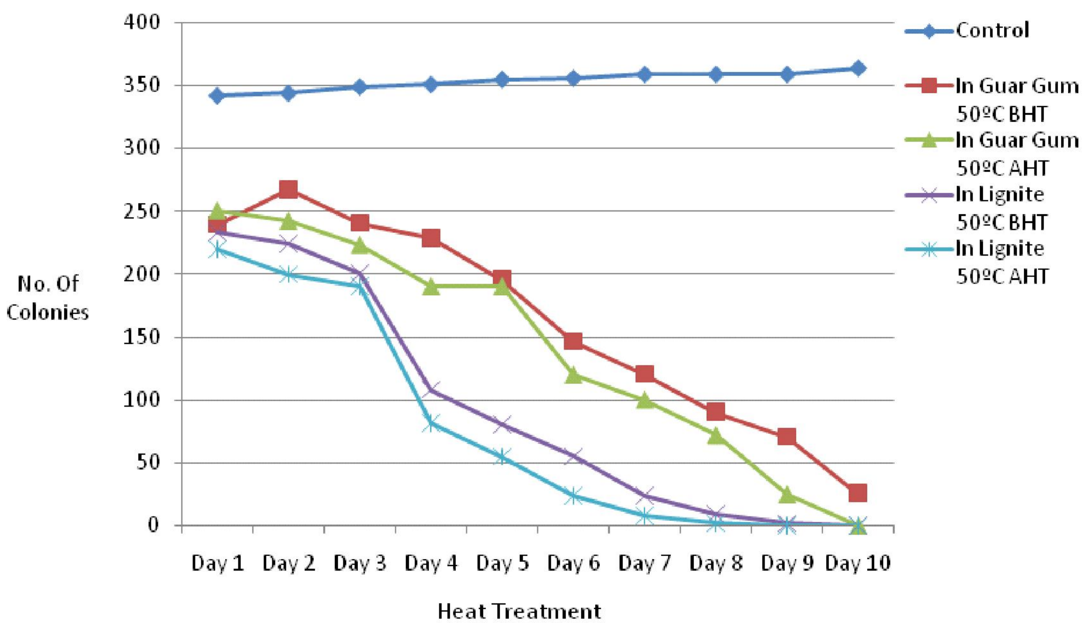
Heat Treatment	Control	In Guar gum (50°C)		In lignite (50°C)	
		BHT	AHT	BHT	AHT
Day 1	342 ± 1.16	239 ± 3.61	250 ± 2.65	233 ± 2.08	220 ± 2.65
Day 2	344 ± 1.15	267 ± 1.53	242 ± 3.61	224 ± 2.52	200 ± 2.52
Day 3	349 ± 1.73	240 ± 3.61	223 ± 2.08	200 ± 2.52	190 ± 0.58
Day 4	351 ± 1.15	228 ± 2.08	190 ± 0.58	108 ± 3.61	82 ± 0.58
Day 5	355 ± 1.53	195 ± 1.15	190 ± 0.58	80 ± 1.15	55 ± 2.52
Day 6	356 ± 2.31	146 ± 2.08	120 ± 1.15	55 ± 2.52	24 ± 1.53
Day 7	359 ± 1.73	120 ± 1.15	100 ± 2.52	24 ± 1.53	8 ± 1.53
Day 8	359 ± 0.58	90 ± 2.08	72 ± 2.08	9 ± 1.53	2 ± 1.53
Day 9	359 ± 1.73	70 ± 1.15	25 ± 1.15	2 ± 1.53	0 ± 0.00
Day 10	364 ± 4.36	25 ± 1.15	0 ± 0.00	0 ± 0.00	0 ± 0.00

BHT=Before heat treatment

AHT=After heat treatment

Data are mean of three replicates.

GRAPH 4 :



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