Optimization of fermentation conditions through response surface methodology for Indole-3-acetic acid production by Marine Sponge Callyspongia diffusa associated Pseudomonas fluorescens BCPBMS-1

Vasanthabharathi Venkataraman (vasanthab2505@gmail.com)
CAS in Marine Biology,Faculty of Marine Sciences, Annamalai University
LakshmiNarayanan Ramasamy
CAS in Marine biology,Faculty of marine sciences
Jayalakshmi Singaram
CAS in marine biology,Faculty of marine sciences

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Abstract

In the Present study we focused on the production and statistical optimization of media components for Indole-3 acetic acid (IAA) production from *P.fluorescens* BCPBMS-1, it was isolated from Marine sponge *Callyspongia diffusa* . This sponge was collected from Gulf of Mannar, Southeast coast of India. We optimized the low-cost agricultural residue based medium for IAA production. In our study Soya bean husk used as a substrate for Indole-3-acetic acid (IAA) production. Fermentation variables were selected based on the Plackett-Burman design and were optimized by response surface methodology. The maximum IAA concentration 1.7474 µg/ml was predicted in medium containing 3.1064% Soya bean husk, Yeast extract 0.8323%, Salinity 9.0765 ppt, pH 6.3108 temperature 27.5524℃, incubation time 64.7475hrs. These predicted values were also verified through experiments as a result we got 1.74 µg/ml. The excellent correlation observed between predicted and measured values of this statistical model hence, this method may recommend for the industrial purpose.

Introduction

Indole-3-acetic acid (IAA) is the main member of the auxin family that controls many important processes including cell elongation and division, tissue differentiation in plants (Teale et al. 2006). It is a well-studied hormone produced by many microbes. There are many researches in IAA by many terrestrial, freshwater, wetland plant rhizosphere microbes, (Haida-Alija,2003) such as *Pseudomonas fluorescens Bacillus subtilis and Klebsiella pneumonia*, *Flavobacterium sp. Flavobacteria, Enterobacter sp. Rhizobium sp., Stenotrophomonas sp. Herbaspirillum sp.* and *Agrobacterium sp* (Dhungana, et al (2018). but, IAA from marine microbial biota researches were very scanty.

The ocean represents a rich resource for even more novel compounds with great potential as pharmaceutical, nutritional supplements, cosmetics, agrichemicals, and enzymes, where each of these marine bioproducts have a strong potential market value (Konig,1994 and Fenical, 1997). The marine environment has high salt, poor nutrition and less microbial growth. (Choudhary et al. 2017). Past two decades onwards the scientific society have recognized that the oceans are rich source of natural products and potential drugs (Cooper, 2004 and Dincalci et al. 2004 and Simmons et al. 2005) It was noticed by many researchers that marine microalgae, cyanobacteria, fungi and heterotrophic bacteria living in association with invertebrates (sponges, tunicates and soft corals) have been identified or strongly suspected, as the true sources of many bioactive and useful constituents (Gordaliza, 2007 and Molinski et al., 2009).

Agro-industrial waste contains high amount of carbohydrates, lipids and hence, can be used as a rich carbon source for microbial metabolite production. Rice bran, wheat bran, lignocellulosic wastes used as substrate for microbial enzyme production (Sivakumar et al .,2012; Annamalai et al ., 2013,2014 and Fernández Núñez, et al .,2017). Sugar cane molasses, beet molasses, bagasse of sugarcane considered as a cheaper carbon source for bio surfactant production (Nitschke et al., 2004; Rashedi et al., 2005; Benincasa, 2007).

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems, in which a response of interest is influenced by several variables (Liu and Fang, 2010).Hence, fractional factorial design like Plackett- Burman becomes a method of choice for initial screening of medium components. This optimization method can be used in the production of microbial compounds at controlled parameter to give a high yield in industries. Our previous study, we have collected

about four different species of sponges. Sponge associated bacteria were isolated and identified. Then screened for IAA production. Among the tested strains maximum IAA production was observed with *Callyspongia diffusa* associated *Pseudomonas fluorescens* BCPBMS-1 (Vasanthabharathi and Jayalakshmi,2017) and also it is a moderately halophilic, strain.

Materials And Methods

Isolation of *P.fluorescens* from marine sponge *Callyspongia diffussa*

P.fluorescens BCPBMS-1 was isolated from Marine sponge *Callyspongia diffusa ,* it was identified by both biochemical and *16S* r DNA sequencing.

Screening and estimation for indole acetic acid (IAA) production

A loop full of *P. fluorescens* was inoculated in 10 ml of Luria broth supplemented with 0.1% L-tryptophan and incubated for 72 hrs at 30°C. Then, the culture was centrifuged at 10,000 x g for 10 min., and the supernatant was collected. One ml of supernatant was allowed to react with 2 ml of Salkowsky reagent at 30°C for 30 minutes. The optical density was read at 535 nm. The recorded OD values were plotted in a standard curve prepared from commercially available IAA (Patten and Glick, 1996).

Optimization of IAA production

Identifying the significant variables using Plackett– Burman design

The Plackett–Burman experimental design is a two factorial design, which identifies the critical physico-chemical parameters required for IAA elevated production by screening n variables in n + 1 experiments (Plackett and Burman, 1946). The variables chosen for the present study were soye bean husk (A), glucose (B), sucrose (C), peptone(D), yeast extract (E). All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameter on IAA production was calculated by the following equation:

$$E = (\sum M_+ - \sum M_-) / N \quad (1)$$

Where E is the effect of parameter under study and M_+ and M_- are responses (IAA production) of trials at which the parameter was at its higher and lower levels respectively and N is the total number of trials.

2.3.2 Optimization by using Response surface methodology

Central composite design (Two level factorial: quarter fraction) consisting of six main critical independent variables viz., temperature (25 to 55 ◦c), P^H (6 to 11), salinity (0 to 35 %), incubation time (24 to 96 hrs.), soya bean husk (0 to 3%) and yeast extract (0 to 1%) were chosen.

For each factor, a conventional level was set to zero as a coded level. These eight factors, each with five coded levels consisting of 53 experimental runs and 2.37841 alpha values were used to analyze the experimental data to allow better estimate of the experimental error and to provide extra information about yields in the interior of

the experimental region. The experimental data were fitted according to Equation as a second-order polynomial regression equation including individual and cross effect of each variable.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

Where Y, β_0, β_i, β_{ii} and β_{ij} are respectively the predicted response, a constant, a linear coefficient, a squared coefficient and an interaction coefficient representing equation was used to build surfaces for variables.

Statistical analysis

Statistical analysis was done by ANOVA for analysis of the results. A probability level of $p < 0.01$ was considered as statistically significant.

Mass scale production of IAA and extraction

Optimized media was prepared, to this about 1 ml of (24 hrs culture) *P. fluorescens* was inoculated in1000ml conical flasks in a shaker at 150rpm incubated at optimized condition. Extraction of IAA was done following the procedure of Ahmad et al (2005).

Results And Discussion

IAA production has been reported in *Pseudomonas* species by many researcher (Xie et al., (1996); Karnwal,(2009)and Jegan et al.,(2018)).In the present study marine *P. fluorescens* BCPBMS-1 which was isolated from the *Callyspongia diffusa* that produced indole acetic acid Likewise Goswami et al (2013) isolated *P. fluorescens* from sea water of Gulf of Khambhat and studied the plant growth promoting efficacy.

Response surface methodology

In the present observation, the significant variables necessary for enhanced IAA production were selected using the Plackett–Burman design. A large variation in IAA production by the Plackett–Burman design experiments suggested the need for further optimization. The Central composite design (Two level factorial: quarter fraction) was employed to study the interactions among the significant factors and also to determine their optimal levels. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process of IAA production. Totally 6 variables (i.e) pH, temperature, salinity, incubation time, soya bean husk and yeast extract were taken for RSM which gave maximal yield in the Plackett–Burman experiments (Table 1).

The analysis of variance (ANOVA) by Fisher's statistical test was conducted for the second-order response surface model and the result showed that the computed F value for linear regression was much greater than the tabulated (F) > P value. Therefore, the model terms temperature, pH, salinity, incubation time, soya bean husk and yeast extract as significant. (Fig.2). The goodness-of-fit of the model was checked by decisive coefficient of determination (R^2) and adjusted R^2. When R^2 is large, then, the regression has accounted for a large proportion of the total variability in the observed value of Y which favors the regression equation model. These results reinforced that the response equation provided a suitable model for the CCD experiment (Table 2).

Y= 1.61911 +0.11479 +0.08808 - 0.11092 -0.19329-0.08918 +0.06639 -0.19224 -0.20727 -0.17987-0.11004 -0.21434 -0.22760 +0.16063-0.05312 -0.02312 -0.02688+0.03812 -0.05438+0.00313 -0.02313 + 0.07188 -0.00187-0.02188 -0.00813+0.08063 -0.04063-0.05312

In the present observation optimum temperature predicted by the model was 27.5524°c (Figure 1). Shokri and Emtiazi (2010) reported that 30°C was the optimum temperature for the production of IAA. Aldesuquy et al. (1998) found that temperatures in the range 25-30°C was suitable for growth and IAA production of *Streptomyces* spp. Apine and Jadhav (2011) also reported 30°C as the ideal temperature for IAA production by *Pantoea agglomerans* strain PVM. Variation from the optimum temperature allowed significant improvement of IAA biosynthesis (Malhotra and Srivastava 2009).

IAA production by *Bacillus* spp. MQH-19 was maximum at pH 6.0 (**Mohite B 2013**). In another research *Paenibacillus* spp. SPT−03, IAA production was maximum at pH 5.0 and Minimum at pH 7.0 (Acuna et al.,2012). Khamna et al. (2010) have shown that pH 7.0 was suitable for maximum IAA production by *Streptomyces* sp. In White-rot fungus gave IAA production at pH 7.5 (Yurekli et al. 2003). We observed optimum pH as 6.3108 (Figure 1). So, in our finding acidic pH medium below 5 and over 7was found to be unfavorable for IAA production by the marine *P.fluorescens* BCPBMS-1 and also it was concluded that optimum pH for IAA production may be influence by other components of the media. In our experimental study, ideal (Figure 1) Salinity was 9.0765 ppt. Nakbanpote et al. (2014), also reported that salt-tolerant *Pseudomonas* sp. PDMZnCd excrete IAA under 8% (w/v) NaCl condition, Whereas Ravikumar et al (2004) observed maximum IAA production at 1% Nacl concentration by *A.brasilense* isolated from mangrove roots. The best concentration found be at 3% for IAA production by Halotolerant bacteria (Jayaprakashvel et al 2014). Sachdev et al (2009) found Nacl concentration of 0.5w/v as the optimum for IAA production in *Klebsiella* spp.

In the present observation 64.7475 hrs (Figure 1) was predicted by RSM method. Cacciari et al. (1989) reported accumulation of IAA from *Azospirillum* and *Arthrobacter* spp. during the stationary phase. The present study also proved the same. Chung and Tzeng. (2004) reported that fungi *U. maydis* synthesized IAA at 3 days of incubation. Shokri and Emtiazi (2010) also observed 72 hrs as the optimum incubation time for IAA production. Khamna et al. (2010) also observed the same in *Streptomyces* spp.

In our experiment 3.1064 % of soya bean husk was used as cheaper substrate for IAA production (Figure 1).Likewise De Oliveira et al. (2012) used citric pulp, soya bran, sugarcane bagasse, soya husk, cassava bagasse and coffee husk for the production of gibberellic acid using soild state fermentation. Lwin et al (2008) used soybean supplement for the production of IAA by *Pseudomonas* spp. Rubio et al. (2000) observed that *Azotobacter* spp, *Enterobacter* spp and *Pseudomona*s spp. preferred soya flour as the source for IAA production. Many researches also revealed that soya bean husk is the one of the cheaper substrates for IAA production. 0.8323% yeast extract was found to be best nitrogen source for IAA production predicted by RSM method. Narayana et al. (2009) also observed highest IAA production from *Streptomyces albidoflavus* in the medium containing yeast extract as nitrogen source.

Mass scale production

The maximum IAA production of 1.7474 µg/ml (Figure 1) was predicted by the model. The suggested medium composition was repeated. Mass scale production was done at optimized condition as a result 1.74µg/ml was

produced. The validation of experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. It endorsed the accuracy of the RSM to optimize the process for I AA production.

Shukla et al. (2005) obtained 1 g/l gibberellic acid produced by solid state fermentation. Khakipour et al. (2008) reported that the production of IAA by *P. fluorescens* strains was 0 to 31.6 mg/l whereas that of *P.putida* was 0 to 24.08 mg/l. Leinhos and Vacek (1994) reported 1.6-3.3 mg/l of IAA production by *P. fluorescens* and Prikryl et al. (1985)observed 0.01-3.93mg/l of auxin production. Nenwani et al. (2010) reported that production of IAA found to be 11.45µgml^{-1}.

Conclusion

The study suggested that that soya bean husk is a potential low-cost substrate; it can be used as a carbon source for IAA production. Statistical optimization by RSM provide useful information about the factors and their interactions. In addition, Marine *P. fluorescens* BCPBMS-1, can be used as bio-inoculants for plant growth promotion.

Abbreviations

IAA- indole -3-acetic acid;

RSM- response surface methodology;

ANOVA-Analysis of variance

CCD- central composite design;

Nacl -Sodium Chloride

Pseudomonas fluorescens- P. fluorescens

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data/ used or analyses during the study are available from corresponding author .

Competing interests

There is no Competing of interest.

Funding

Not applicable

Authors' contributions

VV performed the experiments and wrote the first draft of the manuscript under the constant ethnical guidance of JS .VV revised the manuscript LN provided the analytical help. All authors read and approved the final manuscript.

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Tables

Table 1. Plackett–Burman experiment for screening of significant process variables affecting IAA production

Run	Soyabean husk (%)	Glucose (%)	Sucrose (%)	Peptone (%)	Yeast extract (%)	IAA µg/ml	
						Experimental values	Predicted values
1	3	1	3	0.1	0.1	0.74	0.733333
2	1	1	1	1.0	1.0	0.46	0.466667
3	1	3	3	1.0	0.1	0.70	0.706667
4	3	1	1	0.1	1.0	0.50	0.503333
5	3	3	3	0.1	1.0	0.45	0.446667
6	3	3	1	1.0	0.1	0.86	0.856667
7	1	3	3	0.1	1.0	0.35	0.363333
8	3	3	1	1.0	1.0	0.56	0.560000
9	1	1	3	1.0	1.0	0.42	0.400000
10	1	1	1	0.1	0.1	0.71	0.716667
11	1	3	1	0.1	0.1	0.74	0.726667
12	3	1	3	1	0.1	0.77	0.780000

Table 2. Observed response and predicted values of IAA

Run.	Soya bean husk (%)	Yeast extract (%)	pH	Salinity (%)	Temperature (°c)	Incubation time (hrs)	IAA µg/ml	
							Experiment value	Predicted value
1	1.00000	0.10000	6.0000	5.0000	25.0000	24.000	0.51	0.51055
2	1.0000	0.10000	6.0000	35.000	25.0000	72.000	0.17	0.17175
3	2.00000	0.55000	7.5000	20.000	35.0000	105.082	0.46	0.46136
4	1.00000	0.10000	6.0000	35.000	45.0000	24.000	0.41	0.41258
5	3.00000	0.10000	9.0000	35.000	45.0000	24.000	0.03	0.03344
6	2.00000	0.55000	7.5000	20.000	35.0000	48.000	1.46	1.45972
7	2.00000	0.55000	7.5000	20.000	35.0000	48.000	1.46	1.45972
8	3.00000	0.10000	9.0000	5.0000	25.0000	24.000	0.43	0.42641
9	2.00000	0.55000	11.0676	20.000	35.0000	48.000	0.42	0.42088
10	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
11	3.00000	0.10000	6.0000	35.0000	45.0000	72.000	0.22	0.21701
12	1.00000	0.10000	6.0000	5.0000	45.0000	72.000	0.37	0.36427
13	2.0000	0.5500	7.5000	20.000	11.215	48.000	0.70	0.70138
14	3.00000	0.10000	9.0000	35.0000	25.0000	72.000	0.11	0.10761
15	1.00000	1.00000	9.0000	35.0000	45.0000	24.000	0.01	0.00500
16	3.00000	1.00000	9.0000	35.0000	45.0000	72.000	0.49	0.48943
17	2.00000	0.55000	7.5000	0.0000	35.0000	48.000	1.44	1.44300
18	1.00000	0.10000	9.0000	5.0000	25.0000	72.000	0.69	0.68987
19	1.00000	1.00000	6.0000	5.0000	25.0000	72.000	0.88	0.87654
20	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
21	3.00000	0.10000	6.0000	5.0000	25.0000	72.000	0.89	0.89498
22	3.00000	1.00000	9.0000	5.0000	25.0000	72.000	1.34	1.33740
23	1.00000	0.10000	9.0000	35.0000	25.0000	24.000	0.23	0.22818
24	1.00000	1.00000	6.0000	35.0000	45.0000	72.000	0.32	0.32357
25	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
26	3.00000	1.00000	9.0000	35.0000	25.0000	24.000	0.35	0.35571
27	2.00000	0.00000	7.5000	20.0000	35.0000	48.000	1.02	1.02155
28	1.0000	0.1000	9.0000	35.000	45.000	72.000	0.12	0.12190
29	2.00000	0.55000	7.5000	20.0000	35.0000	0.000	0.44	0.43816

30	4.37841	0.55000	7.5000	20.0000	35.0000	48.000	0.80	0.80046
31	3.00000	1.00000	6.0000	35.0000	25.0000	72.000	1.17	1.16928
32	3.00000	0.10000	6.0000	5.0000	45.0000	24.000	0.38	0.38081
33	2.00000	1.62029	7.5000	20.0000	35.0000	48.000	0.64	0.63965
34	2.00000	0.55000	7.5000	20.0000	58.7841	48.000	0.28	0.27868
35	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
36	3.00000	1.00000	6.0000	35.0000	45.0000	24.000	0.77	0.77011
37	0.00000	0.55000	7.5000	20.0000	35.0000	48.000	0.51	0.50943
38	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
39	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
40	1.00000	0.10000	9.0000	5.0000	45.0000	24.000	0.39	0.39070
41	1.00000	1.00000	9.0000	35.0000	25.0000	72.000	0.19	0.18917
42	3.00000	1.00000	6.0000	5.0000	45.0000	72.000	1.24	1.24180
43	3.0000	0.1000	9.0000	5.0000	45.000	72.000	0.20	0.20013
44	1.00000	1.00000	6.0000	35.0000	25.0000	24.000	0.11	0.10985
45	2.00000	0.55000	3.9324	20.0000	35.0000	48.000	0.95	0.94918
46	1.00000	1.00000	9.0000	5.0000	25.0000	24.000	0.27	0.27297
47	2.00000	0.55000	7.5000	55.6762	35.0000	48.000	0.43	0.42912
48	1.00000	1.00000	6.0000	5.0000	45.0000	24.000	0.25	0.25237
49	3.00000	1.00000	9.0000	5.0000	45.0000	24.000	0.40	0.39823
50	3.00000	1.00000	6.0000	5.0000	25.0000	24.000	1.15	1.14808
51	1.00000	1.00000	9.0000	5.0000	45.0000	72.000	0.20	0.20169
52	2.00000	0.55000	7.5000	20.0000	35.0000	48.000	1.46	1.45972
53	3.00000	0.10000	6.0000	35.0000	25.0000	24.000	0.16	0.15829

Figures

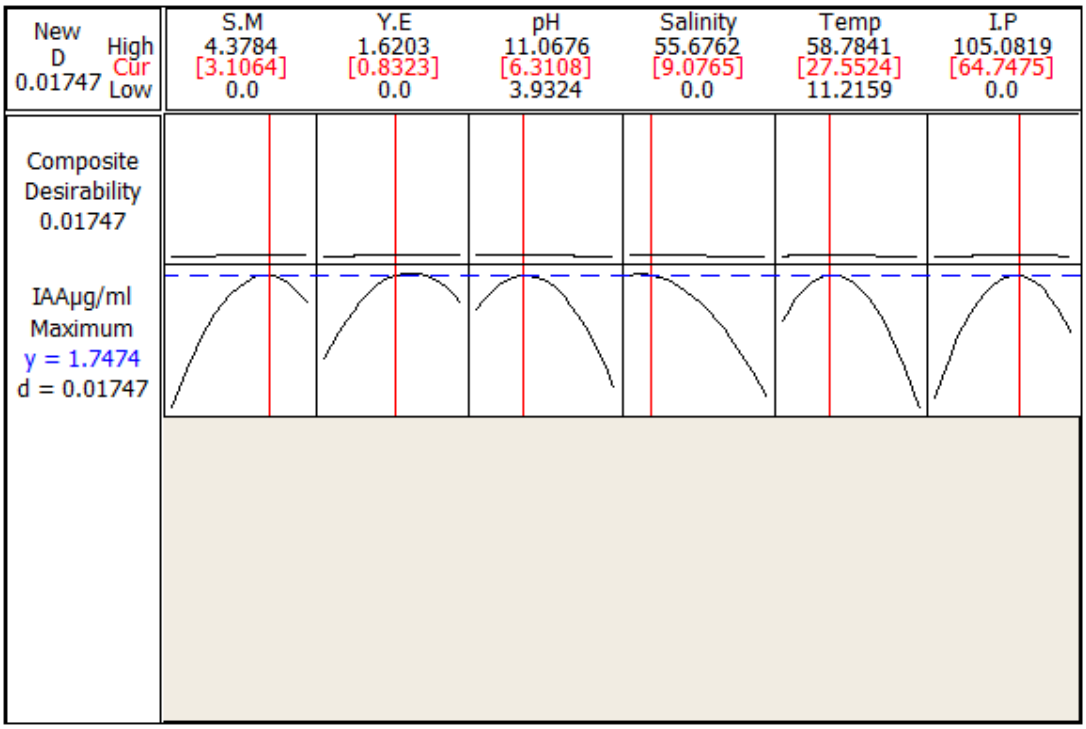

Figure 1

Optimized parameters for IAA production (S.B.H- soya bean husk, Y.E-yeast extract, Temp-temperature, I.P-incubation period (hrs))

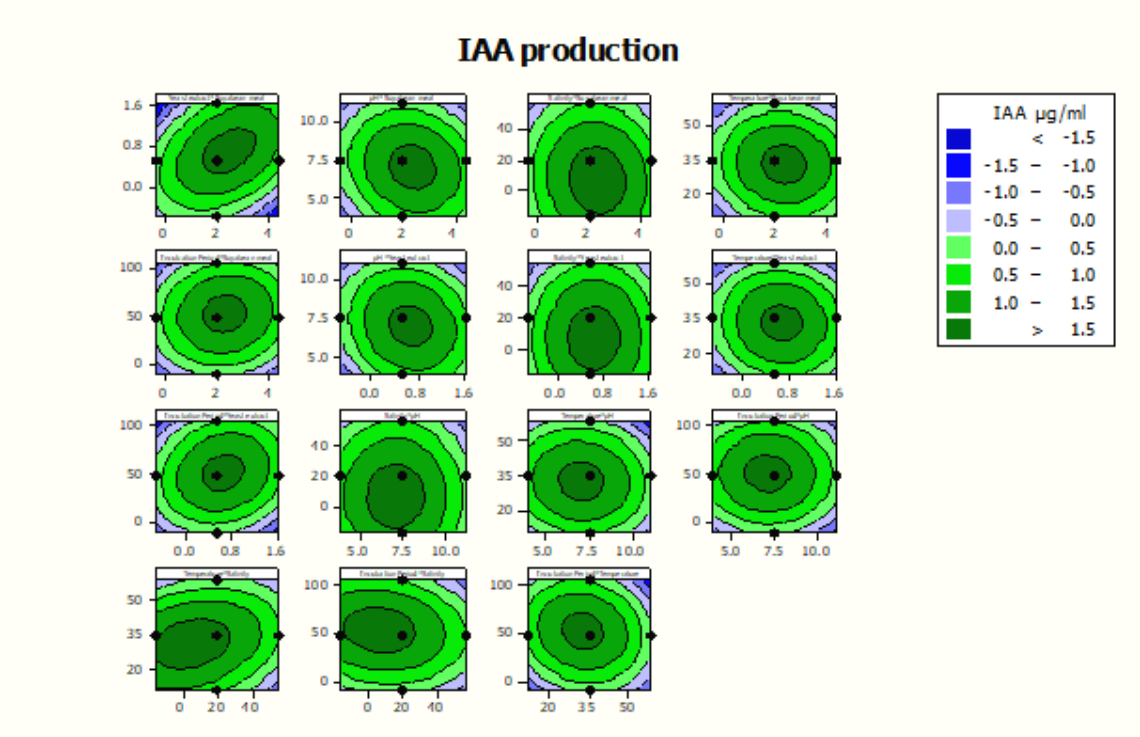

Figure 2

Response contour plots of showing interactions between significant variables

Supplementary Files

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- iaagraabstract.pptx