International Journal of Current Science Research and Review
ISSN: 2581-8341
Volume 06 Issue 04 April 2023
DOI: 10.47191/ijcsrr/V6-i4-47, Impact Factor: 6.789
IJCSRR @ 2023
www.ijcsrr.org

Screening of Different Media and Heat Shock Treatment Regimens for Enhancing Sporulation in *Bacillus licheniformis*

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ABSTRACT: *Bacillus licheniformis* is a spore-producing bacteria. The present study aims to identify maximum sporulation in *Bacillus licheniformis* through induced heat shock conditions using different solidified media and broth media. Suspensions of *Bacillus licheniformis* spores were spread-plated on nutrient agar plates before and after heat shock treatments of 60°C, 80°C and 100°C for 30 minutes. The number of spores was determined by the dilution plate count method. The result indicates that the highest spore production for *Bacillus licheniformis* was observed when the spores were induced in AK (Arret and Kirshbaum) Agar for seven days under a heat shock treatment of 60°C for 30 minutes. Whereas, in the case of Nutrient Broth media, the spore production was highest after four days at heat shock treatment of 80°C for 30 minutes. This study will help find the optimal production of spores from *Bacillus licheniformis*, an industrially beneficial microorganism.

KEYWORDS: *Bacillus licheniformis*, Endospore Staining, Heat shock, Spores, Sporulation

INTRODUCTION

Bacteria are singled cell organisms present in the environment. Most bacteria are classified as gram-negative and gram-positive, depending on their morphology and differential staining properties. Gram-positive bacteria have thicker Peptidoglycan cell walls than gram-negative bacteria. Only gram-positive bacteria can form spores. Spore-forming bacteria produce dormant structures under unfavourable stress and nutrient depletion environments [1]. The most common examples of spore-producing bacteria are the *Bacillus and Clostridium* species. The spores of these species do not have an active metabolism but can carry all the genetic material found in the vegetative form. The bacterial spores can be beneficial or harmful depending on the circumstances. Despite causing food spoilage and poisoning in the food industry because of their heat resistance, the spores play a significant role in many industrial purposes. In the year 1876, Cohn studied bacterial endospores for the first time [2]. The spore of *Bacillus* species is resistant to heat, chemicals and radiation so that they can survive in harsh environments; hence for the last 20 years, its importance has been studied for commercial purposes and scientific research [3, 4]. Sporulation can be induced by nutrient starvation of resources like carbon, nitrogen, and phosphorus [5, 6]. Heat shock conditions for sporulation vary with different species and media [7, 8, 9, 10] The different heat treatments studied on *Bacillus* species reported that the optimal temperature for heat activation was ≤70°C for 15-30 min [11]. *B. coagulans* and *B. subtilis* show the highest spore recovery rate after a heat shock of 68°C for 20 min [12]. The cells of *Bacillus* and *Clostridium* sp. were damaged when exposed to high temperatures and had a sporicidal effect when exposed to 90°C [11, 12, 13, 14]. Whereas spores of *Bacillus stearothermophilus* were induced when subjected to 100°C or less. Still, maximal activation was observed at 110- 115°C [15]. The study of *Clostridium* species has shown that heat treatment enhances sporulation, and enhancing the heat resistance of spores leads to the dormancy and stability of bacillus subtilis [16, 17]. The heat resistance of bacteria like *Bacillus licheniformis* depends on factors like the composition of the media and sporulation temperature. The spores became more heat resistant when induced at high temperatures [18, 19]. Among the Bacillus species, *B.licheniformis* and *B.cereus* sporulated highest when there is a higher level of manganese and calcium, whereas a higher level of manganese and lower level of calcium can enhance the sporulation of *B.subtil*is and *B.coagulans*. But in *B.licheniformis*, it can be seen that media supplemented with calcium alone can improve the sporulation effectively, but when it is supplemented with manganese, it can inhibit the sporulation slightly [20]. The importance of manganese during sporulation has been studied in the past years, so it is commonly used for the sporulation of Bacillus species [21, 22, 23, 24]. The number of viable spores can be checked through the spectrophotometric method [25] but we used the Standard plate count method for our study since we find it more reliable and convenient.

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MATERIALS AND METHODS

Microorganism

Bacillus licheniformis (MTCC 429) was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India, in freeze-dried form. The culture was reconstituted in nutrient broth and kept in an incubator shaker at 37°C for 24 hours.

Induction of Sporulation

Sporulation was induced by inoculating *Bacillus licheniformis* into five types of solid agar media viz; Media 1 (Nutrient Agar), Media 2, Media 3 [26, 27], Media 4 [10] and Media 5 [5, 28] and three types of broth media viz; Nutrient Broth, Nutrient Broth with magnesium sulphate and Sporulation Broth. The inoculated plates and the broth were incubated at 37°C for seven days. The composition of the media is given in Table I and Table II.

Table I: Composition of solidified media viz., Media 1, Media 2, Media 3, Media 4 and Media 5, used to sporulate *Bacillus licheniformis*.

Composition (Gm/l)	**Solidified Media**				
	Media 1 (Nutrient Agar)	**Media 2**	**Media 3**	**Media 4 (AK Agar)**	**Media 5**
Agar	15.00	15.00	15.00	15.00	7.50
Beef extract	10.00	3.00	0.50	1.50	-
Calcium chloride dihydrate	-	-	-	-	0.1
Dextrose	-	-	0.50	1.00	-
Di-ammonium hydrogen phosphate	-	-	-	-	1.00
Ferrous sulphate heptahydrate	-	-	-	-	0.001
Manganese sulphate	-	0.03	0.003	0.30	0.5
Manganese sulphate monohydrate	-	-	-	-	0.05
Pancreatic digest of casein	-	-	-	4.00	-
Pancreatic digest of gelatin	-	-	-	6.00	-
Peptic digest of animal tissue	10.00	-	-	-	-
Peptic digest of gelatin	-	5.00	-	-	-
Peptone	-	-	5.00	-	-
Potassium dihydrogen phosphate	-	-	-	-	5.00
Sodium Chloride	5.00	-	5.00	-	1.00
Sucrose	-	-	-	-	1.00
Yeast extract	-	-	2.00	3.00	-

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Table II: Composition of broth media viz., Nutrient Broth, Nutrient Broth with magnesium sulphate and Sporulation Broth, used to sporulate *Bacillus licheniformis*.

Composition (Gm/l)	**Broth Media**		
	Media 1 (Nutrient Broth)	**Media 2 (Nutrient Broth with Magnesium Sulphate)**	**Media 3 (Sporulation Broth)**
Beef extract	1.00	1.00	1.50
Casein enzymic hydrolysate	-	-	4.00
Dextrose	-	-	1.00
Magnesium sulphate	-	0.50	-
Manganous sulphate	-	-	0.30
Peptic digest of animal tissue	-	-	6.00
Peptone	5.00	5.00	-
Sodium Chloride	5.00	5.00	-
Yeast extract	2.00	2.00	3.00

Preparation of Spore Suspension

Sporulation was monitored by observing at 100x microscopy until fewer vegetative cells remained. Sterile deionized water was added to the agar surface to harvest the spores from the plates with a sterile glass bent rod. The suspension containing spores was centrifuged and rewashed at 10,000 g for 30 mins. The supernatant was discarded, and the pellets were resuspended in 10 ml of cold, sterile deionized water. The spore suspension was then stored at 4°C until further use [26, 29, 30, 31].

Endospore Staining

The main purpose of endospore staining is to differentiate bacterial spores from other vegetative cells. Schaeffer-Fulton's method was performed to observe the bacterial spores [29, 32]

A clean glass slide was taken, and a smear was made. The smear was heat fixed, and it was covered with blotting paper. The blotting paper was saturated with malachite green, and the slide was steamed over a container of boiling water for 5 minutes. The glass slide was washed and counterstained with 0.5% safranin for 30 seconds. The glass slide was washed again with tap water and blotted dry. The slide was then examined under a microscope for the presence of endospores.

Heat Treatment Assay

The spore suspension was heated at 60°C, 80°C, and 100°C for 30 minutes [25, 32,33]. The purpose of this heat shock is to kill vegetative cells and stimulate sporulation.

Spore Plate-Counting Assay

The standard plate count method was used to determine the spore concentrations from the suspension [9, 17, 20, 26, 35, 36]. After heat treatment, the spore suspension was cooled for a few minutes. Serial dilution was performed using distilled water to give maximal plate count [15]. 0.1 ml of each serially diluted suspension was plated in the nutrient agar media by the spread plate technique. The plates were then incubated for 16-18hr at 37°C. The viable counts were observed before and after heat treatment. The number of spores was calculated from triplicate plating. The results were expressed as CFU/ml [25, 29, 34, 37]

Statistical Analysis

The data were presented as mean values with the standard error for all the experiments. All the experiments were replicated thrice, and CFUs were converted to $\log_{10}$ values. The differences among the mean values were detected using the ANOVA test at a significance level of $p \leq 0.5$ using Microsoft excel.

Spore Percentage

The spore Percentage was calculated by observing the Total Viable Count and Heat Resistance Spore Count [38]

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RESULTS AND DISCUSSION

Endospore Staining

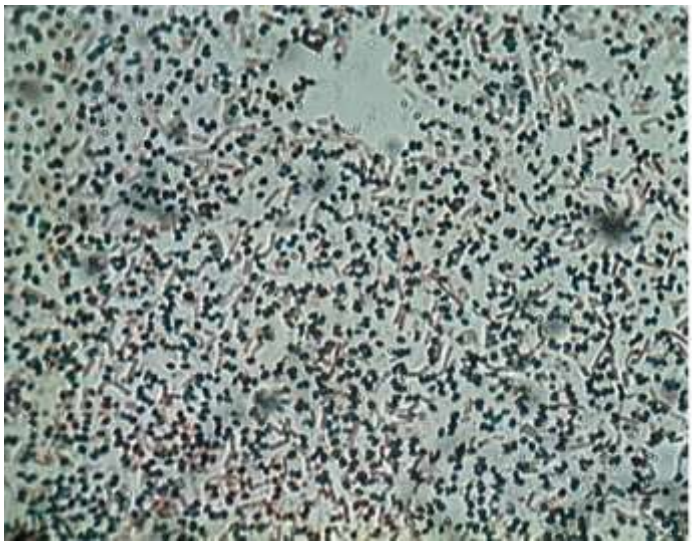

Fig 1: Endospore staining of *Bacillus licheniformis* by Schaeffer-Fulton's Method. The endospores appear green and vegetative cells appear pink in colour.

Malachite green is a weak binding dye that was forced inside the spores by steaming. While washing the glass slide with water, the stain was removed from the vegetative cells, but the endospores retained malachite green. So, when safranin was used as a counterstain, the vegetative cell appeared brownish pink or red, but the endospores appeared green (Fig. 1).

Spore Count

The spore count of *Bacillus licheniformis* induced under different heat shock conditions by using different solidified media is presented in Fig 2. The viable counts of *Bacillus licheniformis* using various solidified media are 14.37, 10.17, 12.68, 13.54, and 11.7 Log CFU/ml, respectively, in control suspension, i.e., without any heat treatments. The heat-resistant spore counts after different heat treatments were significantly different ($p < 0.05$).

The highest spore count was observed at AK Agar when 60°C heat shock was given for 30 mins, and the lowest spore count was observed when 100°C heat shock was given for 30 min on all the media. It seems that strong heat shock conditions damage the cells, which leads to the loss of viability. There has also been a report that AK Agar medium produced maximum sporulation of *Bacillus megaterium* [10]

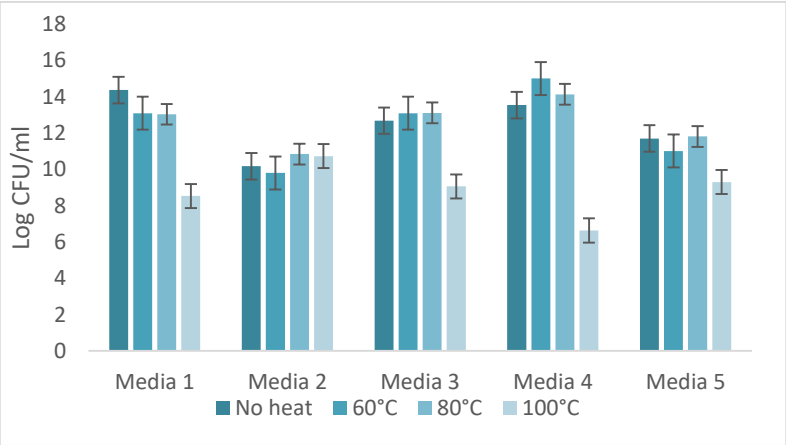

Fig 2: Spore count of *Bacillus licheniformis* under different heat shock conditions in five solidified media after seven days of incubation.

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In the case of broth media, the highest spore count, 15.94 Log CFU/ml, was observed when the spore was kept in the nutrient broth and sporulation broth for six days and seven days, respectively, under no heat shock conditions. The spore count was significantly less at the beginning of the incubation, and a high spore count was observed when there was nutrient depletion in the media. (Fig 3)

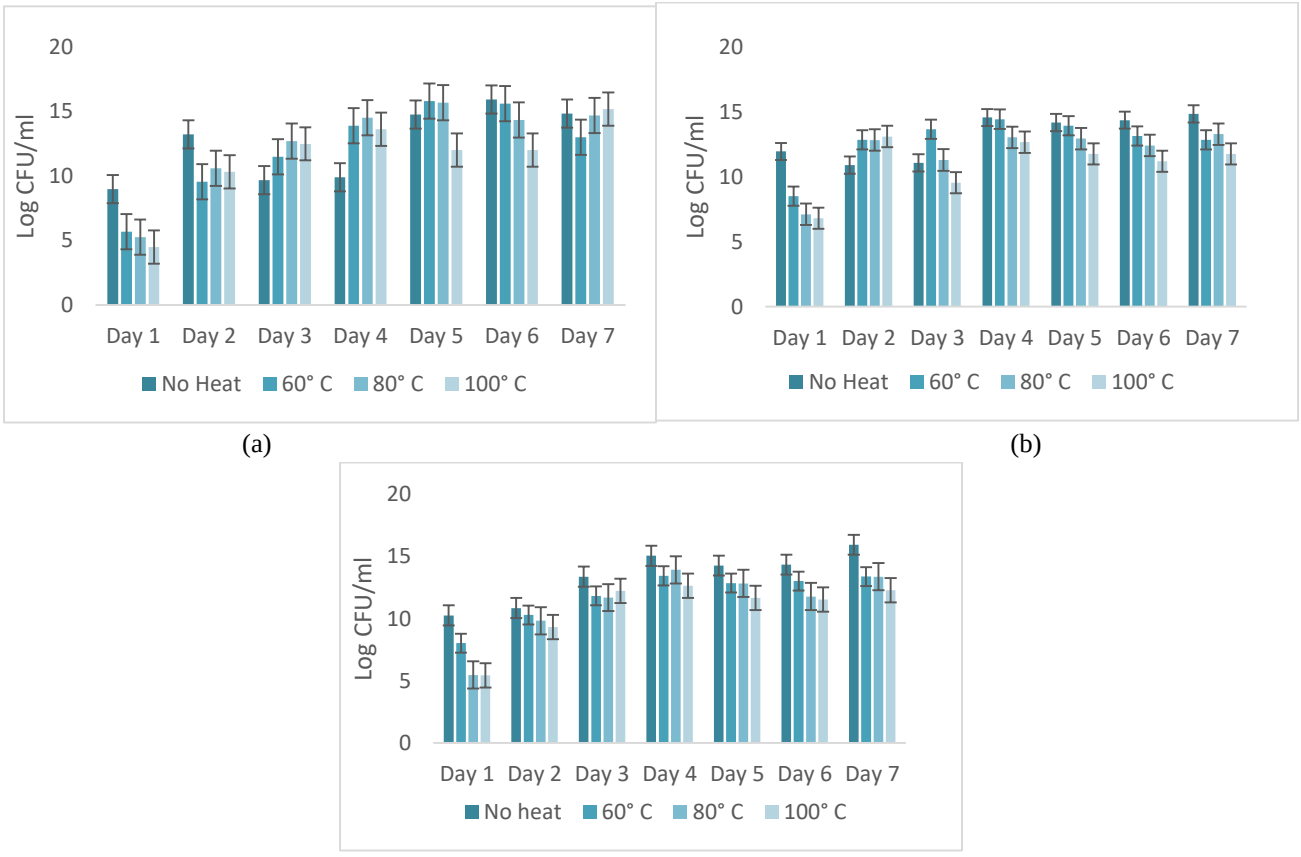

Fig 3: Spore count of *Bacillus licheniformis* at different time intervals from Day 1 to Day 7 under different heat shock conditions at (a) Nutrient broth, (b) Nutrient broth with magnesium sulphate, (c) Sporulation broth. However, as done for the broth media, harvesting of spores every 24 h was not attempted under solid media conditions.

Spore Percentage

The spore percentage was highest at AK Agar for the solidified media when heat treatment of 60°C was given for 30 minutes (110.78%) (Fig 4), whereas, for the broth media, the nutrient broth had the highest spore percentage when 80°C heat shock was given for 30 minutes (146.52%) (Fig 5).

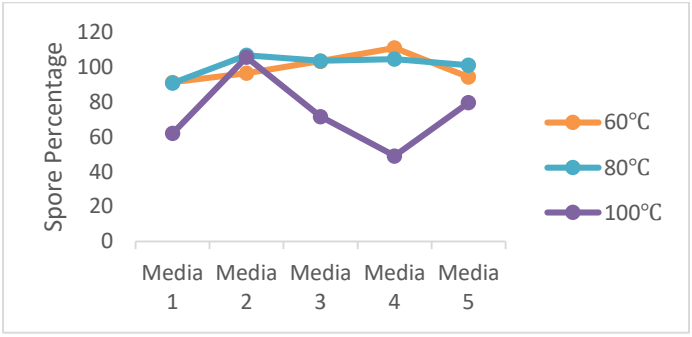

Fig 4: Spore Percentage of *Bacillus licheniformis* at five solidified media viz., Media 1, Media 2, Media 3, Media 4 and Media 5, under different heat shock treatments

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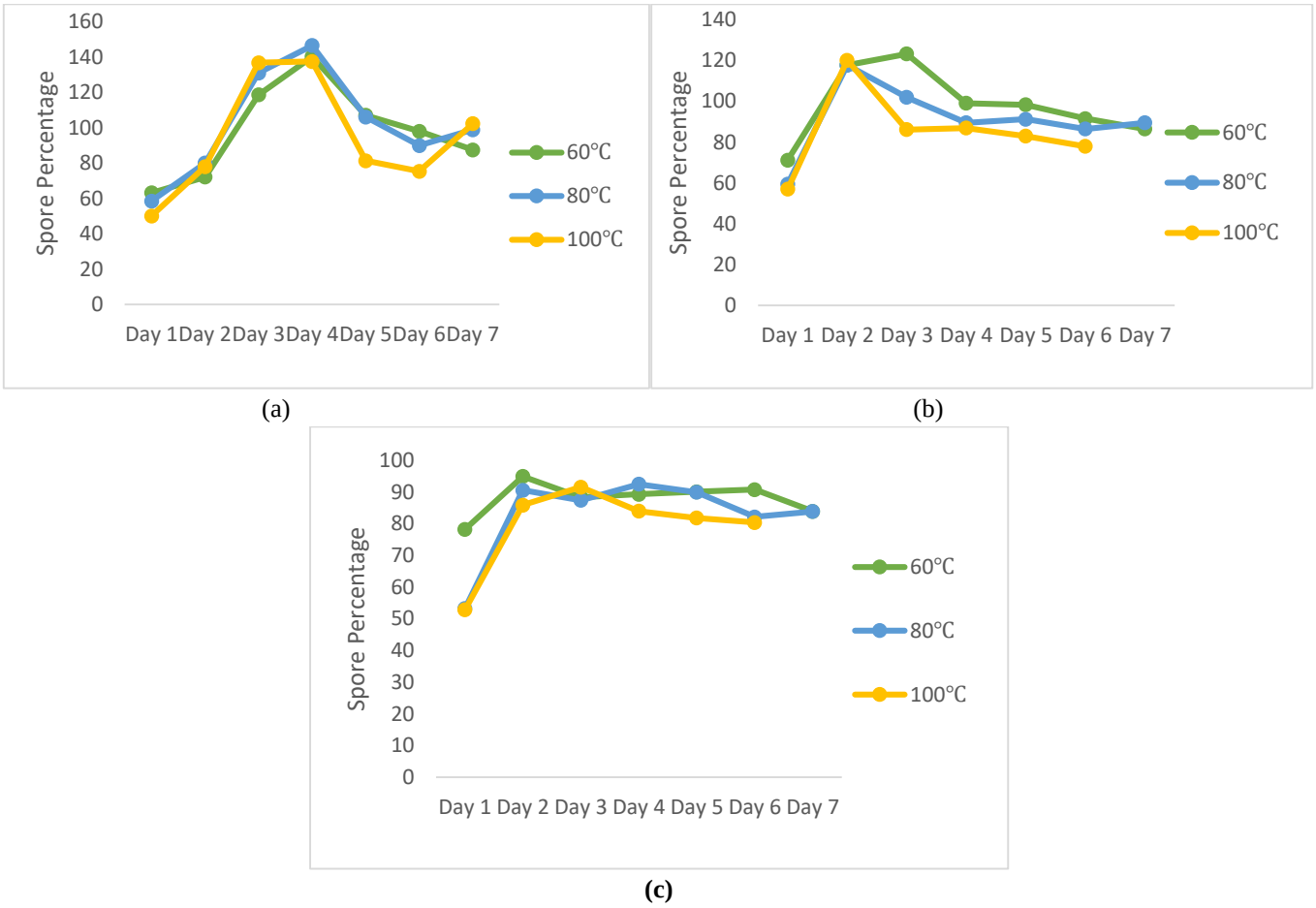

Fig 5: Spore Percentage of *Bacillus licheniformis* under different heat shock conditions at three different broth media (a) Nutrient broth, (b) Nutrient broth with magnesium sulphate, (c) Sporulation broth.

CONCLUSION

Among the five types of solidified media studied, AK Agar, exhibited the highest sporulation under a heat shock of 60℃ for 30 minutes and incubated for seven days, whereas, in the case of broth media, spores inoculated in Nutrient Broth under a heat shock of 80℃ for 30 min and incubated for four days produced the highest number of heat-resistant spores. Omer (2010) indicated that nutrient broth could be used as a basal medium for the generation of high spore yield [38]

Based on the above results, AK Agar and Nutrient Broth media can be selected for large-scale production of *Bacillus licheniformis* spores. The development of methods to effectively enhance the sporulation of Bacillus licheniformis may help in many industrial and research studies. Bacterial spores are generally used for industrial purposes because of their high survival rate when exposed to heat, radiation, freezing and chemicals. Some significant applications include probiotics, biodiesel, biopesticides, food processing, bioremediation, biofertilizer, biomedicine, biological warfare, biosensing and biosorption [1, 39, 40]. *Bacillus licheniformis* is a non-pathogenic bacteria which is not harmful to humans and the environment, hence the spores of this bacteria can be used as biopesticides, like the spores of *Bacillus thuringiensis*, which is used for controlling insects and pests. Bacterial spores are also used in improving concrete, a building material, by solving the problem of cracks, which is a major problem [1]. Spore-based biosensors can also be developed to detect contaminants in the environment. The exploration of bacterial spores is still in progress because of their wide application in various fields.

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ACKNOWLEDGEMENT

The authors are grateful to the North-Eastern Hill University NON-NET Fellowship and UGC Swami Vivekananda Single Girl Child Scholarship for financial assistance and MTCC, CSIR- Institute of Microbial Technology, Chandigarh, India, for supplying the microbial culture.

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Cite this Article: Moirangthem Bidyaswori Devi, Rhea Dhar, Atanu Bhattacharjee, Dibyendu Paul (2023). Screening of Different Media and Heat Shock Treatment Regimens for Enhancing Sporulation in Bacillus licheniformis. International Journal of Current Science Research and Review, 6(4), 2666-2674