Simultaneous Production of Bioemulsifier and Exopolysaccharides by Thermophilic *Brevibacillus Borstelensis* Using Response Surface Model, Genetic Algorithm and NSGA

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Research

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Simultaneous production of bioemulsifier and exopolysaccharides by thermophilic *Brevibacillus borstelensis* using response surface model, genetic algorithm and NSGA

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Abstract:

Bioemulsifier and exopolysaccharides are two of the important biomolecules produced as secondary metabolites by many microorganisms. Bioemulsifiers emulsify hydrophobic substrates allowing their consumption by microorganisms whereas exopolysaccharides provide structural scaffold for the microorganisms to attach to any surface for biofilm formation. Both of these biomolecules have various applications in pharmaceutical and biomedical sectors and hence their production with high yield becomes important. A thermophilic bacterium, *Brevibacillus borstelensis*, has shown to produce bioemulsifier and exopolysaccharides simultaneously with limited yields. Central composite design-response surface methodology was employed as experimental design strategy to maximize the yields of both of these products with concentrations of glucose, monosodium glutamate, yeast extract and magnesium sulphate in the medium as process variables. The concentration of bioemulsifiers, exopolysaccharides and biomass were chosen as responses of the study. A set of 30 experiments were performed as a part of optimization study with 24 runs as non-centre points and 6 runs as centre points. The model obtained from central composite design was further optimized by genetic algorithm and naïve sorted genetic algorithm. The best results obtained from the three modelling and optimization tools were compared and the models were validated.

Keywords: Bioemulsifier; exopolysaccharides; central composite design; response surface model; genetic algorithm; multiobjective optimization; naïve sorted genetic algorithm

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1. Introduction

Bioemulsifiers are high molecular weight amphiphilic molecules that are produced as secondary metabolites by many microorganisms. They have antibacterial, antifungal and antiviral properties and hence can be included in a treatment regime against antimicrobial resistance strains of microorganisms. Due to their emulsifying properties and low or no toxicity compared to chemical surfactants they can be used as stabilizers and emulsifiers in food and cosmetics. The applications of bioemulsifiers in pharmaceutical, food and cosmetics industries require the product to be of very high purity. But the major limitation of microbial production of bioemulsifier is high production cost and low yield of the product.

Exopolysaccharides are also high molecular weight polysaccharides which are synthesized by microorganisms and secreted into the external environment. Exopolysaccharides are also produced by some plants and algae but their production by microorganisms provides the advantages of higher yields and a large variety of structures with unique properties. These can also be produced by renewable resources and are non-toxic and biocompatible (Moscovici 2015). They have applications in food, cosmetics, agriculture and pharmaceutical industries. In agricultural sector, they are used as insecticides and herbicides. Their applications in pharmaceutical sector range from anti-cancer, anti-thrombotic, anti-coagulant and drug delivery agents (Nwodo, Green et al. 2012). They help cells adhere to the surface and protect cells against antimicrobial agents and extreme conditions (Gerba and Bitton 1984).

Process optimization, especially media optimization, is an important parameter for industrial production of biosurfactants. Process optimization helps in increasing production rates and yields. Their production and yields are influenced by concentrations of C, N, P, Mg and trace metal solutions among which carbon and nitrogen sources are the major determinants of the product yield. The major components in medium that require optimization are carbon and nitrogen sources. Carbon sources affect metabolism of bacteria and overall production cost. The quantity and nature of carbon source is an important consideration for production of any biomolecule. Low quantity of carbon will be insufficient for the growth of bacteria whereas high concentration might hinder bacterial growth by substrate inhibition or lead to formation of undesired by-products such as lactates, acetates or 2,3-

butanediols (Glaser et al. 1995, Ramos et al. 2000). The nature of carbon source is important as microorganisms might not be able to degrade complex substrates. The type of bioemulsifier and exopolysaccharide produced with respect to composition and structure also depends on substrate.

The type of carbon source is also dependent on the type of application of biosurfactant. For example, biosurfactants used for bioremediation, microbial enhanced oil recovery and as detergent formulations can be produced using agro-industrial wastes as substrates. These low cost substrates can be used to produce low-value high-volume biosurfactant (Mukherjee et al. 2006). Agro-industrial wastes for biosurfactant production include soybean meal, soy hydrolysate, corn steep liquor, cashew fruit juice, molasses, rice, fish, potato, and cassava wastes.

The ratio of C:N is very significant and depends on bacterial species used for the production and mode of cultivation (Fonseca et al. 2007). The inorganic compounds used as nitrogen source are ammonium nitrate, ammonium sulphate, glutamic sodium, urea and sodium nitrate. The cheaper raw materials such as soybean flour, peptone and casein acid hydrolysate can also be used as nitrogen sources. (Liu et al. 2012). Tryptone and rapeseed meal are some of organic nitrogen sources that promise higher production yields compared to the inorganic sources (Zhao et al. 2013),(Jin et al. 2014).

The determination of concentration of carbon, nitrogen and trace elements to obtain high yields of biosurfactants by one-factor-at-a-time experiments is a tedious process. These experiments also do not study the interaction between different media components. Hence statistical optimization tools are employed to evaluate the concentrations of all the media components.

The optimization methods screen most influential medium components and determine the optimum concentration of each media component. This results in a medium with high product yield and selectivity (Sen et al. 1997, Lee et al. 2004, Mukherjee et al. 2008). The optimization tools such as artificial neural network (ANN), differential evolution (DE), genetic algorithm (GA), particle swarm optimization (PSO) improve biosurfactant production (Eswari et al. 2013, Peng et al. 2014, Chen et al. 2015, Eswari et al. 2016, Eswari et al. 2016, Venkateswarlu et al. 2019). Optimization methods have generally been performed in shake flasks and not have been validated in bioreactors. Some factors such as oxygen transfer rates, aeration and agitation rates are significantly different in shake flasks

and bioreactors. Hence, the expected productivity by optimization techniques is not guaranteed in bioreactor-scale operations.

In the current study, central composite design (CCD) was used to design the experimental runs for simultaneous production of two industrially important products, bioemulsifiers and exopolysaccharides. The responses were generated in terms of bioemulsifier concentration, exopolysaccharide concentration and biomass. These responses were used to develop response surface models (RSM) for yields of bioemulsifier, exopolysaccharides and biomass. The results obtained from response surface models were validated using genetic algorithm (GA) and naïve sorted genetic algorithm (NSGA).

2. Materials and Methods

2.1. Source of microorganism

Thermophilic *Brevibasillus borstelensis* used for the study was previously isolated from a local hot spring in our laboratory (Dhagat et al. 2020). The isolate, preserved at -80°C as glycerol stock, was revived in nutrient broth before each experiment. The broth was incubated at 50°C and 120 rpm for 16-18 hours.

2.2. Fermentation conditions for simultaneous production of bioemulsifier and exopolysaccharides

McKeen medium was used for the production of bioemulsifier and exopolysaccharides simultaneously. The composition of the medium was glucose (10 g/l), monosodium glutamate (10 g/l), yeast extract (3 g/l), MgSO₄.7H₂O (1 g/l), K₂HPO₄ (1 g/l), KCl (0.5 g/l) and trace element solution (1 ml). The trace element solution consisted of MgSO₄.7H₂O (5 g/l), CuSO₄.5H₂O (1.6 g/l) and FeSO₄.7H₂O (0.15 g/l). The cultures were incubated at 50°C and 120 rpm for 24 hours. Post-incubation, the cultures were processed for extraction of bioemulsifier (Y₁) and exopolysaccharides (Y₂), estimation of biomass (Y₃) and emulsification index (Y₄).

2.3. Estimation of biomass

To estimate the biomass of each of the samples, the samples were centrifuged at 10,000 rpm for 20 minutes at 4°C. The cell pellet was washed with distilled water and dried in hot air oven at 60°C until constant weight was obtained. The biomass was calculated in terms of dry cell weight.

2.4. Determination of emulsification ability

One ml of cell-free broth of each of the samples was mixed with equal volume of petrol. The solution was vortexed vigorously for two minutes and allowed to stand at room temperature for the separation of phases. The heights of aqueous and emulsified layers were recorded and emulsification index was determined by the formula

$$EI(\%) = \frac{\text{Height of emulsified layer}}{\text{Total height of liquid}} * 100$$

2.5. Extraction of bioemulsifier

Ten ml of cell-free supernatant was mixed with three-volumes of ice-cold acetone. The mixture was incubated at 4°C overnight to precipitate bioemulsifier. The yellowish precipitate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The precipitate was lyophilized, weighed and stored in airtight glass vials.

2.6. Extraction of exopolysaccharides

Five ml of cell-free supernatant was mixed with two-volumes of absolute ice-cold ethanol. The mixture was incubated overnight at 4°C. The precipitate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was mixed with one-volume of distilled water and two-volumes of absolute ice-cold ethanol. The EPS precipitate was obtained by centrifugation at 10,000 rpm for 20 minutes at 4°C. The exopolysaccharide was lyophilized and stored in airtight vials after weighing.

2.7. Design of experiments- Central Composite Design

Central Composite Design (CCD)- Response Surface Methodology (RSM) was used to design the experiments for optimized biosurfactant production. The experiments were designed using the statistical software Design-Expert 12 version 12.0.12.0. Four process variables which have highest influence on the production of biosurfactant were considered for optimization study. These factors were concentrations of glucose (X_1), monosodium glutamate (X_2), yeast extract (X_3) and $MgSO_4 \cdot 7H_2O$ (X_4). Each of the factors was studied for five levels, that is, $-\alpha$, -1, 0, +1, $+\alpha$. The $-\alpha$ and $+\alpha$ correspond to the axial points, -1 and +1 are the factorial points and 0 is the centre point (Bertrand et al. 2018). The alpha was taken as 2. The values of five levels of the four process variables are mentioned in Table 1.

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Table 1: Five-levels of the four process variables with their values

Factor	Name	Units	- α	-1	0	+1	+ α
X ₁	Glucose	g/L	10	14	18	22	26
X ₂	Monosodium glutamate	g/L	2	6	10	14	18
X ₃	Yeast extract	g/L	0	2	4	6	8
X ₄	MgSO ₄ .7H ₂ O	g/L	0.4	0.6	0.8	1	1.2

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142 The numbers of non-centre and centre points were 24 and 6, respectively making the total number of
 143 experiments as 30 (Table 2). The response was measured in terms of, concentration of bioemulsifier
 144 (Y₁; g/l), concentration of exopolysaccharide (Y₂; g/l), biomass (Y₃; g/l) and emulsification index (Y₄;
 145 %) produced during each run. Once the responses were obtained, ANOVA (Analysis of Variance)
 146 was used to identify the relation between independent variables and dependent variables by a plot of
 147 experimental model.

148

Table 2: The experimental design matrix from Central Composite Design (CCD)

Run	Factors			
	X ₁ : Glucose	X ₂ : Monosodium glutamate	X ₃ : Yeast extract	X ₄ : MgSO ₄ .7H ₂ O
1	22	6	6	1
2	18	10	0	0.8
3	18	10	8	0.8
4	18	18	4	0.8
5	14	14	6	0.6
6	14	14	6	1
7	18	10	4	0.8
8	14	6	2	1
9	14	14	2	0.6
10	22	14	2	1
11	18	10	4	0.8

12	22	14	6	1
13	22	6	2	1
14	14	6	2	0.6
15	14	6	6	0.6
16	22	6	2	0.6
17	22	14	6	0.6
18	10	10	4	0.8
19	18	10	4	1.2
20	18	10	4	0.8
21	14	14	2	1
22	18	10	4	0.8
23	22	6	6	0.6
24	22	14	2	0.6
25	18	10	4	0.8
26	14	6	6	1
27	18	10	4	0.4
28	18	10	4	0.8
29	26	10	4	0.8
30	18	2	4	0.8

2.8. Optimization of process parameters using genetic algorithm (GA)

Genetic algorithm solves both constrained and unconstrained problems. It is based on Darwin's theory of natural selection which is used to study biological evolution. It consists of a population with individual solution and selects random individuals from the population to be parents. These form the basis of next generation as they are used produced children with the help of crossover function. Mutation function enables the transfer of random changes from parents to children. These three rules (selection of individuals as parents, crossover rules and mutation rules) are the most important aspects of genetic algorithm. During this process of crossover and mutation over many generations, the

population will achieve optimal solution. Genetic algorithms are used to solve problems for highly non-linear and discontinuous objective functions.

In the current work, genetic algorithm was performed on MATLAB R2016a with equations obtained from CCD-RSM. The parameters such as concentration of glucose, monosodium glutamate, yeast extract and magnesium sulphate were optimized to obtain maximum production of bioemulsifier, exopolysaccharide and biomass. The population type used was a double vector with 50 as population size. The creation function, mutation and crossover were constraint dependent and the crossover fraction was 0.8.

2.9. Naïve Sorted Genetic Algorithm (NSGA)

Genetic algorithm (GA) when applied to solve a multi-objective problem is known as Naïve and Sorted Genetic Algorithm (NSGA). NSGA is one of the most commonly used algorithm to solve problems on multi-objective optimization as multiple objectives give rise to a set of optimal solutions rather than a single optimal solution. Two or more objective functions might be in competition or conflict. Hence, this problem is solved by a set of optimal solutions known as Pareto optimal solutions or non-dominated solutions. In this method, a population is generated randomly with N individuals and a set of offspring is created from them using mutations and crossovers. Parents and the generated offspring are added to a new set in which a non-dominated sorting is made. This non-dominated sorting is used to acquire various fronts of nondominated after assigning a crowding distance to each solution. The next step is the formation of new parent population from the fronts with better non-dominance with best crowding distance.

In the current study, a multi-objective optimization method was developed by fitting a regression model with genetic algorithm and evaluating it for Pareto optimization using MATLAB R2016a. The advantage of this technique is that it can be used to solve multiple objectives (to obtain responses) for the same set of variables. The input parameters such as concentration of glucose, monosodium glutamate, yeast extract and magnesium sulphate were used to simultaneously evaluate the optimal concentration of bioemulsifier, exopolysaccharide and biomass production. The fitness functions generated from CCD-RSM were run using multiobjective optimization using genetic algorithm as the solver. A

double vector was chosen as the population type with a population size of 50. The selection was based on tournament with a tournament size of 2 and crossover fraction for reproduction was 0.8. Mutation function was constraint dependent whereas crossover function was intermediate. The distance measure function for multiobjective problem was @distancecrowding.

3. Results and Discussion

3.1. Optimization of medium components by CCD-RSM

The efficiency and economics of industrial production of any microbial compound can be improved by optimization of the medium components. One-factor-at-a-time experiment to obtain the best combination of medium components for maximum yield is a tedious and a time taking process and is also used to study the interaction between medium components. Therefore, statistical optimization is a cost-effective and fast approach to determine concentrations of different medium components.

In the current study, a rotatable CCD-RSM was applied to obtain an experimental design with four factors, namely concentrations of glucose, monosodium glutamate, yeast extract and magnesium sulphate and four responses, that is, emulsification index, concentrations of bioemulsifier, exopolysaccharide and biomass produced with a total of 30 runs (24 non-centre points and 6 centre points). The value of α was four for the chosen design. Each factor consisted of five coded levels. The rotatable central composite design is the most preferred method to model second-order response surface. In rotatable central composite design the variance of predicted response is dependent on the distance of point from central point and is determined by the correct choice of alpha. The alpha is calculated as $(n_f)^{1/4}$ wherein n_f is the number of points in a 2^k factorial design. The centre points in this design ranges from three to six (Sahoo et al. 2012).

The relationship between the independent variables and the responses were determined by ANOVA (Analysis of Variance) in terms of polynomial equations. A total of four equations were generated from the experimental results using cubic model for each of the response.

$$Y_1 = 1.16 + 0.4250A - 0.075B + 1.03C + 1.10D - 0.0250AB + 0.4125AC + 0.7AD + 1.41BC - 0.8BD - 0.0375CD + 0.8821A^2 + 0.0821B^2 + 0.3821C^2 + 0.4446D^2 +$$

$$0.075ABC + 0.3375ABD - 0.2ACD - 7.5BCD - 0.1375A^2B - 1.5A^2C - 1.16A^2D + 0.0125AB^2 \quad (1)$$

$$Y_2 = 7.23 + 1.7A - 0.0375B + 13.18C - 0.1D + 0.0813AB + 0.7937AC + 0.7937AD + 1.88BC - 2.99BD - 0.4312CD + 1.55A^2 - 0.8146B^2 + 0.7417C^2 - 0.7458D^2 - 0.0937ABC + 1.58ABD - 1.48ACD - 2.74BCD - 1.56A^2B - 4.38A^2C - 0.5563A^2D - 0.5562AB^2 \quad (2)$$

$$Y_3 = 0.8677 - 0.1875A - 0.1875B + 0.575C + 0.3625D + 0.0031AB + 0.3781AC + 0.0094AD - 0.0281BC - 0.4844BD + 0.0906CD + 0.0755A^2 - 0.037B^2 + 0.1193C^2 + 0.0130D^2 + 0.1406ABC + 0.0719ABD - 0.0656ACD - 0.23444BCD + 0.1969A^2B - 0.3781A^2C - 0.3469A^2D + 0.4031AB^2 \quad (3)$$

$$Y_4 = 27.90 + 6.53A - 2.88B + 11.76C + 9.09D + 0.4574AB + 3.59AC + 7.44AD + 12.44BC - 6.51BD - 1.11CD + 6.61A^2 - 2.93B^2 - 0.1494C^2 + 0.7865D^2 - 0.2088ABC + 3.64ABD - 2.09ACD - 7.51BCD + 0.7902A^2B - 16.46A^2C - 9.2A^2D - 2.28AB^2 \quad (4)$$

In the above equations (1-4), Y_1 , Y_2 , Y_3 and Y_4 represent concentrations (g/l) of bioemulsifier, exopolysaccharide and biomass and emulsification index (%), respectively. The variables A-D correspond to concentrations of glucose, monosodium glutamate, yeast extract and magnesium sulphate, respectively. The results of Analysis of Variance (ANOVA) for bioemulsifier production depict a low p-value of 0.0373 which determined the significance of the model (Table 3). It also shows that the terms C, D, AD, BC, BD, A^2 , BCD and AC^2 are significant terms of the model as their p-values are less than 0.05. The F-value of 3.83 also confirmed that the model is significant and means that the probability of this F-value due to noise is only 3.75%. The adequate precision measures the signal to noise ratio and its value of 6.29 indicated an adequate signal to navigate the design space.

Table 3: Analysis of variance for bioemulsifier production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	112.83	22	5.13	3.83	0.0373	Significant
A-Glucose	1.45	1	1.45	1.08	0.3334	
B-Monosodium Glutamate	0.0450	1	0.0450	0.0336	0.8597	
C-Yeast Extract	8.41	1	8.41	6.28	0.0407	
D-MgSO ₄	9.68	1	9.68	7.23	0.0311	
AB	0.0100	1	0.0100	0.0075	0.9335	
AC	2.72	1	2.72	2.03	0.1969	
AD	7.84	1	7.84	5.86	0.0461	
BC	27.56	1	27.56	20.59	0.0027	
BD	10.24	1	10.24	7.65	0.0279	
CD	0.0225	1	0.0225	0.0168	0.9005	
A ²	21.34	1	21.34	15.94	0.0052	
B ²	0.1848	1	0.1848	0.1380	0.7212	
C ²	4.00	1	4.00	2.99	0.1274	
D ²	5.42	1	5.42	4.05	0.0841	
ABC	0.0900	1	0.0900	0.0672	0.8029	
ABD	1.82	1	1.82	1.36	0.2815	
ACD	0.6400	1	0.6400	0.4780	0.5116	
BCD	9.00	1	9.00	6.72	0.0358	
A ² B	0.1008	1	0.1008	0.0753	0.7917	
A ² C	12.00	1	12.00	8.96	0.0201	

A ² D	7.21	1	7.21	5.38	0.0534	
AB ²	0.0008	1	0.0008	0.0006	0.9808	
Residual	9.37	7	1.34			
Lack of Fit	8.14	2	4.07	16.57	0.0062	Significant
Pure Error	1.23	5	0.2457			
Cor Total	122.20	29				

235 The ANOVA for exopolysaccharide production showed a high p-value of 0.3 referring that
236 the model generated is not significant relative to noise (Table 4). The only significant term of
237 the model is the interaction of concentrations of monosodium glutamate and magnesium
238 sulphate. There is a 34.73% chance that the high F-value of 1.38 is due to noise. An adequate
239 precision value of 4.477 showed adequate signals compared to noise. The results of biomass
240 estimation also showed an insignificant model with p-value (0.3746) greater than 0.05 with
241 BD being the only significant terms of the model (Table 5). Again a high F-value
242 corresponded to a probability of 37.46% that it is due to noise. The F-value of lack of fit
243 (0.45) shows the insignificance of lack of fit revealing the model to be a fit one. The adequate
244 precision value of 4.47 was observed from the experimental design.

245 **Table 4:** Analysis of variance for exopolysaccharide production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	746.77	22	33.94	1.38	0.3473	not significant
A-Glucose	23.12	1	23.12	0.9397	0.3646	
B-Monosodium Glutamate	0.0112	1	0.0112	0.0005	0.9835	
C-Yeast Extract	80.64	1	80.64	3.28	0.1131	

D-MgSO ₄	0.0800	1	0.0800	0.0033	0.9561	
AB	0.1056	1	0.1056	0.0043	0.9496	
AC	10.08	1	10.08	0.4097	0.5425	
AD	10.08	1	10.08	0.4097	0.5425	
BC	56.63	1	56.63	2.30	0.1730	
BD	143.40	1	143.40	5.83	0.0465	
CD	2.98	1	2.98	0.1209	0.7382	
A ²	66.25	1	66.25	2.69	0.1448	
B ²	18.20	1	18.20	0.7398	0.4182	
C ²	15.09	1	15.09	0.6133	0.4592	
D ²	15.26	1	15.26	0.6202	0.4568	
ABC	0.1406	1	0.1406	0.0057	0.9419	
ABD	40.01	1	40.01	1.63	0.2429	
ACD	35.11	1	35.11	1.43	0.2712	
BCD	120.45	1	120.45	4.90	0.0626	
A ² B	12.92	1	12.92	0.5250	0.4922	
A ² C	102.38	1	102.38	4.16	0.0807	
A ² D	1.65	1	1.65	0.0671	0.8031	
AB ²	1.65	1	1.65	0.0671	0.8031	
Residual	172.22	7	24.60			
Lack of Fit	171.95	2	85.97	1572.67	< 0.0001	Significant
Pure Error	0.2733	5	0.0547			
Cor Total	918.99	29				

Table 5: ANOVA for biomass estimation

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	13.76	22	0.6253	1.31	0.3746	not significant
A-Glucose	0.2813	1	0.2813	0.5914	0.4670	
B-Monosodium Glutamate	0.2813	1	0.2813	0.5914	0.4670	
C-Yeast Extract	2.65	1	2.65	5.56	0.0505	
D-MgSO4	1.05	1	1.05	2.21	0.1807	
AB	0.0002	1	0.0002	0.0003	0.9860	
AC	2.29	1	2.29	4.81	0.0644	
AD	0.0014	1	0.0014	0.0030	0.9582	
BC	0.0127	1	0.0127	0.0266	0.8750	
BD	3.75	1	3.75	7.89	0.0262	
CD	0.1314	1	0.1314	0.2763	0.6154	
A ²	0.1564	1	0.1564	0.3289	0.5842	
B ²	0.0375	1	0.0375	0.0789	0.7870	
C ²	0.3902	1	0.3902	0.8205	0.3952	
D ²	0.0047	1	0.0047	0.0098	0.9240	
ABC	0.3164	1	0.3164	0.6653	0.4416	
ABD	0.0827	1	0.0827	0.1738	0.6892	
ACD	0.0689	1	0.0689	0.1449	0.7148	
BCD	0.8789	1	0.8789	1.85	0.2162	
A ² B	0.2067	1	0.2067	0.4347	0.5308	

A ² C	0.7626	1	0.7626	1.60	0.2459	
A ² D	0.6417	1	0.6417	1.35	0.2835	
AB ²	0.8667	1	0.8667	1.82	0.2190	
Residual	3.33	7	0.4756			
Lack of Fit	0.5106	2	0.2553	0.4529	0.6595	not significant
Pure Error	2.82	5	0.5637			
Cor Total	17.08	29				

An insignificant model for emulsification index was obtained from the results of analysis of variance with a p-value of 0.4801 (Table 6) with the interaction of monosodium glutamate with yeast extract being the most significant term of the model. The F-value of 1.1 indicated that the model is not significant to the noise and there is a 48.01% probability that the F-value is due to noise. The lack of fit F-value also points that the model for emulsification index is insignificant and an adequate precision value of 3.88 indicated insufficient signal. Therefore, this model was considered further for neural network fitting.

Table 6: Analysis of variance for emulsification index

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9976.71	22	453.49	1.10	0.4801	not significant
A-Glucose	341.55	1	341.55	0.8316	0.3921	
B-Monosodium Glutamate	66.30	1	66.30	0.1614	0.6998	
C-Yeast Extract	1107.27	1	1107.27	2.70	0.1446	
D-MgSO ₄	661.16	1	661.16	1.61	0.2451	

AB	3.35	1	3.35	0.0082	0.9306	
AC	206.23	1	206.23	0.5021	0.5015	
AD	885.24	1	885.24	2.16	0.1855	
BC	2476.58	1	2476.58	6.03	0.0437	
BD	678.27	1	678.27	1.65	0.2396	
CD	19.65	1	19.65	0.0478	0.8331	
A ²	1198.53	1	1198.53	2.92	0.1313	
B ²	234.77	1	234.77	0.5716	0.4743	
C ²	0.6119	1	0.6119	0.0015	0.9703	
D ²	16.97	1	16.97	0.0413	0.8447	
ABC	0.6976	1	0.6976	0.0017	0.9683	
ABD	211.90	1	211.90	0.5159	0.4958	
ACD	70.00	1	70.00	0.1704	0.6921	
BCD	902.72	1	902.72	2.20	0.1818	
A ² B	3.33	1	3.33	0.0081	0.9308	
A ² C	1445.30	1	1445.30	3.52	0.1028	
A ² D	451.28	1	451.28	1.10	0.3294	
AB ²	27.67	1	27.67	0.0674	0.8027	
Residual	2875.01	7	410.72			
Lack of Fit	725.91	2	362.95	0.8444	0.4831	not significant
Pure Error	2149.10	5	429.82			
Cor Total	12851.72	29				

Response surface methodology has been used to analyse the interaction of independent variable for any particular response. The concentration of bioemulsifier, exopolysaccharide as well as biomass might be affected by concentrations of four variables and the interaction between them. From the results of response surface model it can be seen that bioemulsifier production is affected by the concentrations of glucose, yeast extract and magnesium sulphate alone or by interaction between glucose-magnesium sulphate, monosodium glutamate-yeast extract, monosodium glutamate-magnesium sulphate, glucose- yeast extract and monosodium glutamate-yeast extract-magnesium sulphate (Figures 1 and 2).

Figure 1: Contour and 3D surface plot depicting the interaction of glucose and MgSO₄ (A-B) and monosodium glutamate and MgSO₄ (C-D) for bioemulsifier

Figure 2: Contour and 3D surface plot depicting the interaction of monosodium glutamate and yeast extract (A-B) and glucose and yeast extract (C-D) for bioemulsifier

Figure 3: Interaction of monosodium glutamate and $MgSO_4$ for the production of exopolysaccharide by contour and 3D surface plot (A-B) and biomass (C-D)

The interaction between monosodium glutamate and magnesium sulphate affects the yield of exopolysaccharide and formation of microbial biomass which is depicted by contour and 3-D surface plots (Figure 3) whereas the emulsification ability is affected by the interaction between monosodium glutamate and yeast extract (Figure 4).

Figure 4: Effect of interaction of monosodium glutamate and yeast extract on emulsification index as shown by contour plot (A) and response surface plot (B)

The maximum concentrations of bioemulsifier, exopolysaccharide and biomass and emulsification index as calculated experimentally were 6.1 g/l, 17.6 g/l, 2.78 g/l and 60%, respectively. The same values for the model predicted by CCD-RSM was found to be 5.3 g/l, 14.9 g/l, 2.6 g/l and 52% for bioemulsifier, exopolysaccharides and biomass concentrations and emulsification index (Table 7). The validation of the current model was performed by genetic algorithm and naïve sorted genetic algorithm to further maximize the yield of all the products.

Table 7: The actual and RSM predicted values for bioemulsifier, exopolysaccharide, biomass production and emulsification ability by *Brevibacillus borstelensis*

Run	Bioemulsifier (g/l)		EPS (g/l)		Biomass (g/l)		EI (%)	
	Actual	RSM	Actual	RSM	Actual	RSM	Actual	RSM
1	4.6	3.8	13.5	10.8	2.6	2.4	47	39
2	0.1	0.6	0.2	3.9	0.1	0.2	0	4
3	4.2	4.7	12.9	16.6	2.4	2.5	47	51

4	0.8	1.3	0.3	3.9	0.2	0.3	7	10
5	4.9	5.2	13.8	12.8	1.1	1.1	50	54
6	1.0	0.2	0.1	0.1	0.1	0.0	13	6
7	1.3	1.2	7.0	7.2	0.1	0.9	14	28
8	5.1	4.3	14.5	11.8	1.2	1.0	53	46
9	4.1	3.3	12.2	9.5	1.9	1.7	40	32
10	3.8	3.0	12.3	9.6	0.6	0.4	47	39
11	0.3	1.2	7.4	7.2	0.3	0.9	13	28
12	3.4	3.7	4.0	3.0	1.3	1.4	36	40
13	5.4	5.7	14.5	13.5	0.9	1.0	53	57
14	5.1	5.4	14.1	13.1	0.6	0.7	53	57
15	0.1	0.7	0.3	2.4	0.1	0.2	6	2
16	5.3	4.5	14.8	12.1	0.8	0.6	53	46
17	6.1	5.3	17.6	14.9	2.8	2.6	60	52
18	3.3	3.8	6.4	10.1	1.4	1.5	38	41
19	4.6	5.1	0.4	4.1	1.5	1.6	45	49
20	1.7	1.2	7.6	7.2	1.7	0.9	18	28
21	0.4	0.7	1.8	0.8	0.7	0.7	6	10
22	1.2	1.2	7.0	7.2	0.3	0.9	13	28
23	0.3	0.6	7.0	6.0	1.0	1.0	6	10
24	0.4	0.7	3.8	2.8	0.7	0.8	5	9
25	1.0	1.2	7.2	7.2	1.1	0.9	55	28
26	1.6	1.9	12.4	11.4	1.7	1.8	17	21
27	0.2	0.7	0.8	4.5	0.1	0.2	9	13

28	1.5	1.2	7.2	7.2	1.8	0.9	55	28
29	5.0	5.5	13.2	16.9	0.7	0.8	64	67
30	1.1	1.6	0.4	4.1	1.0	1.1	18	22

Studying these interactions by one-factor-at-a-time experiments would be a very time consuming and costly process. Hayder et al. (2014) optimized six factors for the production of bioemulsifier. These six factors (incubation time, temperature, pH, carbon and nitrogen source and effect of oil and hydrocarbon) were changed one at time keeping the remaining factors constant (Hayder et al. 2014). A full factorial design (2^4) was used to study the interaction of concentrations of urea, corn oil, potassium dihydrogen orthophosphate and ammonium sulphate for bioemulsifier production by *Candida lipolytica* (Albuquerque et al. 2006). A central composite design with five factors were used to obtain an optimum medium for bioemulsifier production by *Bacillus thuringiensis* pak2310 (Rajendran et al. 2014). Apart from optimization of bioemulsifier production, CCD-RSM has also been employed for the production of exopolysaccharide by microorganisms. CCD-RSM was used for exopolysaccharide production by *Lactobacillus plantarum* (Imran et al. 2016) whereas RSM along with ANN has been used to maximize production of exopolysaccharide by *Lactobacillus* sp (Suryawanshi et al. 2019). CCD-RSM was also employed to optimize process parameters for exopolysaccharide production by *Enterococcus faecium* (Chauhan et al. 2019). The process parameters for production of exopolysaccharide by *Klebsiella oxytoca* was also optimized by Plackett–Burman design (PBD) and Box – Behnken design (BBD) (Moghannem et al. 2017).

3.3.Genetic algorithm for medium optimization

The genetic algorithm solver was applied to optimize medium components for bioemulsifier, exopolysaccharide and biomass production individually with equations 1-3 as fitness functions. The objective function provided the value of the maximum production depending

upon the process variables (glucose, monosodium glutamate, yeast extract and magnesium sulphate). The results of genetic algorithm depend upon the population size, rate of mutation and crossover and the selection function chosen. Considering all these factors, the highest production of bioemulsifier was 7.3 g/l, exopolysaccharide as 17.8 g/l and biomass 3.3 g/l. (Figure 5). These values show the maximum production of all the responses that can be obtained using this model.

Figure 5: The predicted results of bioemulsifier (A), exopolysaccharide (B) and biomass (C) yield by genetic algorithm

Various bioprocesses have been optimized using genetic algorithm (Angelov et al. 1997, Park et al. 1997, Angelova et al. 2011) and hybrid genetic algorithm (Rivera et al. 1993). Hybrid genetic algorithm combines neural network and genetic algorithm. The optimization of bioemulsifier production has not been performed using genetic algorithm whereas only a few reports are available for optimization of exopolysaccharide production by *Burkholderia cepacia* (Strino et al. 2005) and *Lactobacillus plantarum* using genetic algorithm (Desai et al. 2006).

3.4. Process optimization using NSGA

The results of Pareto optimization obtained by naïve and sorted genetic algorithm were analysed using MATLAB R2016a. *Brevibacillus borstelensis* was used for the production of bioemulsifier, exopolysaccharide and biomass in medium with different concentrations of glucose, monosodium glutamate, yeast extract and magnesium sulphate. These conditions were used to build a regression model where maximization of all the three responses was desired. Equations 1-3 were used as fitness function and the objective functions in terms of

the best values of responses were calculated. The Pareto optimal solutions as given by naïve and sorted genetic algorithm is shown in Figure 6. The optimum value of responses as predicted by NSGA for all the three equations were 6.3 g/l, 24 /l and 2.5 g/l of bioemulsifier, exopolysaccharide and biomass, respectively. These values are close to the experimental results which also validates the model. Various statistical tools such as genetic algorithm, artificial neural network, simulated annealing have been used for the process optimization of either of the variables (single objective) but naïve and sorted genetic algorithm for solving multi-objective problem has not been used so far for these responses. Hence, in the current study three objectives were optimized simultaneously for maximization of products.

Figure 6: Multi-objective optimization using NSGA showing the percentage of criteria met and Pareto front between bioemulsifier and exopolysaccharide production

3.5.Validation of the experimental model

The experimental design as provided by CCD was validated by RSM, genetic algorithm and NSGA and the results were compared with those obtained experimentally. The maximum production of bioemulsifier, exopolysaccharide, biomass from experimental run were 6.1 g/l, 17.6 g/l, 2.8 g/l which was obtained for 22 g/l glucose, 14 g/l monosodium glutamate, 6 g/l

yeast extract and 0.6 g/l magnesium sulphate whereas emulsification index (64%) was maximum at 26 g/l glucose, 10 g/l monosodium glutamate, 4 g/l yeast extract and 0.8 g/l magnesium sulphate. The maximum bioemulsifier concentration as predicted by RSM was at 22 g/l glucose, 6 g/l monosodium glutamate, 2 g/l yeast extract and 1 g/l magnesium sulphate and that of exopolysaccharide was 18 g/l glucose, 10 g/l monosodium glutamate, 8 g/l yeast extract and 0.8 g/l magnesium sulphate.

The maximum biomass, both experimentally and as predicted by RSM, was formed when 22 g/l glucose, 14 g/l monosodium glutamate, 6 g/l yeast extract and 0.6 g/l magnesium sulphate were used as the factors for production. The RSM-predicted value for emulsification index also coincides with that obtained experimentally. The results of genetic algorithm and NSGA were generated in terms of objective function which gives the output value of responses. The objective functions obtained by GA for all the responses were 7.3, 17.8 and 3.3. These values correspond to the concentrations (g/l) of bioemulsifier, exopolysaccharide and biomass, respectively. These values are slightly higher than that found experimentally as GA maximizes the function and provides the best value for a given model. Similarly, the objective function for NSGA yielded 6.3, 24 and 2.5 for bioemulsifier (g/l), exopolysaccharide (g/l) and biomass (g/l), respectively which also resembles the experimental values. But the advantage of NSGA is its ability to optimize multiple responses at once. The predicted results of all the statistical tools, namely RSM, GA and NSGA confirm that they are very close and in agreement to the experimental values. Hence, the models formed by different variable for multiple responses are valid and fit well with the experimental ones.

4. Conclusion

Central composite design-response surface model was used to optimize the medium components for maximum bioemulsifier, exopolysaccharide and biomass production by

Brevibacillus borstelensis. Genetic algorithm and naïve and sorted genetic algorithm were used to validate the model obtained from RSM. The highest yield for all the products obtained by RSM varied for different variables and responses which was 6.1 g/l (bioemulsifier), 17.6 g/l (exopolysaccharide) and 2.8 g/l (biomass). The maximum yield predicted by GA was 7.3, 17.8 and 3.3 whereas the predicted results by NSGA were 6.3 g/l, 24 g/l and 2.5 g/l. The predicted values were found to be better than the experimental values due to the ability of these tools to maximize objective functions. The study concludes that ANN is a better optimization tool when compared with response surface model while NSGA is most suitable to optimize multiple responses simultaneously. This study also provides a solution to the problem of enhanced bioproduct synthesis as it involves a fast and cost-effective approach for maximum yield.

Declarations

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References

Albuquerque, C. D., A. M. Filetti and G. Campos-Takaki (2006) Optimizing the medium components in bioemulsifiers production by *Candida lipolytica* with response surface method. *Canadian Journal of Microbiology* 52: 575-583.

406 Angelov, P. and R. Guthke (1997) A genetic-algorithm-based approach to optimization of
 407 bioprocesses described by fuzzy rules. *Bioprocess Engineering* 16: 299-303.

408 Angelova, M. and T. Pencheva (2011) Tuning genetic algorithm parameters to improve
 409 convergence time. *International Journal of Chemical Engineering* 2011.

410 Bertrand, B., F. Martínez-Morales, N. S. Rosas-Galván, D. Morales-Guzmán and M. R.
 411 Trejo-Hernández (2018) Statistical Design, a Powerful Tool for Optimizing Biosurfactant
 412 Production: A Review. *Colloids and Interfaces* 2: 36.

413 Chauhan, A. and S. S. Kanwar (2019) Optimization of exopolysaccharide production by
 414 response surface methodology from *Enterococcus faecium* isolated from the fermented foods
 415 of Western Himalaya. *International Journal of Scientific Research in Biological Sciences* 6:
 416 01-11.

417 Chen, F., H. Li, Z. Xu, S. Hou and D. Yang (2015) User-friendly optimization approach of
 418 fed-batch fermentation conditions for the production of iturin A using artificial neural
 419 networks and support vector machine. *Electronic Journal of Biotechnology* 18: 273-280.

420 Desai, K., S. Akolkar, Y. Badhe, S. Tambe and S. Lele (2006) Optimization of fermentation
 421 media for exopolysaccharide production from *Lactobacillus plantarum* using artificial
 422 intelligence-based techniques. *Process Biochemistry* 41: 1842-1848.

423 Dhagat, S. and S. E. Jujjavarapu (2020) Isolation of a novel thermophilic bacterium capable
 424 of producing high-yield bioemulsifier and its kinetic modelling aspects along with proposed
 425 metabolic pathway. *Brazilian Journal of Microbiology* 51: 135-143.

426 Eswari, J. S., M. Anand and C. Venkateswarlu (2013) Optimum culture medium composition
 427 for rhamnolipid production by *Pseudomonas aeruginosa* AT10 using a novel multi-objective
 428 optimization method. *Journal of Chemical Technology & Biotechnology* 88: 271-279.

429 Eswari, J. S., M. Anand and C. Venkateswarlu (2016) Optimum culture medium composition
 430 for lipopeptide production by *Bacillus subtilis* using response surface model-based ant colony
 431 optimization. *Sadhana* 41: 55-65.

432 Eswari, J. S. and C. Venkateswarlu (2016) Multiobjective simultaneous optimization of
 433 biosurfactant process medium by integrating differential evolution with artificial neural
 434 networks. *Indian Journal of Chemical Technology (IJCT)* 23: 335-344.

435 Fonseca, R., A. Silva, F. De França, V. Cardoso and E. Sérvulo (2007) Optimizing
 436 carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain.'In': *Applied*
 437 *biochemistry and biotechnology*, Springer.

438 Glaser, P., A. Danchin, F. Kunst, P. Zuber and M. M. Nakano (1995) Identification and
 439 isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*.
 440 *Journal of bacteriology* 177: 1112-1115.

441 Hayder, N. H., S. Alaa and H. Abdulmalik (2014) Optimized conditions for bioemulsifier
 442 production by local *Streptomyces* sp. SS 20 isolated from hydrocarbon contaminated soil.
 443 *Romanian Biotechnological Letters* 19: 8979-8993.

444 Imran, M. Y. M., N. Reehana, K. A. Jayaraj, A. A. P. Ahamed, D. Dhanasekaran, N.
 445 Thajuddin, N. S. Alharbi and G. Muralitharan (2016) Statistical optimization of
 446 exopolysaccharide production by *Lactobacillus plantarum* NTMI05 and NTMI20.
 447 *International Journal of Biological Macromolecules* 93: 731-745.

448 Jin, H., X. Zhang, K. Li, Y. Niu, M. Guo, C. Hu, X. Wan, Y. Gong and F. Huang (2014)
 449 Direct bio-utilization of untreated rapeseed meal for effective iturin A production by *Bacillus*
 450 *subtilis* in submerged fermentation. *PLoS One* 9: e111171.

451 Lee, B.-S. and E.-K. Kim (2004) Lipopeptide production from *Bacillus* sp. GB16 using a
 452 novel oxygenation method. *Enzyme and microbial technology* 35: 639-647.

453 Liu, X., B. Ren, H. Gao, M. Liu, H. Dai, F. Song, Z. Yu, S. Wang, J. Hu and C. R. Kokare
 454 (2012) Optimization for the production of surfactin with a new synergistic antifungal activity.
 455 PloS one 7: e34430.

456 Moghannem, S. A., M. M. Farag, A. Shehab and M. S. Azab (2017) Media optimization for
 457 exopolysaccharide producing *Klebsiella oxytoca* KY498625 under varying cultural
 458 conditions. International Journal of Advanced Research in Biological Sciences 4: 16-30.

459 Mukherjee, S., P. Das and R. Sen (2006) Towards commercial production of microbial
 460 surfactants. TRENDS in Biotechnology 24: 509-515.

461 Mukherjee, S., P. Das, C. Sivapathasekaran and R. Sen (2008) Enhanced production of
 462 biosurfactant by a marine bacterium on statistical screening of nutritional parameters.
 463 Biochemical Engineering Journal 42: 254-260.

464 Park, L., C. Park, C. Park and T. Lee (1997) Application of genetic algorithms to parameter
 465 estimation of bioprocesses. Medical and biological engineering and computing 35: 47-49.

466 Peng, W., J. Zhong, J. Yang, Y. Ren, T. Xu, S. Xiao, J. Zhou and H. Tan (2014) The artificial
 467 neural network approach based on uniform design to optimize the fed-batch fermentation
 468 condition: application to the production of iturin A. Microbial Cell Factories 13: 54.

469 Rajendran, D., P. Venkatachalam and J. Ramakrishnan (2014) Response surface
 470 methodology: optimisation of antifungal bioemulsifier from novel *Bacillus thuringiensis*. The
 471 Scientific World Journal 2014.

472 Ramos, H. C., T. Hoffmann, M. Marino, H. Nedjari, E. Presecan-Siedel, O. Dreesen, P.
 473 Glaser and D. Jahn (2000) Fermentative metabolism of *Bacillus subtilis*: physiology and
 474 regulation of gene expression. Journal of Bacteriology 182: 3072-3080.

475 Rivera, S. and M. Karim (1993) Use of micro-genetic algorithms in bioprocess optimization.
 476 IFAC Proceedings Volumes 26: 245-248.

477 Sahoo, P. and T. K. Barman (2012) ANN modelling of fractal dimension in machining.'In':
478 Mechatronics and Manufacturing Engineering, Elsevier.

479 Sen, R. and T. Swaminathan (1997) Application of response-surface methodology to evaluate
480 the optimum environmental conditions for the enhanced production of surfactin. Applied
481 Microbiology and Biotechnology 47: 358-363.

482 Strino, F., A. Nahmany, J. Rosen, G. J. Kemp, I. Sá-correia and P.-G. Nyholm (2005)
483 Conformation of the exopolysaccharide of Burkholderia cepacia predicted with molecular
484 mechanics (MM3) using genetic algorithm search. Carbohydrate research 340: 1019-1024.

485 Suryawanshi, N., S. Naik and J. S. Eswari (2019) Extraction and optimization of
486 exopolysaccharide from Lactobacillus sp. using response surface methodology and artificial
487 neural networks. Preparative Biochemistry and Biotechnology 49: 987-996.

488 Venkateswarlu, C. and S. E. Jujjavarapu 2019 Stochastic Global Optimization Methods and
489 Applications to Chemical, Biochemical, Pharmaceutical and Environmental Processes.
490 Elsevier.

491 Zhao, P., C. Quan, L. Jin, L. Wang, J. Wang and S. Fan (2013) Effects of critical medium
492 components on the production of antifungal lipopeptides from Bacillus amyloliquefaciens Q-
493 426 exhibiting excellent biosurfactant properties. World Journal of Microbiology and
494 Biotechnology 29: 401-409.

495

Figures

Figure 1

Contour and 3D surface plot depicting the interaction of glucose and MgSO4 (A-B) and monosodium glutamate and MgSO4 (C-D) for bioemulsifier

Factor Coding: Actual

Bioemulsifier (g/l)

● Design Points

0.1 6.1

X1 = B

X2 = C

Actual Factors

A = 18

D = 0.8

(A)

Factor Coding: Actual

Bioemulsifier (g/l)

● Design Points

● Above Surface

○ Below Surface

0.1 6.1

X1 = B

X2 = C

Actual Factors

A = 18

D = 0.8

(B)

Factor Coding: Actual

Bioemulsifier (g/l)

● Design Points

0.1 6.1

X1 = A

X2 = C

Actual Factors

B = 10

D = 0.8

(C)

Factor Coding: Actual

Bioemulsifier (g/l)

● Design Points

● Above Surface

○ Below Surface

0.1 6.1

X1 = A

X2 = C

Actual Factors

B = 10

D = 0.8

(D)

Figure 2

Contour and 3D surface plot depicting the interaction of monosodium glutamate and yeast extract (A-B) and glucose and yeast extract (C-D) for bioemulsifier

Figure 3

Interaction of monosodium glutamate and MgSO₄ for the production of exopolysaccharide by contour and 3D surface plot (A-B) and biomass (C-D)

Figure 4

Effect of interaction of monosodium glutamate and yeast extract on emulsification index as shown by contour plot (A) and response surface plot (B)

Figure 5

The predicted results of bioemulsifier (A), exopolysaccharide (B) and biomass (C) yield by genetic algorithm

Figure 6

Multi-objective optimization using NSGA showing the percentage of criteria met and Pareto front between bioemulsifier and exopolysaccharide production

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