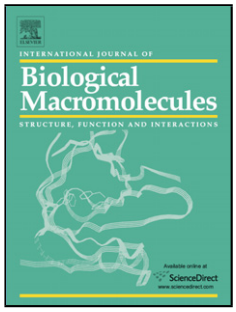

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Optimization of rhamnolipid production by biodegrading bacterial isolates using Plackett-Burman design

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Abstract
Biosurfactants are biological surfactants produced by microorganisms. *Pseudomonas* species are well known for the production of the rhamnolipid biosurfactant. In this work, the production of rhamnolipid biosurfactant by *Pseudomonas* spp. was investigated and further optimized. Two Plackett-Burman designs to study the effect of carbon source, nitrogen source, C/N ratio, iron concentration, magnesium concentration, phenol toxicity, pH, temperature, agitation and sampling time were tested. The first design revealed an optimization that increased biosurfactant productivity by almost two to five folds for the tested isolates. However, using the second design showed no remarkable increase in biosurfactant productivity. An additional validation run was adopted using the predicted optimal medium with predicted optimal conditions. The validation run showed remarkable increase in the productivity of the tested isolates.
The use of microorganisms with biodegradation ability coupled with optimization of the parameters affecting productivity provide an efficient strategy for biosurfactant production.

Keywords: Multifactorial; Plackett-Burman; Rhamnolipid

1. Introduction

Biosurfactants are biological surfactants produced by different microorganisms as bacteria, yeasts and fungi. They are totally or partially extracellular amphipathic polymers containing polar and non-polar moieties. This amphipathic property allow

them to accumulate at interface of liquids with different polarities as water and oil, hence, reduce surface tension and facilitate hydrocarbon emulsification [1].
In comparison with synthetic surfactants, biosurfactants have many features which have made them gain more and more attention. The superior properties of biosurfactants include high biodegradability, low toxicity, ecological acceptability, low cost, selectivity and high specific activity at extreme temperatures, pH and salinity [2]. On the other hand, synthetic surfactants currently used are toxic and are hardly degraded by microorganisms causing damage to the environment [3]. Based on these properties, biosurfactants provide environmentally friendly green alternatives to chemical surfactants used in many industrial and medical applications. Biosurfactants have applications in many fields including environmental remediation processes, food industry, pharmaceutical and cosmetic industry and also they play an important role in enhanced oil recovery, mining and metallurgical industries [4].
Rhamnolipids are glycolipid biosurfactants that have been extensively investigated in the literature [5]. Rhamnolipid biosurfactant is commonly known to be produced by *Pseudomonas* species when grown on various substrates [4], hence they can be considered as promising candidates for large scale production of biosurfactants. Coping with the flow of green chemistry and creating an ecological friendly society, this study aims to use multifactorial Plackett-Burman design in an attempt to optimize the production of rhamnolipid biosurfactant by two biodegrading *Pseudomonas* strains. Since successful bioprocessing will occur only when all the essential factors are drawn together, the proper optimization of biosurfactant production by biodegrading microorganisms would provide an interesting strategy for the ecological practice of modern industrial processes.

2. Materials and Methods:

Unless otherwise specified, all experiments were conducted under aseptic conditions and in triplicates. The represented data equal mean ± standard error of conducted replicates.

2.1 Microorganisms

A total of ten biodegrading bacterial isolates were previously investigated for dual property (biodegradation and biosurfactant production) [6], from which two isolates (M2H2 1 and M2H2 14) were selected for further investigation in the present study. The two isolates were molecularly identified by 16S rDNA gene sequencing to belong to *Pseudomonas* species, and particularly *Pseudomonas aeruginosa*. Interestingly M2H2 1 and M2H2 14 tolerated high phenol concentrations up to 1500 and 1300 mgl^{-1} respectively, and both tolerated cyclohexanol up to 1000mgl^{-1}. Moreover, high concentrations of rhamnolipids biosurfactant reaching 15.2 and 10.7 rhamnose gl^{-1} by isolates M2H2 1 and M2H2 14, respectively, were achieved. Therefore, these two isolates were considered as promising candidates for optimization of biosurfactant production in the present study.

2.2 Medium and culture condition

The basal medium used for biosurfactant production was mineral salt medium (MSM) [7], containing: K_2HPO_4, 4 g; KH_2PO_4, 4 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4.7H_2O$, 0.5g; $CaCl_2$, 0.01g and $FeSO_4 \cdot 7H_2O$, 0.01g per one liter distilled water. Erlenmeyer flasks of 250 ml volume, containing 100 ml MSM with 2% (w/v) coconut oil as substrate were inoculated by 5% (v/v) of bacterial culture suspension (10^6-10^7 CFU ml^{-1}) and incubated for 6 days at 180 rpm and 37 °C.

2.3 Characterization of produced rhamnolipid

The glycolipid nature of the produced rhamnolipid was assessed using Blue agar plates Method [8], concurrently, the concentration of the sugar moiety in rhamnolipid was assayed by rhamnose test [9]. Isolates were also molecularly screened for genes involved in rhamnolipid synthesis by PCR. The screened genes were rhamnosyltranseferase chain A gene (*rhlA*), transcriptional regulator gene (*rhlR*) and auto-inducer synthesis protein (*rhlI*) [5, 10].The sequences of primers used were 5'CGAGACCGTCGGCAAATA3' & 5'GACTCCAGGTCGAGGAAATG3' with PCR fragment size 294 bp, 5'GCGATACCAGATGCAGAACTAC3' & 5' GCTCAGGATGATGGCGATTT3' with PCR fragment size 409 bp and 5' GACCAGGAATTCGACCAGTT3' & 5' TAGCGCAACAGCATCTCC3' with PCR fragment size 431 bp for genes *rhlA*, *rhlR* and *rhlI* respectively.

2.4 Optimization of rhamnolipid production

Plackett-Burman experimental design was applied for optimization of rhamnolipid production by isolates M2H2 1 and M2H2 14. Plackett-Burman experimental design is an efficient statistical technique to demonstrate the relative importance of the studied factors and predict their optimum values [11, 12]. Ten independent factors were included in the design. Factors affecting the medium composition were carbon source, nitrogen source, C/N ratio, iron concentration and magnesium concentration. Factors affecting physiological condition were pH, temperature and agitation rate. Also sampling time and toxicity factor (phenol concentration) were included in the design. For each factor, a high (+) and low (-) level was tested. The design included 12 runs of various combinations of levels of the assessed factors, in addition to a 13^{th} run under the basal conditions. All runs were done in triplicates and a total of 39 runs were applied according to the Plackett-Burman design matrix (Tables 1&2).The optimization of rhamnolipid production was done by applying two designs by which in design A different carbon sources were assessed (Table 3), while in design B, the percentage of the used carbon source was assessed (Table 4). In design A, the response measured was biosurfactant activity by emulsification assay expressed as emulsification unit/ml ($EUml^{-1}$), whereas in design B, the response measured was biosurfactant productivity by rhamnose test expressed as concentration gl^{-1}.

2.5 Analysis of production and activity

2.5.1 Blue agar plates Method (CTAB Agar Plate)

The blue agar plates were prepared by adding 0.2 g cetyltrimethylammonium bromide (CTAB) and 0.005 g methylene blue (MB), 15 g agar and 20 g glycerol as

a substrate to 1 liter of the MSM [13]. Shallow wells were cut on the agar plate surface [14], where 10 µl of the inoculum were added. The plates were incubated for 48 h at 37 ^{o}C and then stored in the refrigerator for at least 24 h. Formation of productive colonies, surrounded by dark blue halos indicates positive results. Examination using UV transilluminator was used to light the plates for easier detection [8].

2.5.2 Rhamnose Test

Rhamnolipids concentration was assayed by colorimetric method as rhamnose content (gl^{-1}) using the method of Dubois et al 1956 [9]. A volume of 0.5 ml of cell supernatant was mixed with 0.5 ml of 5% phenol solution and 2.5 ml of sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm. Standard curves were prepared with L-rhamnose.

2.5.3 Emulsification Assay

Aliquot of 3 ml of the supernatant was vortexed with 0.5 ml castor oil for 2 min. The mixture was left undisturbed for 1 h. at 28 ± 3 ^{o}C to separate aqueous and oil phases. The aqueous phase was collected and measured at 400 nm using spectrophotometer (T80 uv/vis spectrophotometer, USA). The units of the emulsification activity were calculated according to the following equation [15].

EU ml^{-1} = absorbance at 400 nm x dilution factor / 0.01

2.6 Statistical analysis Statistical analysis and graphical presentation of data was done using Minitab® software (version 16.1.0).

3. Results

3.1 Characterization of produced rhamnolipid

The nature of the produced biosurfactants was preliminary identified to be glycolipid, where both isolates showed dark blue haloes in the CTAB agar plates indicating the production of rhamnolipid (Fig. 1). This was further confirmed when both isolates M2H2 1 and M2H2 14 showed positive reactions in the PCR for the genes involved in rhamnolipid synthesis (Fig. 2).

3.2 Optimization of rhamnolipid production

The two Plackett-Burman experimental designs were applied and the values of measured responses were recorded (Tables 1&2). For both designs, the standardized effect of each factor (E value) was calculated using minitab software, the magnitude of the "E" value of the tested factor indicate its effect or its significance in affecting the response[12], while the positive and the negative sign of the "E" value are indicative of its positive and negative influence on the responses respectively. The standardized effects of magnitude and significance for each factor of both designs are shown in Pareto charts (Fig. 3). Run no. 13 represents the basal medium composition under the basal conditions. Isolates M2H2 1 and M2H2 14 recorded emulsification activity of 337 and 138 Emulsification Unit/ml ($EUml^{-1}$) and rhamnolipid production of 15.2 and 10.7 rhamnose gl^{-1}, respectively, using the basal medium under the basal conditions.

Interestingly, Isolate M2H2 1 showed remarkable increase in emulsification activity in runs no. 1 &11 of design A by almost 2 fold increase and recording 528 and

593EUml^{-1} in both runs respectively. Even better enhancement in emulsification activity was achieved by isolate M2H2 14 in runs no. 1, 3, 5, 10 and 12 of design A by almost 5 fold increase and recording up to 691 EU ml^{-1} (run no 5).

Based on design A, run no. 11 had the best enhancement in emulsification activity by isolate M2H2 1 and was of the following medium composition; glycerol, 20g; K_2HPO_4, 4 g; KH_2PO_4, 4 g; $NaNO_3$, 4.74 g; $MgSO_4.7H_2O$, 1g; $CaCl_2$, 0.01g; $FeSO_4 \cdot 7H_2O$, 0.001 g and phenol, 0.1g per 1000ml distilled water, at pH 8 and incubation at 42°C and 110 rpm. On the other hand, as per isolate M2H2 14, run no. 5 recorded the highest emulsification activity with medium composition of; glucose, 20g; K_2HPO_4, 4 g; KH_2PO_4, 4 g; $NaNO_3$, 4.85 g; $MgSO_4.7H_2O$, 0.05g; $CaCl_2$, 0.01g; $FeSO_4 \cdot 7H_2O$, 0.05 g and phenol, 0.5g per 1000 ml distilled water, at pH 8 and incubation at 30°C and 110 rpm. For isolate M2H2 1, the significant factors affected emulsification activity were phenol concentration and pH (Fig. 3-A), where for higher emulsification activity low concentration of phenol and high pH are favored (Fig. 4-A). As it appears in (Fig. 5-A) that the highest emulsification activity of 593 EUml^{-1}was achieved at phenol concentration of 100mgl-1 and pH 8 (Fig. 5-A). As for isolate M2H2 14, the same factors (phenol concentration and pH) also significantly affected emulsification activity, however, stronger significance was recorded by temperature, iron concentration, magnesium concentration and C/N ratio on emulsification activity as was observed (Fig. 3-B). Temperature and iron concentration had the highest significance on emulsification activity by isolate M2H2 14, where their relationship was observed in (Fig. 5-B), emulsification activity of 691 EUml^{-1} was recorded at the low temperature (30°C) and high iron concentration (0.05gl$^{-1)}$.

On the contrary, in design B, none of the 12 runs showed remarkable increase in rhamnolipid productivity than the basal run with both isolates. Temperature, pH and agitation rate were the significant factors affecting rhamnolipids production by isolates M2H2 1 and M2H2 14 (Fig. 3-C & 3-D). The highest rhamnolipids production by both isolates was achieved at low temperature (30°C), high pH (8) and high agitation rate (250 rpm) (Fig 4-C & 4-D). Consequently, additional validation run was adopted using the predicted optimal medium under the predicted optimal conditions. Based on the results obtained from design B, two optimal media were predicted, media MMH1 and MMH14 for isolates M2H2-1&M2H2-14, respectively. The composition medium MMH1 was as follow; coconut oil, 30g; K_2HPO_4, 4 g; KH_2PO_4, 4 g; $NaNO_3$, 13.36 g; $MgSO_4.7H_2O$, 0.05g; $CaCl_2$, 0.01g; $FeSO_4 \cdot 7H_2O$, 0.05 g and phenol, 0.1g per 1000ml distilled water, at pH 8 and incubation at 30°C and 250 rpm. Medium MMH14 had the same composition of medium MMH14 except for $NaNO_3$ which was substituted by peptone, 21.4 g/1. The validation run showed remarkable increase in the productivity with isolate M2H2-1 as rhamnolipid concentration reached 18.7 & 22.9 rhamnose gl^{-1} on day 6, and 21& 24 rhamnose gl^{-1} on day 8 using media MMH1 and MMH14, respectively. This was conformed when the same pattern was observed with isolate M2H2-14

were the productivity reached 17.9 & 20 rhamnose gl^{-1} on day 6, and 23.3 & 20.6 rhamnose gl^{-1} on day 8 using media MMH1 and MMH14, respectively.

Normal probability plot of residuals of both designs for both isolates was used to examine the goodness of model fit (Fig. 6), the blue line in the graph is a graphical representation of the mathematical regression equation and since the red points in the plots generally formed a straight line, then the residuals are considered normally distributed.

4. Discussion

Rhamnolipids biosurfactants have various applications in diverse fields [10, 16], many studies have focused on rhamnolipids production by *Pseudomonas aeruginosa* strains [10, 17]. From this prospective, this study focused on optimization of rhamnolipid production by two *Pseudomonas* strains previously investigated for their production of high concentration of rhamnolipid and high tolerance of organic pollutants. The selection of these two isolates M2H2 1 & M2H2 14 for maximal production of rhamnolipids was even more supported when their productivity was compared to rhamnolipids productivity of other *Pseudomonas* strains in previous studies.

In an approach to building a strong reliable design for optimization of rhamnolipid production, the key factors affecting bioproduction process were selected as follows; factors related to medium composition, factors related to physiological conditions, in addition to toxicity factor was included. All the factors included in the design were previously proven to have considerable influence on rhamnolipid production [18-20].

The results showed that the highest rhamnolipid production occurred by using coconut oil as substrate at concentration 3%w/v, such a high value is in agreement with previously published work where despite both water soluble and water-insoluble substrates are used for rhamnolipids production, yet the use hydrophopic carbon sources especially vegetable oils is always accompanied by high rhamnolipids productivity [10]. Also the high percent of coconut oil is needed to provide the energy required for production as rhamnolipids synthesis involves two central metabolic pathways [21].

Regarding nitrogen source, many studies reported nitrates as the best nitrogen source for rhamnolipid production [10, 22], others stated that peptone as nitrogen source was accompanied by high rhamnolipid production [23]. The two predicted optimal media differ only in the used nitrogen source as medium MMH1 included NaNO3 at C/N ratio 10, while medium MMH14 included peptone at C/N ratio 10. Yet, the results didn't show much discrimination between the two nitrogen sources used, as the highest produced ramnolipids concentration was 24.1 & 23.3 rhamnose gl^{-1} for peptone and NaNO3, by isolates M2H2 1 & M2H2 14 respectively. This variation in favoring organic nitrogen source over inorganic nitrogen source or the opposite may be strain dependent.

Both isolates favored low magnesium concentration for high rhamnolipids production indicating that magnesium limitation stimulates

rhamnolipids synthesis [18, 24], as the protein synthesis is blocked and the cellular metabolism is shifted to carbohydrates synthesis and rhamnolipids production.

With respect to physiological conditions, based on the previous results, temperature, pH and agitation rate had significant effect on rhamnolipids production (Fig 3). The highest rhamnolipids production by both isolates was achieved at low temperature (30°C), high pH (8) and high agitation rate (250 rpm). Increasing the agitation rate up to 250 rpm increased rhamnolipids production, owing to the increase in both oxygen and medium component transfer to the medium which is needed for effective rhamnolipids production [20]. The favorable temperature for rhamnolipids production ranges from 30-37 °C [20, 25]. Based on our results, temperature of 30°C was favored over 42°C for rhamnolipids productions, suggesting that high temperature might have undesirable effect on the production.

5. Conclusion

The recorded results support the efficiency of multi-factorial experimental designs in elucidating the significant factors and predicting their optimum settings for achieving maximum productivity.

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Figure Legends

Fig. 1: Blue agar plates (CTAB agar plates), 10 micro liters of the inoculum were added into each well, plates were incubated for 48 h at 37 ^{o}C and then stored in the refrigerator for 24 h. Dark blue haloes were then detected using UV transilluminator.

Fig. 2: Gel electrophoresis for amplified sequences of genes: *rhlA*, *rhlI* and *rhlR*. Lane 1: 100 bp ladder, lane 2-4: PCR fragment of *rhlA* gene by standard *Pseudomonas* strain, isolate M2H2 1 and isolate M2H2 14 respectively, lane 5-7: PCR fragment of *rhlI* gene by standard *Pseudomonas aeruginosa* strain, isolate M2H2 1 and isolate M2H2 14 respectively, lane 8-10: PCR fragment of *rhlR* gene by standard *Pseudomonas* strain, isolate M2H2 1 and isolate M2H2 14 respectively.

Fig. 3: Pareto chart ranking the standardized effects of the factors. 3-A & 3-B: Pareto chart of isolates M2H2 1 & M2H214 in design A, respectively. 3-C & 3-D: Pareto chart of isolates M2H2 1 & M2H214 in design B, respectively. The red line in the chart represents a reference line, any factor that extends past this line is of significant effect at P-value <0.5.

Fig. 4: Main effect plot comparing the magnitude of the main effects of all factors. 4-A & 4-B: main effect plot of isolates M2H2 1 & M2H214 in design A, respectively. 4-C & 4-D: main effect plot of isolates M2H2 1 & M2H214 in design B, respectively. The steeper the slope of the line, the greater the magnitude of the main effect. When the line is horizontal (parallel to the x-axis), then there is no main effect present.

Fig. 5: 3D surface plot showing the relationship between the most significant factors and their effect on the measured response. 5-A: 3D surface plot for the effect of phenol concentration and pH on emulsification activity (EU/ml) by isolate M2H2 1. 5-B: 3D surface plot for the effect of iron concentration and temperature on emulsification activity (EU/ml) by isolate M2H2 14. 5-C: 3D surface plot for the effect of pH and temperature on rhamnose concentration (gl^{-1}) by isolate M2H2 1. 5-D: 3D surface plot for the effect of pH and agitation rate (rpm) on rhamnose concentration (gl^{-1}) by isolate M2H2 14.

Fig. 6: Normal probability plot of residuals. 6-A: Normal probability plot of residuals by isolate M2H2 1 in design A. 6-B: Normal probability plot of residuals by isolate M2H2 14 in design A. 6-C: Normal probability plot of residuals by isolate M2H2 1 in design B. 6-D: Normal probability plot of residuals by isolate M2H2 14 in design B. Normal probability plot of residuals is used to examine the goodness of model fit in regression. The line is a graphical representation of the mathematical regression equation. The points in the plots generally form a straight line showing that the residuals are normally distributed.

Highlights

· Two Plackett-Burman designs were tested to optimize rhamnolipid productivity.

· Biosurfactant productivity was increased two to five folds for the tested isolates.
· Multi-factorial experiments can elucidate significant factors affecting production.

Table 1: The Plackett-Burman design matrix representing the coded values for independent factors and the values of measured response in Design A

Run	Factors											Response	
	C	N	C/N	Ph	Fe	Mg	pH	Temp	Rpm	S	D	*Emulsification Unit/ml	
												Isolate M2H21	Isolate M2H214
1	+	-	+	-	-	-	+	+	+	-	+	528	245
2	+	+	-	+	-	-	-	+	+	+	-	0	0
3	-	+	+	-	+	-	-	-	+	+	+	278	563
4	+	-	+	+	-	+	-	-	-	+	+	189	68
5	+	+	-	+	+	-	+	-	-	-	+	35	691
6	+	+	+	-	+	+	-	+	-	-	-	0	0
7	-	+	+	+	-	+	+	-	+	-	-	154	158
8	-	-	+	+	+	-	+	+	-	+	-	77	118
9	-	-	-	+	+	+	-	+	+	-	+	143	157
10	+	-	-	-	+	+	+	-	+	+	-	375	669
11	-	+	-	-	-	+	+	+	-	+	+	59	50

Run	Factors											Response	
	C	N	C/N	Ph	Fe	Mg	pH	Temp	Rpm	S	D	*Rhamnose conc gl^{-1}	
												Isolate M2H21	Isolate M2H214
1	+	-	+	-	-	-	+	+	+	-	+	8.4	11.3
2	+	+	-	+	-	-	-	+	+	+	-	0	0
3	-	+	+	-	+	-	-	-	+	+	+	11.2	5.6
4	+	-	+	+	-	+	-	-	-	+	+	6.1	2.3
5	+	+	-	+	+	-	+	-	-	-	+	14.8	11.8
6	+	+	+	-	+	+	-	+	-	-	-	0	0
7	-	+	+	+	-	+	+	-	+	-	-	8.2	7.4
8	-	-	+	+	+	-	+	+	-	+	-	2.3	1.5
9	-	-	-	+	+	+	-	+	+	-	+	5.8	4.5
10	+	-	-	-	+	+	+	-	+	+	-	11.6	10.3
11	-	+	-	-	-	+	+	+	-	+	+	7.4	4.1
12	-	-	-	-	-	-	-	-	-	-	-	5.3	1.7
13	0	0	0	0	0	0	0	0	0	0	0	15.2	10.7
12	-	-	-	-	-	-	-	-	-	-	-	3.166	37.4
13	0	0	0	0	0	0	0	0	0	0	0	33.37	13.8

*All the values represent the mean of three independent experiments. "0" represents the original concentration of each component under the control conditions, "-" represents the low level and "+" represents the high level for each factor. C= carbon source, N= nitrogen source, C/N= carbon/nitrogen ratio, Ph= phenol concentration, Fe= iron concentration, Mg= magnesium concentration, S= sampling time and D= dummy factor.

Table 2: The Plackett-Burman design matrix representing the coded values for independent factors and the values of measured response in Design B

* All the values represent the mean of three independent experiments. "0" represents the original concentration of each component under the control conditions, "-" represents the low level and "+" represents the high level for each factor. C= carbon source, N= nitrogen source, C/N= carbon/nitrogen ratio, Ph=

phenol concentration, Fe= iron concentration, Mg= magnesium concentration, S= sampling time and D= dummy factor.

Table 3: Experimental range and levels of independent factors in the Plackett-Burman Design A

Factor	Unit	Symbol	Levels		
			Low -	Center 0	High +
Type of carbon Source	-	C	glycerol	coconut oil	glucose
Type of nitrogen Source	-	N	Organic Urea	Inorganic NH_4SO_4	Inorganic $NANO_3$
C/N Ratio		C/N	10	34.5	60
Phenol (toxicity factor)	mgl^{-1}	Ph	100	0	500
Ferrous sulfate Concentration	gl^{-1}	Fe	0.001	0.01	0.05
Magnesium sulfate Concentration	gl^{-1}	Mg	0.05	0.5	1
Initial pH	pH	pH	5	7	8
Temperature	C^0	Temp	30	37	42
Agitation	Rpm	Rpm	110	180	250
Time of sampling	day	S	3	6	8
*Color of label (independent dummy Factor)	-	D	Red	Blue	Black

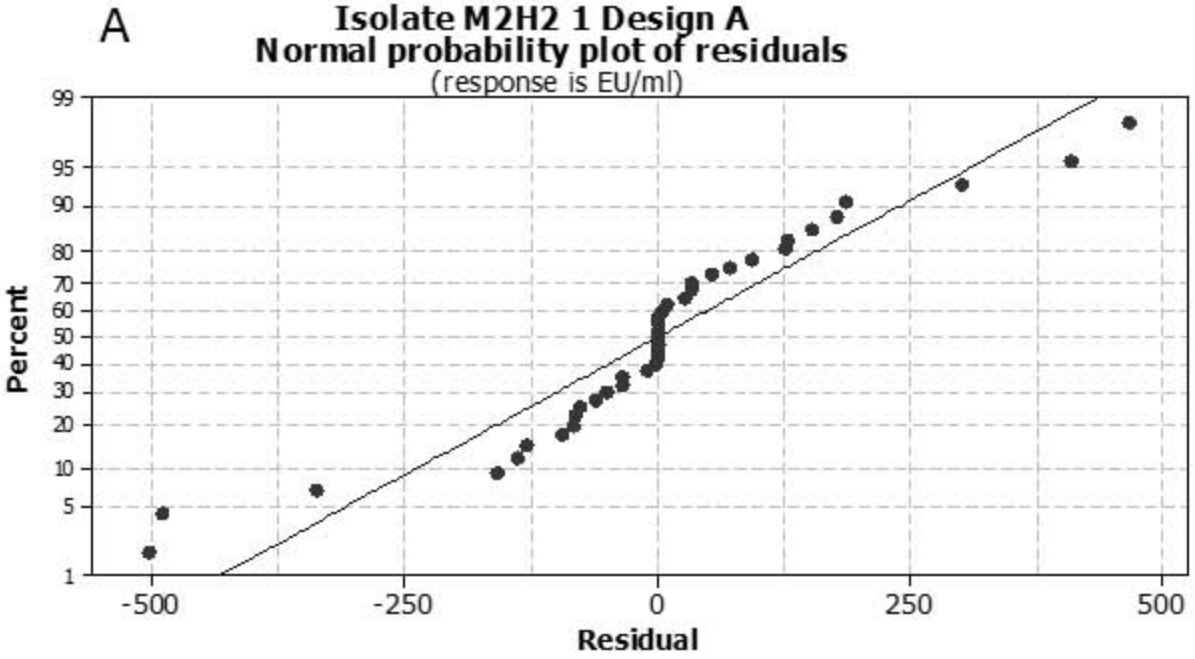
*Since we wanted to study just 10 real factors, an 11 factor design was used, the final factor being a dummy one; this may be a variable that has no influence on the experiment, such as the color of label used.

Table 4: Experimental range and levels of independent factors in the Plackett-Burman Design B

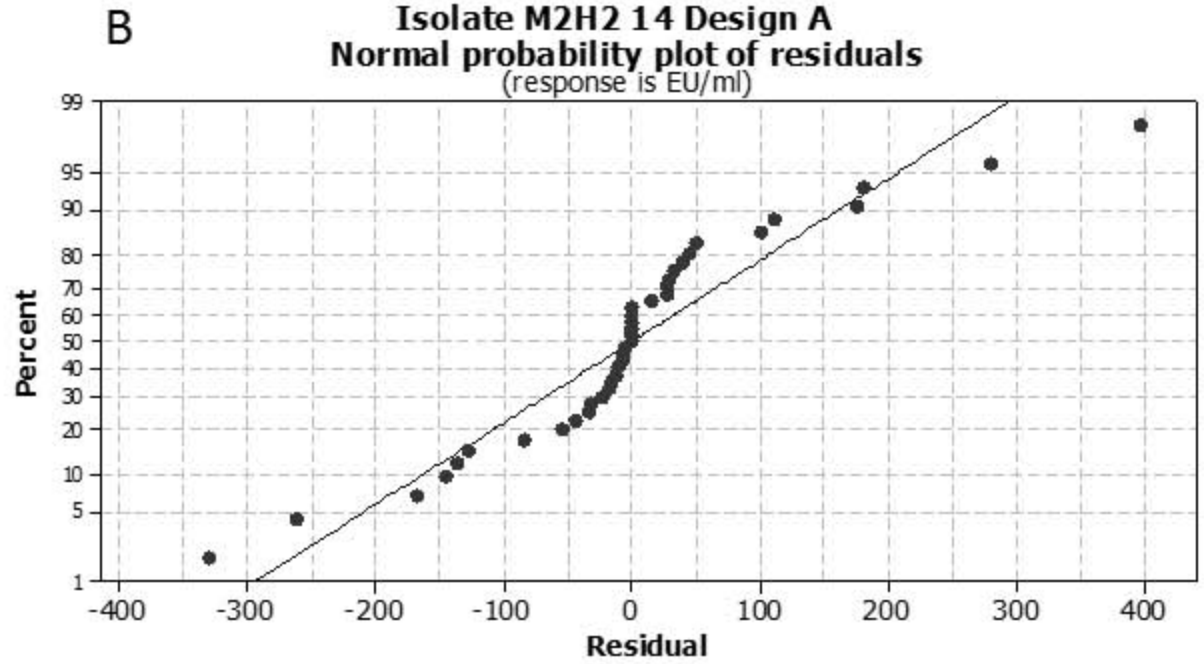
*Since we wanted to study just 10 real factors, an 11 factor design was used, the final factor being a dummy one; this may be a variable that has influence the no on experiment, such as the color of label used.

Factor	Unit	Symbol	Levels		
			Low -1	Center 0	High 1
Concentration of Carbon Source	% w/v	C	Coconut oil 1%	Coconut oil 2%	Coconut oil 3%
Type of nitrogen Source	-	N	Organic Peptone	Inorganic NH_4SO_4	Inorganic $NANO_3$
C/N Ratio		C/N	10	34.5	60
Phenol (toxicity factor)	mgl^{-1}	Ph	100	0	500
Ferrous sulfate Concentration	gl^{-1}	Fe	0.001	0.01	0.05
Magnesium sulfate Concentration	gl^{-1}	Mg	0.05	0.5	1
Initial pH	pH	pH	5	7	8
Temperature	C^0	Temp	30	37	42
Agitation	Rpm	Rpm	110	180	250
Time of sampling	day	S	3	6	8
*Color of label (independent dummy Factor)	-	D	Red	Blue	Black

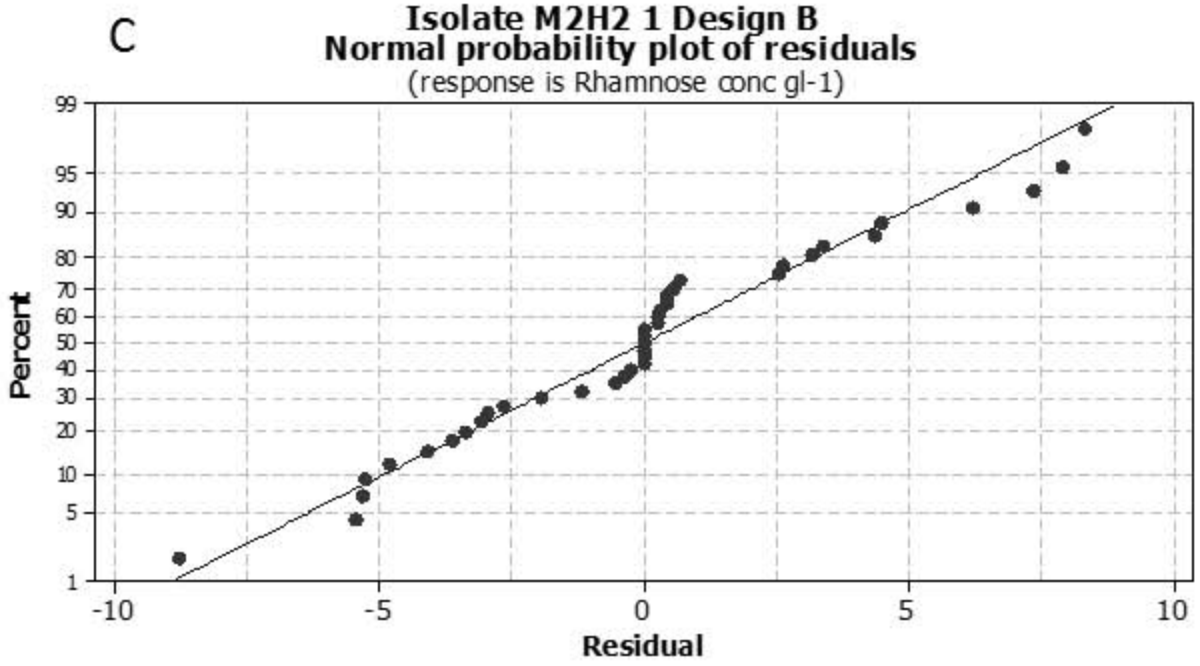
A


Isolate M2H2 1 Design A
Normal probability plot of residuals
(response is EU/ml)
Percent
Residual
99
95
90
80
70
60
50
40
30
20
10
5
1
-500
-250
0
250
500

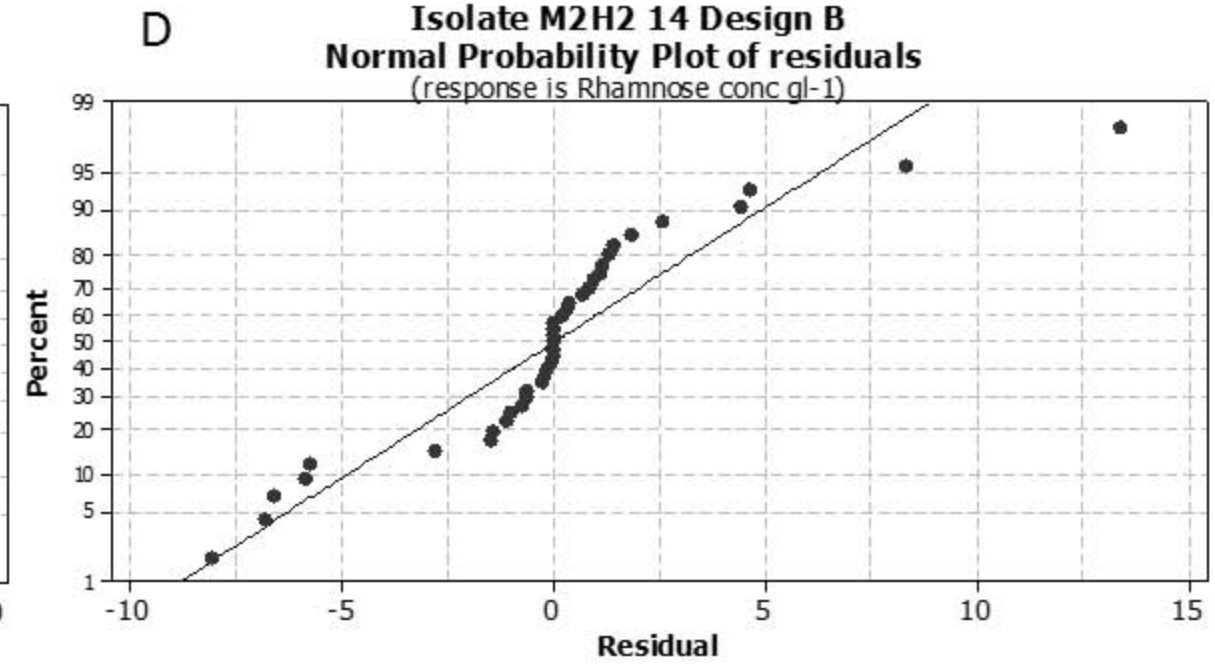
B


Isolate M2H2 14 Design A
Normal probability plot of residuals
(response is EU/ml)
Percent
Residual
99
95
90
80
70
60
50
40
30
20
10
5
1
-400
-300
-200
-100
0
100
200
300
400

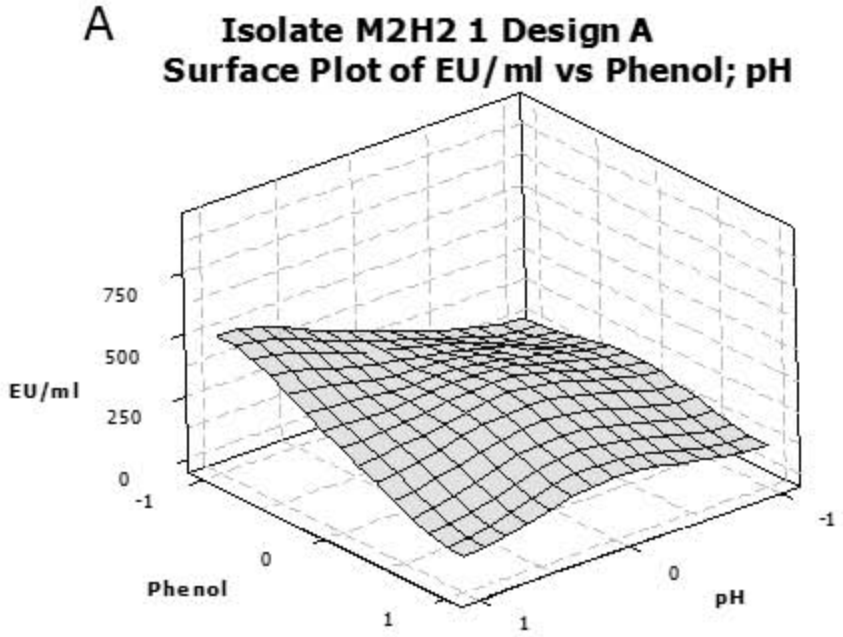
C


Isolate M2H2 1 Design B
Normal probability plot of residuals
(response is Rhamnose conc gl-1)
Percent
Residual
99
95
90
80
70
60
50
40
30
20
10
5
1
-10
-5
0
5
10

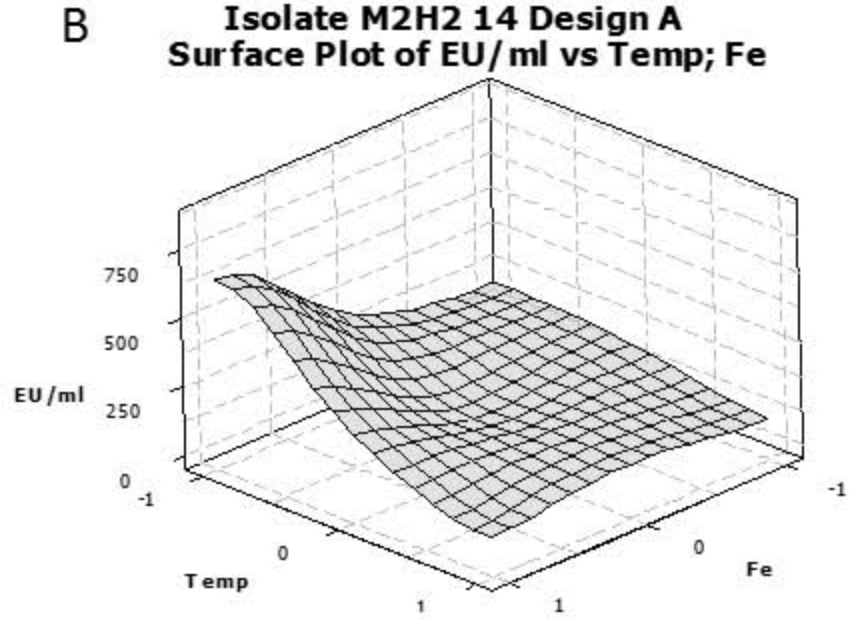
D


Isolate M2H2 14 Design B
Normal Probability Plot of residuals
(response is Rhamnose conc gl-1)
Percent
Residual
99
95
90
80
70
60
50
40
30
20
10
5
1
-10
-5
0
5
10
15

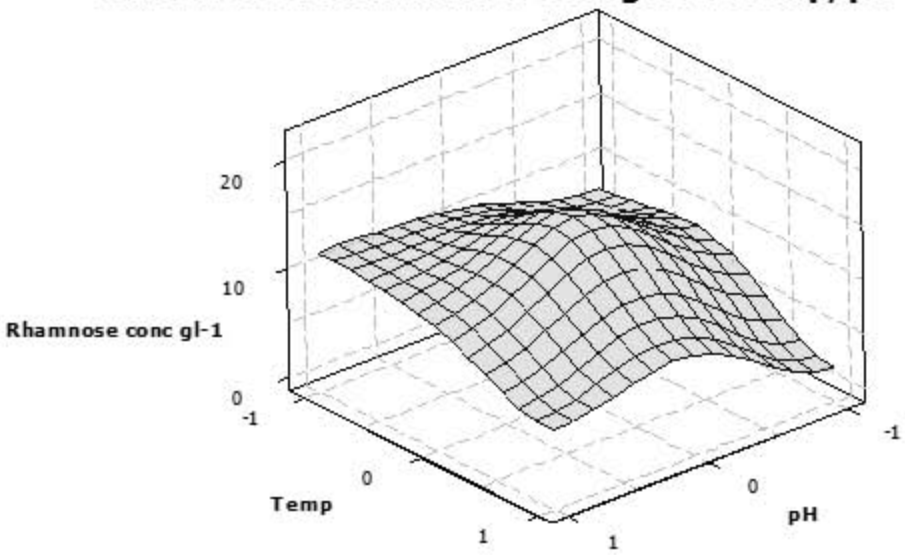
A


Isolate M2H2 1 Design A
Surface Plot of EU/ml vs Phenol; pH
EU/ml
750
500
250
0
-1
0
1
Phenol
1
0
-1
pH

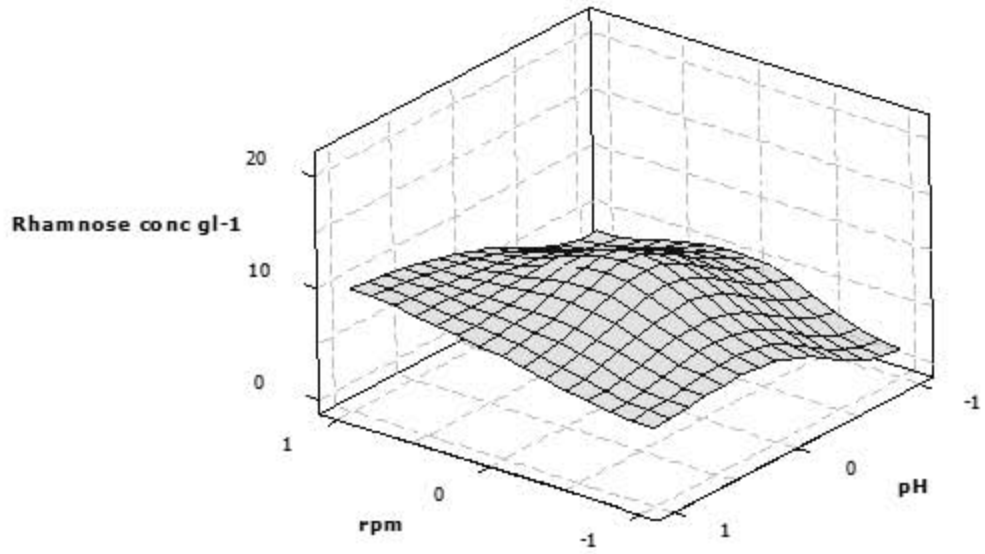
B


Isolate M2H2 14 Design A
Surface Plot of EU/ml vs Temp; Fe
EU/ml
750
500
250
0
-1
0
1
Temp
1
0
-1
Fe

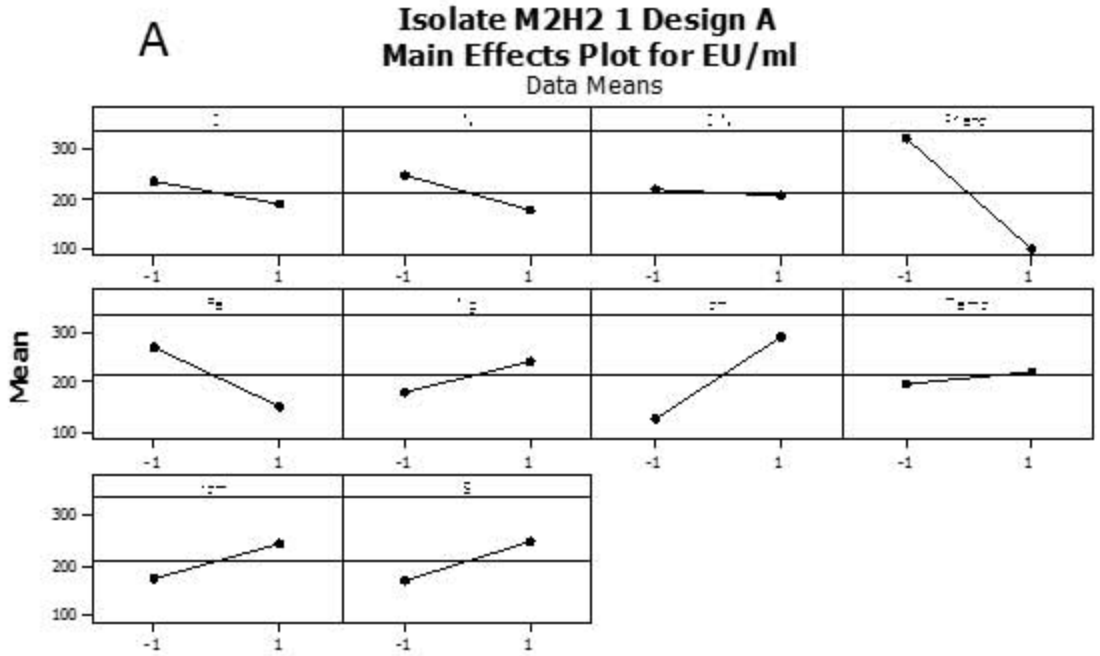
C


Isolate M2H2 1 Design B
Surface Plot of Rhamnose conc gl-1 vs Temp; pH
Rhamnose conc gl-1
20
10
0
-1
0
1
Temp
1
0
-1
pH

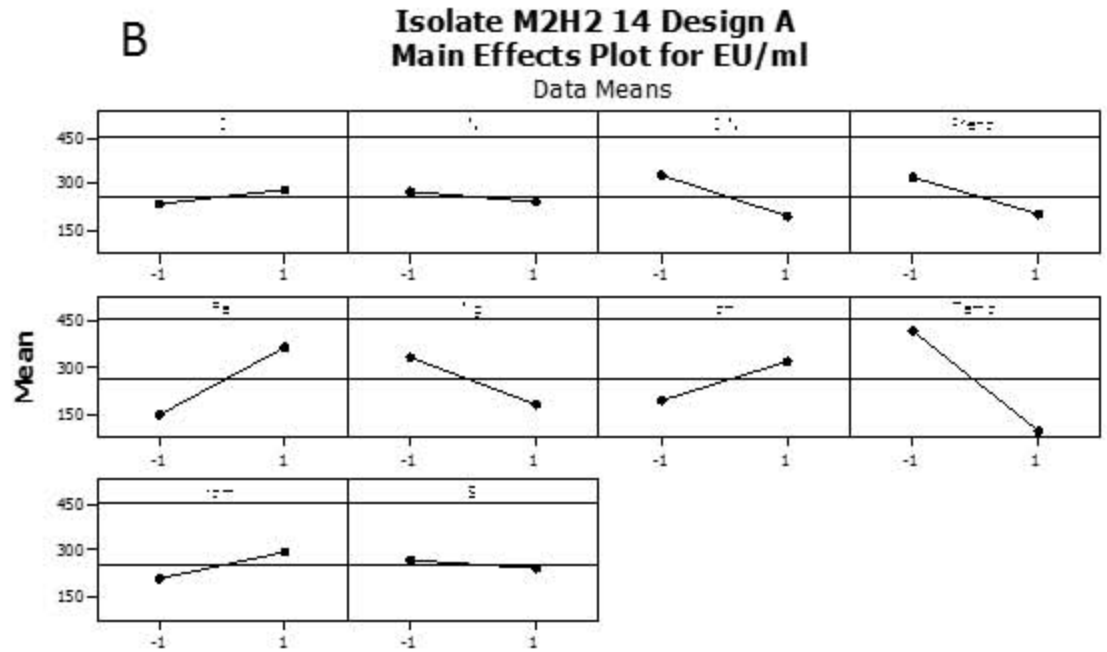
D


Isolate M2H2 14 Design B
Surface Plot of Rhamnose conc gl-1 vs pH; rpm
Rhamnose conc gl-1
20
10
0
1
0
-1
rpm
1
0
-1
pH

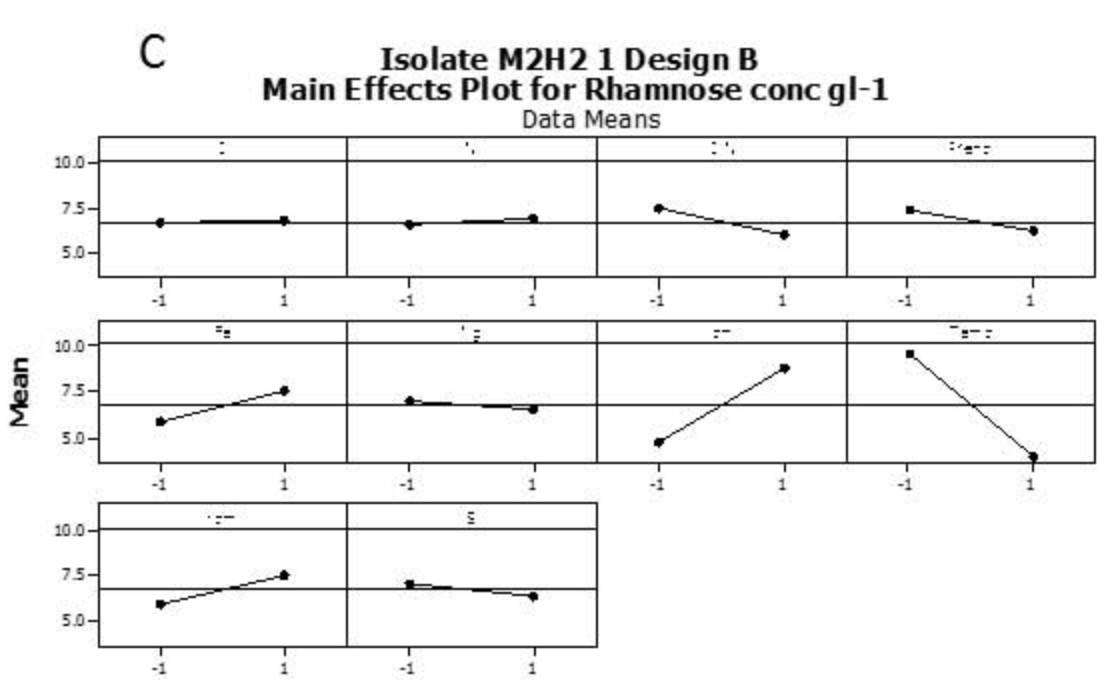
A


Isolate M2H2 1 Design A
Main Effects Plot for EU/ml
Data Means
Mean

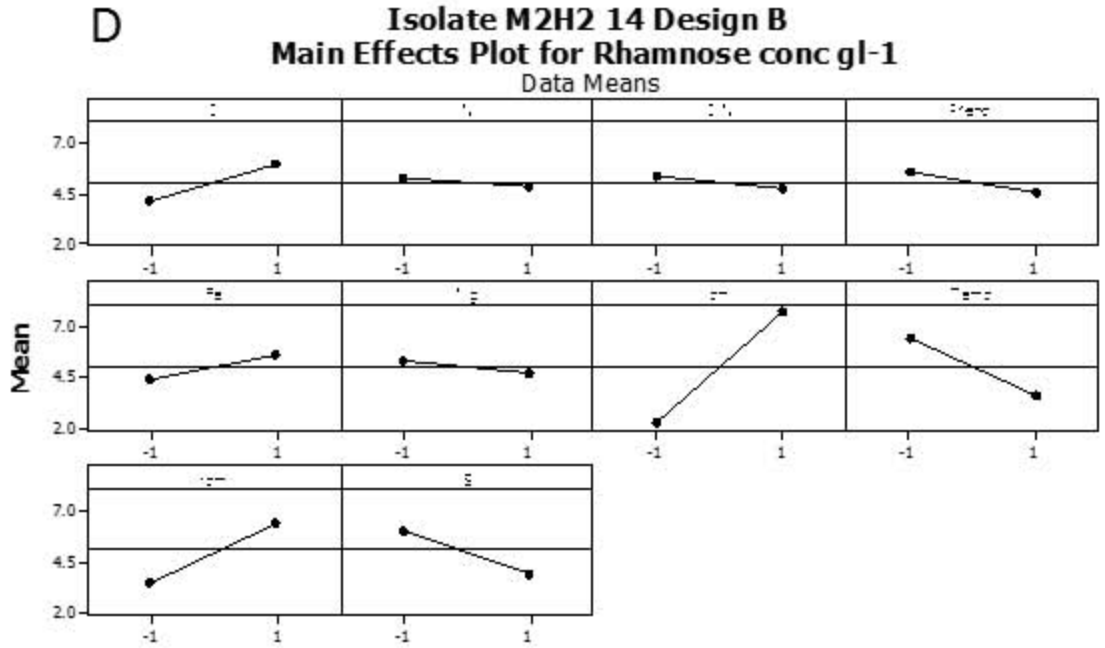
B

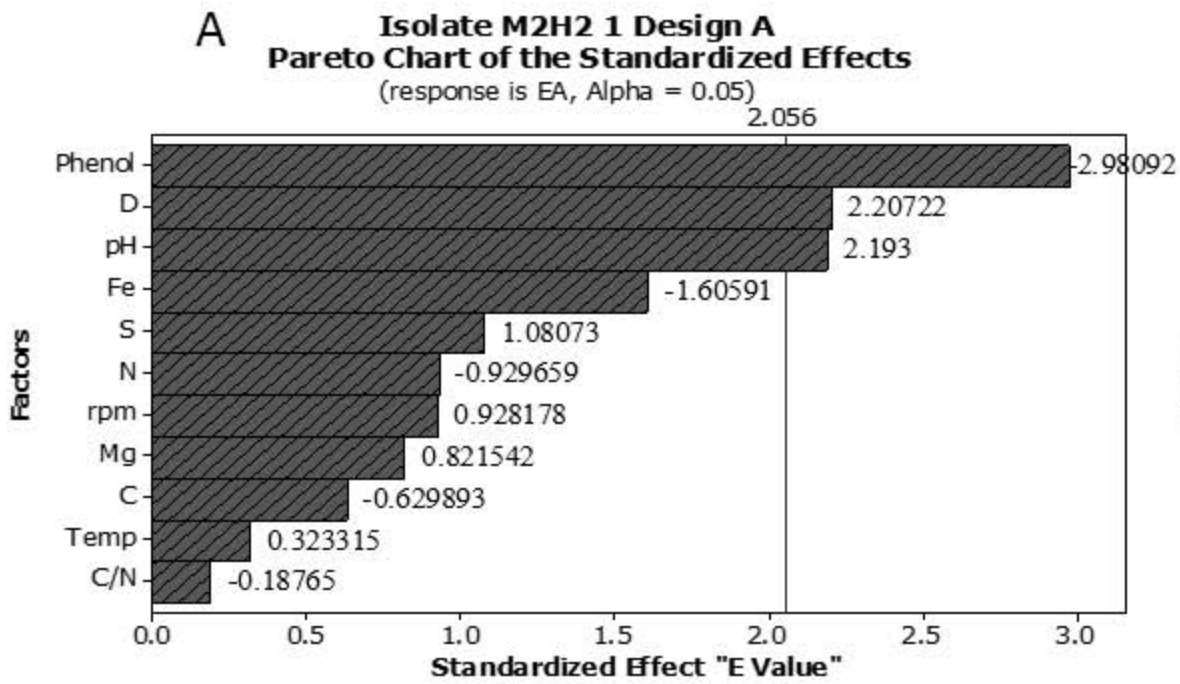

Isolate M2H2 14 Design A
Main Effects Plot for EU/ml
Data Means
Mean

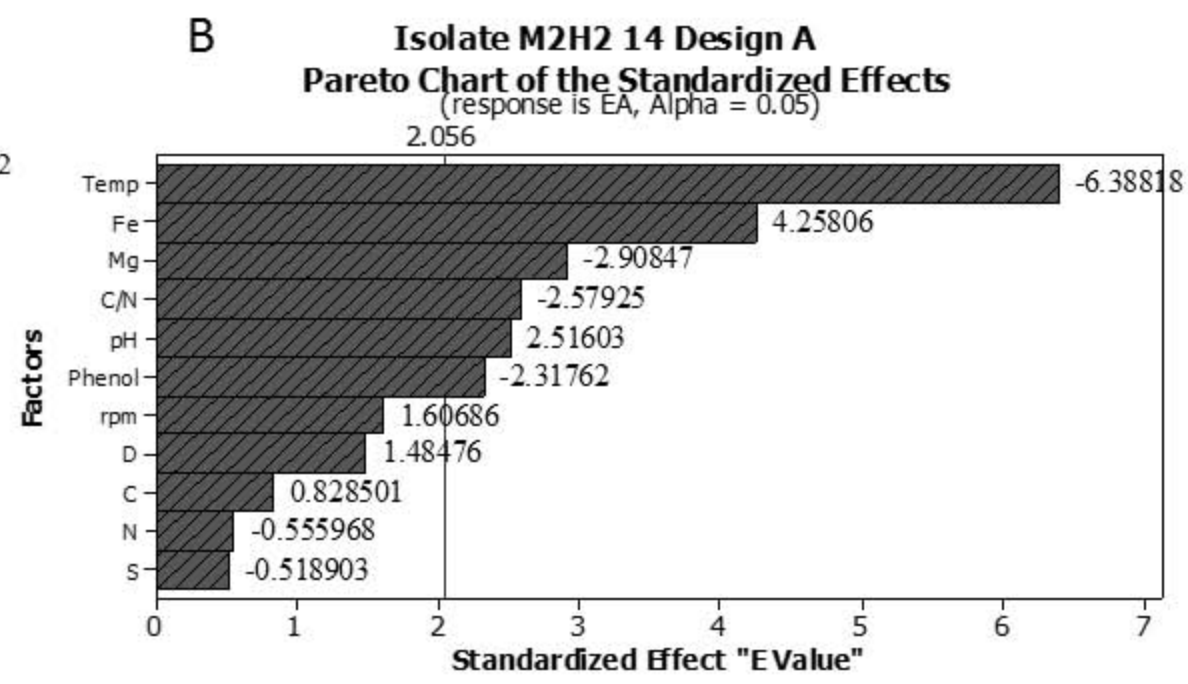
C

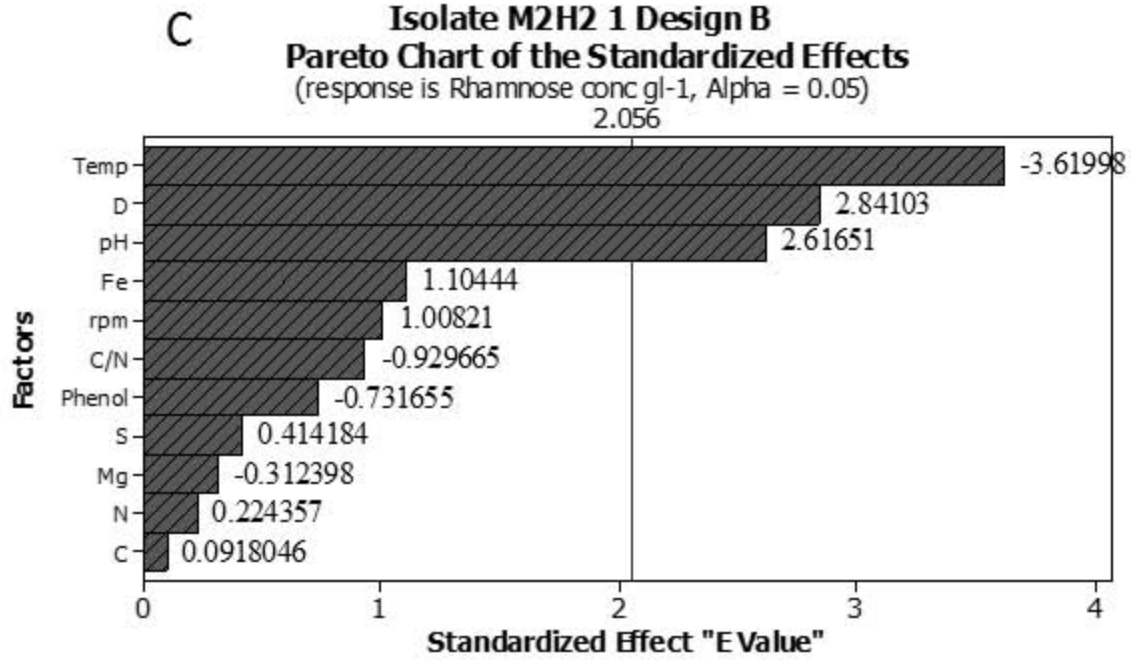

Isolate M2H2 1 Design B
Main Effects Plot for Rhamnose conc gl-1
Data Means
Mean

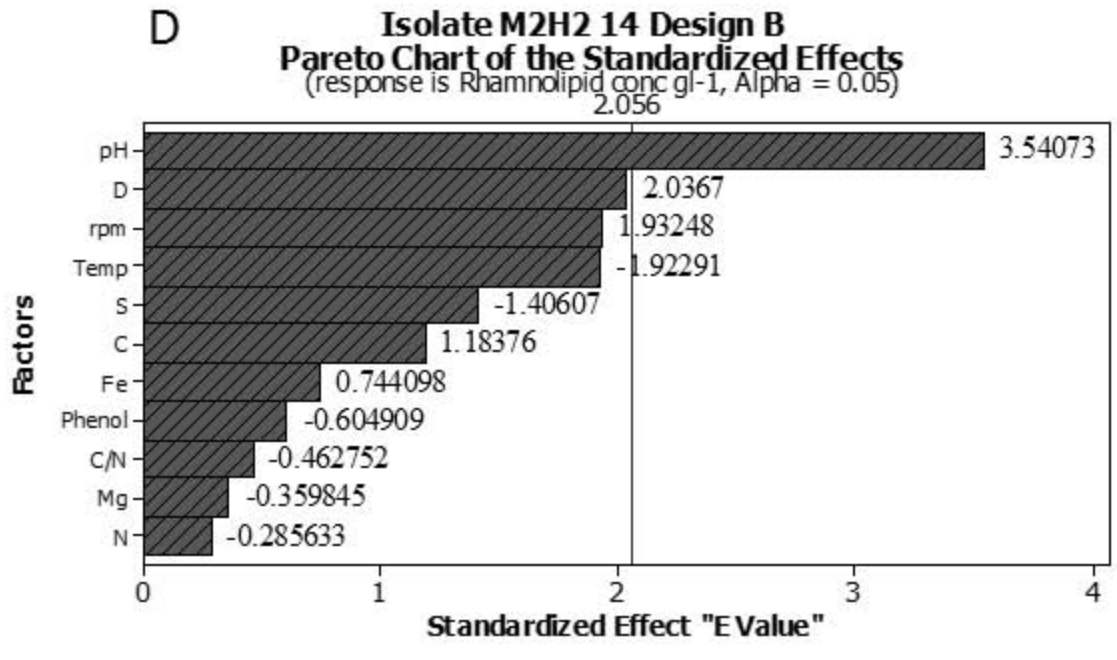
D

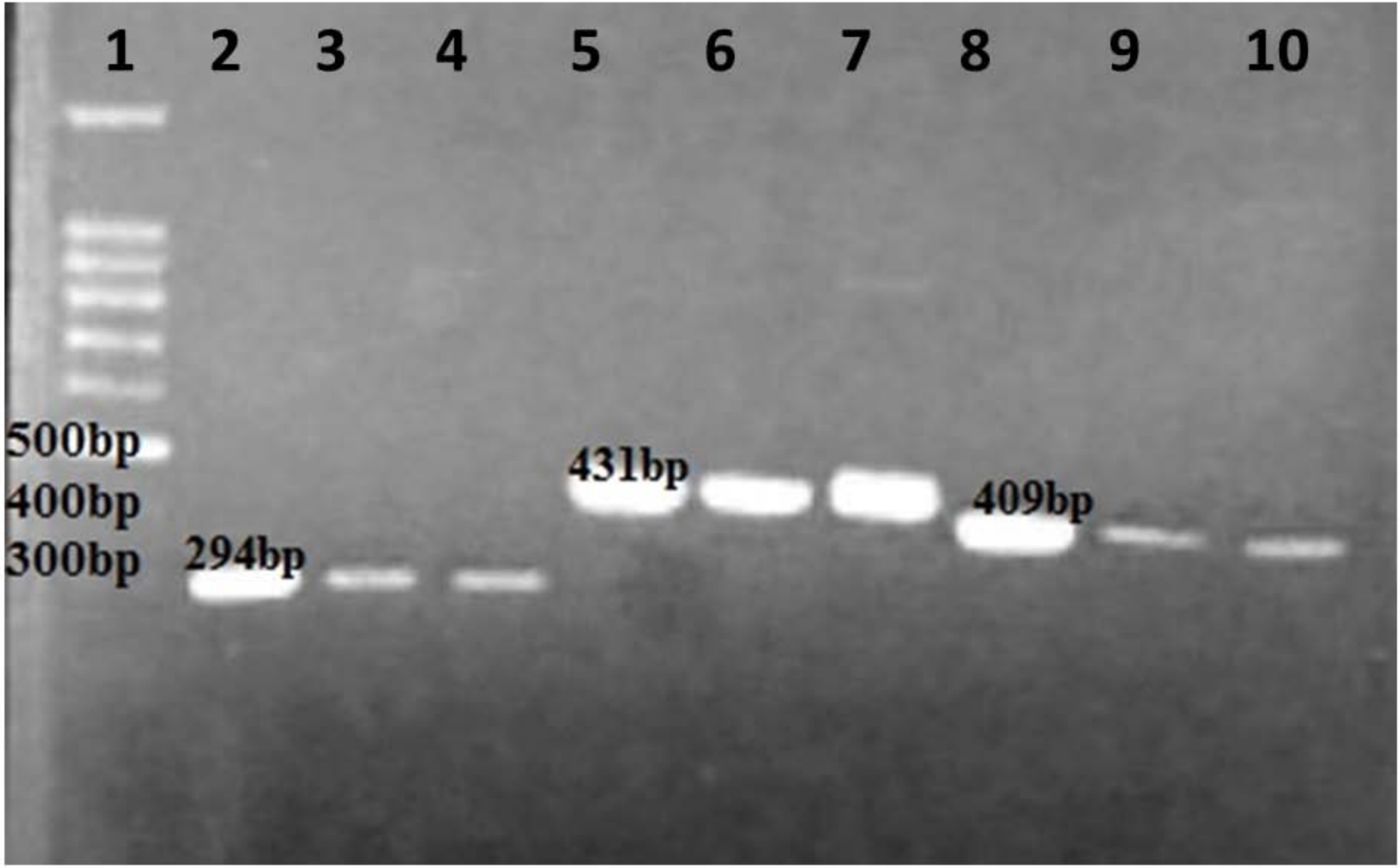

Isolate M2H2 14 Design B
Main Effects Plot for Rhamnose conc gl-1
Data Means
Mean


A
Isolate M2H2 1 Design A
Pareto Chart of the Standardized Effects
(response is EA, Alpha = 0.05)
2.056
Phenol
D
pH
Fe
S
N
rpm
Mg
C
Temp
C/N
-2.98092
2.20722
2.193
-1.60591
1.08073
-0.929659
0.928178
0.821542
-0.629893
0.323315
-0.18765
Factors
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Standardized Effect "E Value"


B
Isolate M2H2 14 Design A
Pareto Chart of the Standardized Effects
(response is EA, Alpha = 0.05)
2.056
Temp
Fe
Mg
C/N
pH
Phenol
rpm
D
C
N
S
-6.38818
4.25806
-2.90847
-2.57925
2.51603
-2.31762
1.60686
1.48476
0.828501
-0.555968
-0.518903
Factors
0
1
2
3
4
5
6
7
Standardized Effect "E Value"


C
Isolate M2H2 1 Design B
Pareto Chart of the Standardized Effects
(response is Rhamnose conc gl-1, Alpha = 0.05)
2.056
Temp
D
pH
Fe
rpm
C/N
Phenol
S
Mg
N
C
-3.61998
2.84103
2.61651
1.10444
1.00821
-0.929665
-0.731655
0.414184
-0.312398
0.224357
0.0918046
Factors
0
1
2
3
4
Standardized Effect "E Value"


D
Isolate M2H2 14 Design B
Pareto Chart of the Standardized Effects
(response is Rhamnolipid conc gl-1, Alpha = 0.05)
2.056
pH
D
rpm
Temp
S
C
Fe
Phenol
C/N
Mg
N
3.54073
2.0367
1.93248
-1.92291
-1.40607
1.18376
0.744098
-0.604909
-0.462752
-0.359845
-0.285633
Factors
0
1
2
3
4
Standardized Effect "E Value"


1
2
3
4
5
6
7
8
9
10
500bp
400bp
300bp
294bp
431bp
409bp


1B
1S