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ORIGINAL ARTICLE

Plackett–Burman randomization method for Bacterial Ghosts preparation form *E. coli* JM109

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Abstract Plackett–Burman randomization method is a conventional tool for variables randomization aiming at optimization. Bacterial Ghosts (BGs) preparation has been recently established using methods other than the *E* lysis gene. The protocol has been based mainly on using critical concentrations from chemical compounds able to convert viable cells to BGs. The Minimum Inhibition Concentration (MIC) and the Minimum Growth Concentration (MGC) were the main guide for the BGs preparation. In this study, *Escherichia coli* JM109 DEC has been used to produce the BGs following the original protocol. The study contained a detail protocol for BGs preparation that could be used as a guide.

1. Introduction

Bacteria which lose their internal macromolecules have been given the name BGs if they have correct 3D structure. BGs found their way in many applications especially as a drug delivery system and in immunization (Kany and Curtiss, 2003; Jalava et al., 2002a,b).

E lysis gene for BGs preparation has been well established. Simply, *E* lysis gene follows the Bacteriophage strategy for lysis of the cells and in fact, it is one of the Φx174 phage genes (Hutchinson and Sinsheimer, 1966; Haidinger et al., 2003). But, using such genes might have some risk factors in applications concerning the humans. For that, Amara et al. (2013)

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have been introducing a protocol for BGs preparation using only chemical compounds. The protocol has been given the name "Sponge-Like" protocol (Amara et al., 2013). The protocol has been based mainly in chemical, physical, biological and statistical tools to enable mapping the best conditions for BGs preparation. For more details, refer to Amara et al. (2013) and the references therein. Microbes have many differences in their structure and responses to various conditions. Such diversity could be within the same species. For that differentiated strains might have some minor or major differences (Satyanarayana et al., 2005; Amara et al., 2012). For that, it is important to prove the effect of such strain differences on the BGs Sponge-Like protocol (BGsSLP). Experimental design is a powerful tool for optimizing different non-homogenous parameters and conditions. Plackett–Burman enables randomizing different variables aiming to get the best conditions where each variable coordinates with other variables to give the best-expected results (Plackett and Burman, 1946). This is mainly by using the maximum and minimum amounts of each variable in separate experiments. This ensures that each variable will be suitable to be used in its minimum or maximum value as well as the other variables. Plackett–Burman enable optimizing any type of variable if there is a possibility to put them together in one experiment and give one or more responses (results). Such an experiment could be run in one-step or more than one-step as described by Amara (2011). Simple tools could be used to conduct complicated target if the correct variables have been used or if successful alternative variables are used as well. Using cloned or genetically modified elements could have some risk factors. Genetic elements could by one or other way interact with others that pose a risk when any of them entered into our bodies (Makino et al., 1998). For that it is better to apply process used non-genetically based steps whenever that is possible, or insure the absence of the existence of any of the genetic materials. SDS and NaOH have been reported for their ability to interfere with the bacterial cell wall. H_2O_2 is well known for its ability, as an oxidant that degrades the DNA (Amara et al., 2013). This study aims to prepare the BGs from *Escherichia coli* JM109 using a Sponge-Like protocol for BGs preparation. A model for the expected effect has been included. Plackett–Burman, MIC, MGC, and light and electron microscope were used for the BGs preparation. The protocol has succeeded to prepare the BGs and the study included statistical and logical analysis for the data.

2. Materials and methods

2.1. Bacterial strain

E. coli recombinant cells JM109 (Promega) was used in this study. The *E. coli* genotype is: endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F′, traD36, proAB, laqI q ZΔM15] (Yanish-Perron et al., 1985; Jalava et al., 2002a,b).

2.2. BGs preparation protocol

2.2.1. Determination of the MIC and MGC for NaOH, SDS, and H_2O_2

Standard experiment for determining the MIC for each of NaOH, SDS, and H_2O_2 was conducted (Andrews, 2001). The MIC value for each compound was calculated as well as the concentration which allows first bacterial growth which is abbreviated as MGC (the concentration which shows first growth after the MIC (Amara et al., 2013)). Only $CaCO_3$ MIC and MGC were not determined and had to be used in quantities equal to +1 which was 1.05 μg/mL while −1 was 0.35 μg/mL as described by Amara et al. (2013).

2.2.2. Bacterial biomass preparation, collection and cleaning

The *E. coli* JM109 was cultivated in a one liter flask containing 500 mL NB under static conditions. The bacterial cells have been allowed to grow for 72 h at 37 °C. After that the biomass has been collected using centrifugation at 4000 rpm/min, and washed with 0.5% saline. The cells biomass was then collected and re-suspended in distilled water to give a final concentration equal nearly 10^6 CFU/mL.

2.2.3. Preparation of the +1 (MGC) and −1 (MIC) values for each variable

Five time concentrations of each of the calculated MGC and the MIC have been prepared for each of the used chemical compounds which represent NaOH, H_2O_2, and SDS. Five times $CaCO_3$ has been prepared from the quantities as described above.

2.2.4. Plackett–Burman design

Twelve experiments representing the Plackett–Burman design have been designed to conduct the randomization step as in Table 1 and to a correctly enable the regression analysis. Each experiment contains only either the +1 or −1 value of each variables. However, it contains an entirely different variable represented by +1 or −1. None of the twelve experiments is similar to the other All the twelve experiment constituents have been prepared by using one ml of each of the five-concentration stocks of NaOH, and SDS and $CaCO_3$ and finally one ml taken from the bacterial biomass was added as well as 1 ml of H_2O to each preparation to reach a final concentration equal to 1x. H_2O_2 has been used in separate step with concentration equal to 1x/ml. The fifth variable was representing two different physical parameters combined to each other to represent one variable; they are the shaking rate and the temperature. The +1 of the fifth variable represents 37 °C and 100 rpm while the −1 represents 30 °C and 50 rpm. The fifth variable represents the physical condition of the BGs preparation.

2.2.5. The BGs preparation experiment steps

The BGs preparation was conducted in three steps: The first step contains all the variables except H_2O_2. The BGs first mixture has been incubated for 1 h either at 30 °C and 50 rpm or at 37 °C and 100 rpm in the presence of each of the SDS, NaOH and $CaCO_3$. After that the cells of each of the twelve experiments have been collected using centrifugation at 4000 rpm/min followed by saline/water washing, and re-centrifugation at 4000 rpm. After the collection of the pellet for each experiment, the cells were washed by 0.5% saline. Then re-centrifuged and re-suspended in 1 ml H_2O to readjust the bacterial volume to its original volume (1 ml). The second step contains only H_2O_2. One ml of the bacterial subsection has been diluted by adding three ml of distilled H_2O followed by adding 1 ml of H_2O_2 and then incubated statically or using shaking as above. After that the cells of each of the twelve experiments have been

Table 1 Plackett–Burman experimental design.

Exp. No.	SDS	H_2O_2	$CaCO_3$	NaOH	Shacking rate–Temperature	Basic experiment Protein mg/ml	Basic experiment DNA mg/ml	H_2O_2 Protein mg/ml	H_2O_2 DNA mg/ml	Ethanol Protein mg/ml	Ethanol DNA mg/ml	BGQ
1	1	1	1	–1	1	2.6	0.16	0.19	0.012	0.06	0.027	10
2	–1	–1	–1	–1	–1	2.5	0.10	0.14	0.003	0.16	0.013	10
3	1	–1	1	1	–1	2.9	0.95	0.09	0.003	0	0.067	8
4	–1	–1	–1	1	1	2.6	0.07	0.11	0	0.32	0.17	10
5	–1	1	1	1	–1	1.8	0.10	0.31	0.023	0	0.037	10
6	1	1	–1	1	–1	4.2	0.12	0.26	0.028	0.03	0.088	10
7	–1	1	–1	–1	–1	2.1	0.07	0.84	0.045	0.1	0.0345	10
8	–1	–1	1	1	1	2	0.12	0.15	0.005	0.03	0.0119	8
9	1	1	–1	1	1	2.9	0.08	0.79	0.038	0.04	0.038	10
10	–1	1	1	–1	1	2.1	0.11	0.1	0.045	0.12	0.0145	10
11	1	–1	–1	–1	1	4.2	0.09	0.38	0.27	0.07	0.006	10
12	1	–1	1	–1	–1	2.1	0.11	0.29	0.008	0	0.016	0

collected using centrifugation at 4000 rpm/min followed by saline/water washing, and re-centrifugation. Finally, the cell pellets were re-suspended in 60% Ethanol and left at room temperature for 30 min with gentle vortexing each 5 min for 30 s. The cell pellets were then collected and washed with distilled water as above. Then, after washing the cells were re-centrifuged and the supernatant for each was discarded and the wet cell used for either the light or electron microscopes examination.

2.2.6. Determination of the DNA concentration

The concentration of the DNA was determined by measuring the absorption at 260 nm. Quartz cuvette was used. Extinction corresponds to 50 μg dsDNA mL^{-1} (Sambrook et al., 1989; Amara et al., 2013).

2.2.7. Determination of the protein concentration

Protein analysis of each experiment (the different supernatants) was determined using the spectrophotometer at 280 nm. Quartz cuvette was used. Different concentrations of protein were derived from Bovine Serum Albumin standard curve (Sambrook et al., 1989; Amara et al., 2013).

2.2.8. BGs evaluation using light microscope

Bacterial smear for each treatment was prepared using standard criteria. Then the smears have been stained using crystal violet. The cells from each experiment were investigated by the aid of the light microscope. The quality of the cells has been determined based on the bacterial structure as either being correct or deformed. BGQ has given a number out of 10. 10 means that all the ten tested cells are correct BGs.

2.2.9. Sample preparation for electron microscope examination

For further study as to the quality of BGs, electron microscope was used to scan the bacterial cells. Dry bacterial smear for each preparation was prepared and the smear surface then coated with about 15 nm gold (SPI-Module Sputter Coater).

2.2.10. Scanning of the BGs surface

The golden-coated sample then has been scanned by analytical scanning electron microscope (Jeal JSM-6360LA) with secondary element at 10 kv acceleration voltage at room temperature. The digital images then were adjusted to 8500x and saved.

2.2.11. Determination of E. coli viability

The various BG preparations were investigated for the possibility of the presence of any viable cells by subjecting them to grow in NA plates, where 25 μL from each sample was transferred to the surface of NA. The plates then were incubated at 37 °C for 5 days (Amara et al., 2013).

2.3. Statistical analysis of the data from the Plackett–Burman design

2.3.1. Determination of the main effect of the five used variables

The experimental design using Plackett–Burman method was produced using +1 and −1 for each variable as in Table 1 where twelve experiments have been conducted. The results are summarized as BGQ. The mean of +1 experiments has been calculated using the following formula: $(\sum +1)/n_{(+1)}$. While the mean of −1 experiments has been calculated using the following formula: $(\sum -1)/n_{(-1)}$. The main effect of both of +1 and −1 for each variable has been calculated from the following formula: $\textbf{Main effect} = \sum(+1)/n_{(+1)} - \sum(-1)/n_{(-1)}$. The different main effects of the different variables for BGQ have been summarized in Table 2. In the case of BGQ using Plackett–Burman design under the various experiments, the values that appear upper to the *x*-axis in the main graphs as in Fig. 1 have positive effects on the BGQ, while those that appear under the *x*-axis have negative effects on the BGQ.

2.3.2. Multiple linear regression analysis and ANOVA test of Plackett–Burman design

The results of the Plackett–Burman design experiments were applied to linear multiple regression analysis using Microsoft Excel 2002. The linear multiple regression analysis was conducted for the twelve experiments in Table 1 and the BGQ has been taken as the response (dependant variable). The statistical analysis of the data in Table 1 has been summarized in Table 3. The variables whose confidence levels % were ⩾ 90% were considered to significantly affect the BGQ. Variables with confidence level% less than 90% till 70% were considered as

being effective (Stowe and Mayer, 1966). While the Plackett–Burman analysis has been performed on the BGQ as responses, a multiple linear regression analysis for the data of the BGQ has been performed to study the relationship between different variables and their level of significance regarding to BGQ as a response. From the analysis of the Coefficient, Standard error, *T* Statistic, *P*-value and Confidence level % for each has been calculated as in Tables 3. The confidence level has been calculated from the formula ***The confidence level% = 100*(1 − P-value).*** The *P*-value from the ANOVA analysis for BGQ response was determined to analyse the relationship between the variables at the 90% or higher confidence level as in Table 4.

2.3.3. Generating 1st order-model

The model created from the analysis of Plackett–Burman experimental design using multiple regression analysis is based on the 1st order-model $\boldsymbol{Y = \beta 0 + \sum \beta i\ Xi}$.where *Y* is the predicted response, β_0 model intercept, β_i variables linear coefficient. ANOVA test was generated for each response to determine the relationship between the variables at the 90% or higher confidence level.

3. Results and discussion

BGs drew increasing interest in the recent years particularly aiming to be used as an immunostimulant or as a drug delivery carrier. For that, a method based on *E* lysis gene has been established. The *E* lysis gene is coded by a temperature sensitive promoter, so at the correct time after reaching the critical biomass, the temperature of the viable cells is adjusted and for that, the internal component of the cells are lysed and the cells turn to be unviable or BGs. However, using such genetic based tools might still have some type of risk. Different genetic elements could by one or other routes be passed to our genetic material and might be harmful. For that, it is important to find alternative methods for BGs preparation not based on the use of the genetic elements. Amara et al. (2013), and for the first time introduce a fully described and optimized protocol for BGs preparation. The protocol is based on using different tactics for reaching the target of the BGs. The cells have been aged to give a thicker cell wall. The chemicals have been used in two concentrations showing minimum effect on the bacterial cells. They are the MIC and the MGC. Moreover, Experimental Design tools which are represented in Plackett–Burman have been used to map the best conditions and guarantee the best production for the BGs. The BGQ is evaluated using light and electron microscope as in Fig. 2. In this study, precisely, we follow the original protocol for preparing the BGs from another *E. coli* strain the *E. coli* JM109. The BGs preparation that has been summarized in a protocol enable better propagation upon following or those conducted by any. *E. coli* JM109 prove to be more sensitive to SDS than *E. coli* BL21 (DE3), where the (+1, −1) values were 0.24 mg/mL and 0.03 mg/mL of SDS respectively. The MIC and MGC of *E. coli* JM109 for each of the NaOH and H_2O_2 have been as same as those of *E. coli* BL21 (DE3) and are represented by 0.0138 N and 0.00231 N (+1, −1) for NaOH and 40.8 μL/mL and 5.83 μL/ml (+1, −1) from 30% H_2O_2 for H_2O_2 respectively. This might be an indication about the cell wall variation; a tool that might be used in future for the determination of the competent cells rigidity and transferability. In the case of $CaCO_3$ the used amount of +1 value was 1.05 μg/mL while −1 value was 0.35 μg/mL. The twelve experiments which contain either the +1 or −1 value for each variable in each experiment in random arrangement have been conducted at the same time to get the best results and to enable the best possible comparison. The BGQ has been given for the 100% quality as 10, while ten cells have been evaluated as either bad or good. This will decrease the range of the

Table 2 Main effect of each variable.

Variable name	Main effect = $\sum(+1)/n_{(+1)} - \sum(-1)/n_{(-1)}$
SDS	−1.667
H_2O_2	2.3333
$CaCO_3$	−2.333
NaOH	1
Shacking rate and Temperature	1.6667

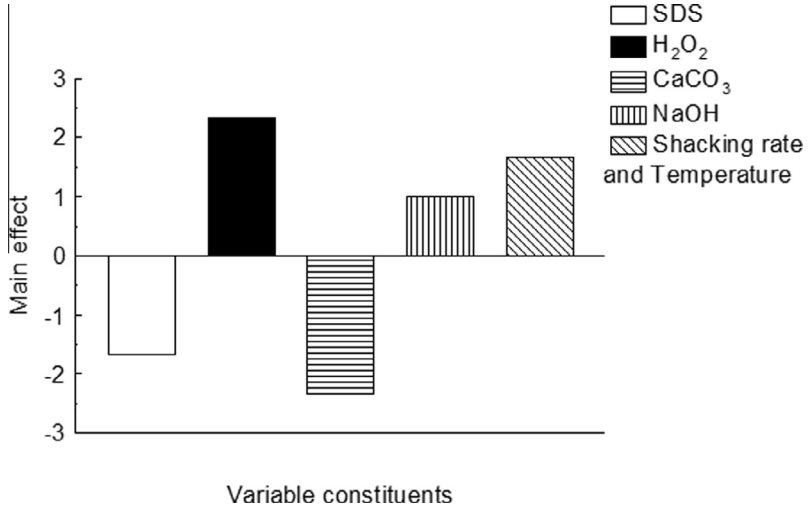

Figure 1 Main effect of the five used variables.

Table 3 Logical analysis for the BGQ obtained from the experiment number twelve using each of experiments numbers, one, two and three.

Exp. No.	SDS	H_2O_2	$CaCO_3$	NaOH	Shacking rate-Temperature	Basic experiment Protein mg/ml	Basic experiment DNA mg/ml	H_2O_2 Protein mg/ml	H_2O_2 DNA mg/ml	Ethanol Protein mg/ml	Ethanol DNA mg/ml	BGQ (Good out of 10 cells)
1	1	1	1	−1	1	2.6	0.16	0.19	0.012	0.06	0.027	10
2	−1	−1	−1	−1	−1	2.5	0.10	0.14	0.003	0.16	0.013	10
3	1	−1	1	1	−1	2.9	0.95	0.09	0.003	0	0.067	8
12	1	−1	1	−1	−1	2.1	0.11	0.29	0.008	0	0.016	0

differences if we use %. Unexpectedly, *E. coli*, which is more sensitive to the SDS than *E. coli* BL21 (DE3) gives better results with most of the experiments. Nine experiments give the number 10 out of the twelve experiments. Two give the number eight and only one gives the number 0 which means very poor preparation. In experiment twelve which give the number 0 SDS and $CaCO_3$ have been used in the +1 value. It is clear that SDS and $CaCO_3$ might coordinate to damage the cell wall. Logical analysis of the differences was done and analysed why experiment twelve gives 0 quality BGs? To understand what happened to make the cells in experiment twelve completely damaged a special comparison between the experiment twelve and experiment one and three has been generated and extracted from the main Plackett–Burman Table 1 and summarized in Table 5. In experiment twelve, SDS and $CaCO_3$ are the only factors that might affect the cells severely in quality while they have been used in their +1 value. In experiment number three SDS, $CaCO_3$ and NaOH have been used in their +1 which might be responsible for the loss in the quality. In experiment number one NaOH has been used in −1 which might be responsible for obtaining the highest quality score. If similar variables in experiment one and three compared with experiment twelve are ignored, one variable (+1 H_2O_2) in experiment one and two variables in experiment three (+1 NaOH and −1 Shacking rate–Temperature) are still different. It must be that, low temperature and shaking rate in the presence of +1 SDA has a negative effect on the bacterial ghost preparation in the condition of experiment twelve. H_2O_2 (−1) if used in the condition of experiment number twelve will also reduce the BGs quality. Additionally, low temperature and shaking rate might enhance SDS (if represented in high amount +1) to damage the cells. For doing more unbiased analysis, the Main effect of the variables have been determined as in Fig. 1. The main effect clearly supports our argument in the logical analysis of the data. Clearly, H_2O_2, NaOH and Shaking rate–temperature positively affect the cells quality when used in higher concentrations SDS proved in the experiment number twelve that it is able to negatively affect the BGs quality. $CaCO_3$ does the same but in a stronger way as in the above Figure. In the previous study made by Amara et al. (2013) who used the same tools each of the NaOH and Shaking rate–Temperature negatively affects the BGs quality. Here and unexpectedly, they are positively affecting the BGs quality. That might explain the fact that both *E. coli* strains are different in their behaviors and responses to various treatments and that *E. coli* JM109 is more sensitive to the changes in the chemical compounds as proved by the MIC and MGC in a positive way. It seems that JM109 might have more ability to neutralize the effect of the SDS if a larger shaking rate has been used. This might enable faster release of the internal protein content, which will react with the SDS and neutralize it. But in the case of low temperature and shaking rate it might be that this condition makes the cells unable to get rid of their protein content and the level of the SDS remains high and continues in its attack on the cell wall. Low amount of protein reading using a spectrophotometer might be apparently measured in the case of higher temperatures and shaking rate (even expected high release of the protein) might be due to that the SDS-Protein complex is not detectable at 280 nm or precipitated. An observation must be investigated in future studies. Meanwhile, one should compare our data in this study with those in Amara et al. (2013), for a clearer image about the effect of various

Table 4 Linear multiple regression analysis of Plackett–Burman.

Parameter	Estimate	Standard error	Statistic	*P*-Value	Confidence level %
Constant	8.83333	0.739119	11.9512	0.0000	100
$CaCO_3$	−1.16667	0.739119	−1.57846	0.1655	83.45
H_2O_2	1.16667	0.739119	1.57846	0.1655	83.45
NaOH	0.5	0.739119	0.676481	0.5239	47.61
SDS	−0.833333	0.739119	−1.12747	0.3026	69.74
Shacking rate and temperature	0.833333	0.739119	1.12747	0.3026	69.74

R-squared = 57.0909 percent; *R*-squared (adjusted for d.f.) = 21.3333 percent; Standard error of Est. = 2.56038; Mean absolute error = 1.38889; Durbin–Watson statistic = 1.54237 (*P* = 0.2872); Lag 1 residual autocorrelation = −0.0451977.

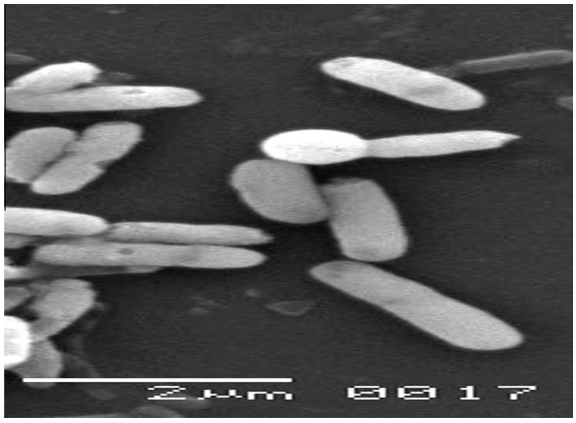

Figure 2 Scanning electron microscope for *E. coli* BG cells.

used chemical compounds on the different *E. coli* strains used. Main effect analysis, is a simple but a powerful tool for determining which variables positively and which negatively affected the BGs quality.

4. Multiple regressions – BGQ

One dependent variable, the BGQ and five independent variables, $CaCO_3$, H_2O_2, NaOH, SDS and Shaking rate + Temperature have been subjected to multiple regression analysis. The multiple linear regression models describe the relationship between BGQ and the five independent variables. The equation of the fitted model is: BGQ = 8.83333–1.16667*$CaCO_3$ + 1.16667*H_2O_2 + 0.5*NaOH − 0.833333*SDS + 0.833333*(Shaking rate + Temperature).

The *R*-Squared statistic indicates that the model as fitted explains 57.0909% of the variability in BGQ. The adjusted *R*-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 21.3333%. The standard error of the estimate shows the standard deviation of the residuals to be 2.56038. The mean absolute error (MAE) of 1.38889 is the average value of the residuals. The Durbin–Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. Since the *P*-value is greater than 0.05, there is no indication of serial autocorrelation in the residuals at the 95.0% confidence level. As in the multiple regression analysis table of the data, $CaCO_3$ and H_2O_2 are clearly effective factors on the BGs preparation with

Table 5 ANOVA test.

Source	Sum of squares	Df	Mean square	*F*-Ratio	*P*-Value
Model	52.3333	5	10.4667	1.60	0.2912
Residual	39.3333	6	6.55556		
Total (Corr.)	91.6667	11			

confidence levels equal to 83.45. However, $CaCO_3$ is negatively effective while H_2O_2 is positively effective. This agrees with the Main effect analysis as well as the logical analysis of the data. SDS, which has a confidence level nearly equal to 70%, might also be considered as effective. SDS negatively affects the BGs quality. The same could be taken into consideration for Shaking rate and Temperature but it positively effects the BGs preparation. Shaking rate and Temperature in +1 might coordinate with other variables to improve the BGs quality as described also in the logical analysis of the data. NaOH is an insignificant factor with the lower confidence level % which might be due to some unique properties for the *E. coli* JM109 or due to the type of the overall reaction which might by one way or other interfere with the NaOH effect. The ANOVA test and since the *P*-value in the ANOVA Table is greater or equal to 0.05, there is not a statistically significant relationship between the variables at the 95.0% or higher confidence level. For that, Plackett–Burman might be a correct choice while it could randomize those variables collectively, to give the best formula that could guarantee the best BGs preparation under the experimental conditions.

Since the *P*-value in the ANOVA Table is greater or equal to 0.05, there is not astatistically significant relationship between the variables at the 95.0% or higher confidence level.

5. Conclusion

BGs have many useful applications. For that, it is a subject for different kinds of investigation aiming at better preparation and better use. In vivo use might require materials free of genetic elements. For that, a chemical and physical protocol has been introduced by Amara et al. (2013) and given the name "Sponge-Like protocol for BGs preparation". In this study another *E. coli* has been investigated, the JM109. The used strain proved more suitable for BGs preparation and proved the strain variation within similar microbes from the same species. JM109 is more sensitive to the SDS than BL21 as proved in this study. Unexpectedly, NaOH and Shaking rate + Temperature have positive effects on the BGQ which disagree with the

data obtained from BL21 Amara et al. (2013). Plackett–Burman design proves to be a powerful tool for optimizing inhomogeneous variables. The study is an additional step toward understanding the conditions for the preparation the BGs using chemical compounds and conditions could lead to the release of the cells constituents without deforming the cell wall 3D structure. Such a safe protocol could open the way for safe applications using BGs.

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