Multi-response optimization of rhamnolipid production using grey rational analysis in Taguchi method

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A B S T R A C T

The present paper envisages the multi-response optimization of certain process parameters like total sugars concentration, C/N ratio and incubation time on rhamnolipid yield, surface tension reduction, biomass formation and substrate utilization, in rhamnolipid production by a *Pseudomonas aeruginosa* mutant strain grown on clarified blackstrap molasses, under L_9 orthogonal array in Taguchi design. The results have been analyzed by using grey relational analysis for the identification of an optimum level of process parameters. The validity of the results was checked though confirmation experiment, under grey relational analysis. Subsequently, the rhamnolipid yield improved from 1.45 to 1.50 g/L, substrate utilization reduced from 26 to 14% (w/v) and lesser biomass was formed. Moreover, the volumetric productivity of the process improved from 0.0086 to 0.0208 g/L/h by 142%. Furthermore, using analysis of variance method, significant contributions of process parameters were determined.

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1. Introduction

Emulsifiers and surfactants are widely used in the petroleum, pharmaceuticals, cosmetics, foods, environmental protection and crude oil recovery. The biosurfactants are making their place in surfactants market due to their lower toxicity, biodegradability, selectively and specific activity at extreme environmental conditions [19]. They are actually extracellular biopolymers produced by bacteria, yeast and fungi and in particular by native and recombinant bacteria when grown on distant carbon sources [25,27]. Nevertheless, a cost-effective production of biosurfactant is a major challenge which necessitates the study of low-price carbon sources for enhanced quantity without compromise in quality of biosurfactant. Some studies dealt with the use of plant-derived oils, oily wastes and lactic whey as carbon sources [2].

Specifically, *pseudomonas* strains are well known for their ability to produce rhamnolipid type of biosurfactants when grown on various renewal resources, especially agro-industrial wastes, such as molasses, for biosurfactants production. This leads to the greater possibility for economical production and reduced pollution caused by those wastes [26]. The main reasons for widespread use of molasses as substrate are their low price compared to other sources of sugar and their possession of several other compounds

and vitamins [8,10,15]. The production of a biosurfactant by various bacterial strains is being well studied today, and studies on optimizing the conditions of biosurfactant production, including temperature, pH, salinity, non-hydrocarbon and hydrocarbon substrates, nitrogen source type, and the C/N ratio had been treated as the most important aspects of this field [6]. Nevertheless, no significant literature is available regarding the statistical modeling, including Taguchi design, for rhamnolipids production on renewable substrates.

Taguchi design undertakes orthogonal arrays to reduce the number of experiments required to determine the optimal setting of process parameters. The effectiveness of the Taguchi method for improving quality in industry has extensively been verified. However, most of the Taguchi applications concerned with the optimization of only one response, while most of the industrial problems are concerned with multiple responses [28]. Whereas, grey relational analysis (GRA), based on grey system theory, is the solution for solving the problem of complicated interrelationships among the multi-responses. The term 'Grey' lies between 'Black' (symbols no information) and 'White' (symbols full information), and it symbolizes that the information is partially available. It is suitable to unascertained problems with poor and incomplete information.

This method transforms multiple quality characteristics into single grey relational grades. By comparing the computed grey relational grades, the arrays of respective quality characteristics are obtained in accordance with response grades to select an optimal set of process parameters. This methodology has been

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widely applied in many industries such as biotechnology, food processing, molecular biology, wastewater treatment, and bioremediation [4,9].

In this study, using the grey relational method, different process parameters for the best multiple quality characteristics have been investigated. The input factors were total sugars concentration, C/N ratio and time of incubation, and the responses being sugars utilization, biomass formation, rhamnolipids yield and surface tension. The microbial growth and product formation kinetics were also studied by evaluating different yield parameters such as: the product yields related to substrate consumption and to biomass, biomass yield related to substrate consumption, and volumetric productivity of the fermentation system.

The present study is the extension of our previous work [24] with the purpose to assess and multi-response optimize the best consistent conditions for rhamnolipid production by *Pseudomonas aeruginosa* mutant strain grown on molasses on the basis of grey relational analysis in Taguchi design. Lower number of experiments, minimization of variation in response results and presentation of results with higher applicability are such substantial advantages of this method [31]. The molasses, rich in various nutrients and one of the main byproducts of sugar industry, was evaluated as the cheapest substrates to produce value-added products such as rhamnolipids. Finally analysis of variance (ANOVA) and confirmation test have been conducted to validate the experimental results.

2. Materials and methods

2.1. Growth substrate

The growth substrate of sugar cane blackstrap molasses was obtained from a local sugar industry. The molasses was clarified according to a modified method [14]. The pre-treated samples were stored in separate glass jars at 4 °C until needed for analyses and/or rhamnolipid production.

2.2. Total organic carbon

Total organic carbons (TOCs) in clarified molasses were determined by a modified colorimetric method [11].

2.3. Total sugars

Total sugars (TS) in clarified molasses were determined by the standard dinitrosalicylic acid (DNS) method [16]. Each test was conducted in triplicate and the values of averages are reported.

2.4. Bacterial strain

The present work investigates the growth behavior of hydrocarbon utilizing gamma ray-induced mutant strain, *P. aeruginosa* EBN-8 [25]. The strain was first adapted to molasses, and then a single bacterial colony was transferred to nutrient broth (Oxoid) and incubated at 37 ± 1 °C and 100 rpm in an orbital shaker for 48 h. The cells were harvested by centrifugation (at 8000 rpm and 4 °C for 15 min), washed with filter-sterilized normal saline (0.89% w/v, NaCl) and re-suspended in it to set an absorbance of 0.7 at 660 nm. This cell suspension was used as inoculum for inoculation in further shake flask experiments.

2.5. Plan of investigation

Two experimental setups were established using clarified molasses as carbon source to produce biosurfactants. In the first setup, varying concentrations of molasses (without NaNO₃ addition) on the basis of total sugars (1–3% w/v) were used as the carbon source (at native C/N ratio of 30). The carbon contents (C) in the media are adjusted on the basis of TOCs. In the second setup, NaNO₃ was added to the respective concentrations of molasses to adjust the C/N ratio of 20 or 10 of the media. The pH value of the media was set at 7.0, followed by sterilization.

The Taguchi method uses a special deign of orthogonal array (OA) with the grey relational analysis in order to study the entire parameter space with a small number of experiments. The full factorial design could require $3^3 = 27$ experimental runs, which would make the effort and experimental cost prohibitive and unrealistic. However, the experimental design of an OA required only nine experiments. The factors and their levels considered in this study are shown in Table 1. The experiments were conducted with three factors each at three levels and hence a three level L_9 OA was chosen, as shown in Table 2. Only main effects were considered, whereas interaction effects were assumed to be negligible.

The production experiments were conducted in three independent replicates and data reported are the mean values of three readings. The chemicals were of analytical grade, and used as received from the supplier without further purification.

2.6. Fermentation process follow-up

Various process parameters were monitored, during the tenure of rhamnolipid production on molasses under shake flask condition; the most considerable of them were the changes in surface tension, residual substrate, dry cell biomass (DCBM) and rhamnolipid contents. According to Zhang and Miller [34], three-way interaction between the biosurfactant, substrate and cells is very critical to achieve an enhanced production rate and to understand the kinetics of fermentation process.

2.7. Biomass estimation

The DCBM in the culture medium was determined after harvesting the cells by centrifugation ($7740 \times g$, 15 min) the culture broth in a centrifuge machine (Beckman; T2-HS Centrifuge with Rotor JA-20). The cell pellet was desiccated at 60 °C to a constant mass. The cell-free culture broth (CFCB) alongside obtained was saved to determine its substrate utilization, rhamnolipid contents and surface tension.

2.8. Measurement of surface tension

The equilibrated surface tension of the CFCB was measured by using a Theta lite Optical Tensiometer (Biolin, Finland).

2.9. Extraction of rhamnolipids

Crude biosurfactants were extracted from the CFCB by acid precipitation followed by liquid–liquid extraction by using a solvent system of chloroform/methanol (2:1, v/v) mixture [34]. The resultant solvent extracts were transferred to a round-bottom flask connected to a rotary evaporator. The concentration process was continued at 40 °C until a consistently viscous precipitate of crude biosurfactant was obtained, which was then freeze-dried.

Table 1
Factors and levels.

Factor	Code	Unit	Levels		
			1	2	3
TS	A	% (w/v)	1	2	3
C/N ratio	B	–	10	20	30
Incubation time	C	days	3	5	7

Table 2
L_9 Orthogonal array with factors and responses.

Run	TS % (w/v)	C/N ratio –	Incubation time (days)	Utilized TS % (w/v)	DCBM (g/L)	RL (g/L)	ST (mN/m)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{X/S}$ (g/g)	P_V (g/L/h)
1	1	10	3	24	0.65	0.80	32.0	3.33	1.23	2.71	0.0111
2	1	20	5	39	1.11	0.90	31.0	2.31	0.81	2.85	0.0075
3	1	30	7	47	1.10	0.88	31.0	1.87	0.80	2.34	0.0052
4	2	10	5	19	1.30	1.00	30.0	2.63	0.77	3.42	0.0083
5	2	20	7	26	1.50	1.45	28.0	2.79	0.97	2.88	0.0086
6	2	30	3	13	1.20	1.20	29.0	4.62	1.00	4.61	0.0167
7	3	10	7	19	0.85	0.95	31.0	1.67	1.12	1.49	0.0056
8	3	20	3	10	1.21	0.90	29.5	3.00	0.74	4.03	0.0125
9	3	30	5	15	1.00	1.04	30.0	2.31	1.04	2.22	0.0087

2.10. Colorimetric quantification of rhamnolipids

For rhamnolipids estimation, the crude extract was re-dissolved in distilled water at the neutralized pH value to determine its rhamnose equivalents by the standard orcinol method [5]. The rhamnose concentration was calculated by comparing the data with a standard curve of L-rhamnose and the rhamnolipids as 3.4 times the rhamnose contents [3].

2.11. Kinetics of fermentation process

The kinetics of fermentation experiments was studied in terms of the product yields related to substrate consumption ($Y_{P/S}$, g/g) and to biomass ($Y_{P/X}$, g/g), biomass yield related to substrate consumption ($Y_{X/S}$, g/g), and volumetric productivity (P_V, g/L/h) of the culture media. The measurements were repeated thrice and their average values were used for calculation.

2.12. Taguchi method

In Taguchi method, the experimental results were transformed into S/N ratios to measure the quality characteristics deviating from the desired value. Regardless of the category of the quality characteristic, a greater S/N ratio corresponded to better quality characteristics [18]. The method of calculating the S/N ratio depends at each run of the experiment on whether the quality characteristic is lower-the-better, higher-the-better, or nominal-the-better [30]. Accordingly, the three cases with respective equations are narrated below:
(a) Upper-bound effectiveness (i.e., higher-the-better)

$$\frac{S}{N}\text{ ratio} = -10\log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_{ij}^2}\right) \tag{1}$$

where y_{ij} = ith replicate of jth response, n = number of replicates = $1, 2, \cdots, n$; $j = 1, 2, \cdots, k$.

Eq. (1) is applied for problem where maximization of the quality characteristic of interest is required.
(b) Lower-bound effectiveness (i.e., lower-the-better)

$$\frac{S}{N}\text{ ratio} = -10\log\left(\frac{1}{n}\sum_{i=1}^{n}y_{ij}^2\right) \tag{2}$$

Eq. (2) is applied for the problem where minimization of the quality characteristic is required.
(c) Moderate effectiveness (i.e., nominal-the-best)

$$\frac{S}{N}\text{ ratio} = 10\log\left(\frac{\bar{y}^2}{s^2}\right) \tag{3}$$

where, $\bar{y} = \frac{y_1 + y_2 + y_3 \cdots + y_n}{n}$ and $s^2 = \frac{\Sigma(y_i - \bar{y})^2}{n-1}$

A nominal-the-best type of problem is one where minimization of the mean squared error around a specific target value is desired.

Adjusting the mean on target by any means renders the problem to a constrained optimization problem.

2.13. Grey relational analysis

This sub-section illustrates step-by-step the theory and methodology of GRA.

Step 1: Calculated the S/N ratios for the corresponding responses using one of the formulae (Eqs. (1–3)) depending upon the type of quality characteristic.

Step 2: Normalized the Y_{ij} as Z_{ij} ($0 \leq Z_{ij} \leq 1$) by the following formula to avoid the effect of using different units and to reduce variability. The normalization is a transformation performed on a single input to distribute the data evenly and scale it into acceptable range for further analysis. Haq et al. [12] recommended that the S/N ratio should be used to normalize the data in GRA. For further analysis, normalization is applied on each response to distribute the data evenly and in acceptable range [7].

$$Z_{ij} = \frac{Y_{ij} - \min(Y_{ij}, i = 1, 2, \cdots, n)}{\max(Y_{ij}, i = 1, 2, \cdots, n) - \min(Y_{ij}, i = 1, 2, \cdots, n)} \tag{4}$$

Eq. (4) was used for the S/N ratio with higher-the-better case.

$$Z_{ij} = \frac{\max(Y_{ij}, i = 1, 2, \cdots, n) - Y_{ij}}{\max(Y_{ij}, i = 1, 2, \cdots, n) - \min(Y_{ij}, i = 1, 2, \cdots, n)} \tag{5}$$

Eq. (5) was used for the S/N ratio with lower-the-better case.

$$Z_{ij} = \frac{|Y_{ij} - \text{Target}| - \min(|Y_{ij} - \text{Target}|, i = 1, 2, \cdots, n)}{\max(|Y_{ij} - \text{Target}|, i = 1, 2, \cdots, n)} \\ -\min(|Y_{ij} - \text{Target}|, i = 1, 2, \cdots, n) \tag{6}$$

Eq. (6) is applicable for the S/N ratio with nominal-the-better case.

Step 3: Determined quality loss functions by using the eq. $\Delta = (\text{quality loss}) = |y_o - y_{ij}|$.

Step 4: Computed the grey relational coefficient (GC) for the normalized S/N ratio values.

$$GC_{ij} = \frac{\Delta_{\min} + \delta\Delta_{\max}}{\Delta_{ij} + \delta\Delta_{\max}} \begin{cases} i = 1, 2, \cdots, n \\ j = 1, 2, \cdots, k \end{cases} \tag{7}$$

where

(a) GC_{ij} = grey relational coefficient for the ith replicate of jth response,
(b) Y_{oj} = optimum performance value of the jth response,
(c) Y_{ij} = the ith normalized value of the jth response,
(d) Δ = quality loss = $|y_o - y_{ij}|$,
(e) $\Delta_{\min}$ = minimum value of Δ,
(f) $\Delta_{\max}$ = maxmum value of Δ,
(g) δ = distinguishing coefficient which is defined in the range $0 \leq \delta \leq 1$ (the value may be adjusted on the practical needs of the system.

Step 5: Computed the grey relational grade (G_i)

$$G_i = \frac{1}{m} \sum GC_{ij} \qquad (8)$$

Step 6: Determined the optimal factor and its level combination. Higher the grey relational grade, better the quality of the product is and vice versa. The factor effect and the optimal level for a controllable factor could be determined on the basis of grey relational grade. For each level of j of each factor i, we calculated the average of grade values $(AGV)_{ij}$, then the effect of E_i is defined as:

$$E_i = \max(AGV)_{ij} - \min(AGV)_{ij} \qquad (9)$$

For the controllable factor i, the optimum level, j^*, is taken by:

$$j^* = \max(AGV)_{ij} \qquad (10)$$

Step 7: Finally, examined the validity of grey relational analysis.

2.14. Analysis of variance

The ANOVA was performed to find out the statistical significance of the rhamnolipid production parameters. The results were examined to determine the main effects of all the factors. With the grey relational analysis and ANOVA, the optimum combination of the process parameters could be predicted. Finally, a confirmation experiment was conducted to verify the optimal process parameters obtained from the production process design.

3. Results and discussion

The Taguchi method is a systematic approach for design and analyzes the experiments to improve the product quality. This method could simplify the optimization of process parameters for multiple performance characteristics. Rashedi and Assadi [23] used the Taguchi method to optimize rhamnolipid production. Wei et al. [32] used Taguchi method to optimize the trace elemental composition of minimal media for surfactin production by a *Bacillus subtilis* strain. Salehizadeh and Mohammadizad [29] used the Taguchi method to optimize the biosurfactants production by using *Alcaligenes faecalis* strain. Khalifeh et al. [13] used this method to optimize the application of biosurfactants for oily polluted waters clearance recovery. Mnif et al. [17] also investigated the soil washing potency by using Taguchi method in order

to enhance the bioavailability of hydrophobic contaminants for bioremediation.

3.1. Cause and effect

The possible factors and sub-factors which could affect the production process and the yield of rhamnolipid surfactants are shown in Fig. 1. The rhamnolipid yield obtained through a fermentation process generally depends on the microbiology and growth requirements of native or recombinant microbes. In addition, environmental and process factors also contribute to affect the net outcome of rhamnolipid yield. Some of the key factors have been under taken in the present study.

3.2. Production of rhamnolipid surfactant

At the first glance, by changing three factors (i.e., TS concentration, C/N ratio and incubation time), the rate of rhamnolipid produced in 3-level experiments was determined by the orcinol method. The experiments were conducted using L_9 OA and the response values hence obtained are given in Table 2. The results show that the highest rhamnolipid yield of 1.45 g/L, when the TS, C/N ratio and incubation time were 2% (w/v), 20 and 7 days, respectively, under run 5; while the lowest value (corresponding to 0.80 g/L) the TS, C/N ratio and incubation time were 1% (w/v), 10 and 3 days, respectively, under run 1. The amounts of rhamnolipid yields under other conditions have been represented in Table 2. Maximum and minimum values of DCBM were obtained as 1.50 and 0.65 g/L, respectively.

3.3. Surface activity of rhamnolipid

The effectiveness of a biosurfactant is estimated by its ability to lower the ST of the medium. Due to the presence of biosurfactant, less work is required to bring a molecule to the surface, hence the ST of the media decreases. The lowest value of 28 mN/m and the highest value of 32 mN/m of surface tension are related to the run number 5 and 1, respectively (Table 2).

3.4. Rhamnolipid production kinetics

In the present study, maximum ST reduction (50–28 mN/m) of the CFCB coincided the maximum rhamnolipid yield (1.45 g/L) after 7 days of incubation, when the C/N ratio of the molasses
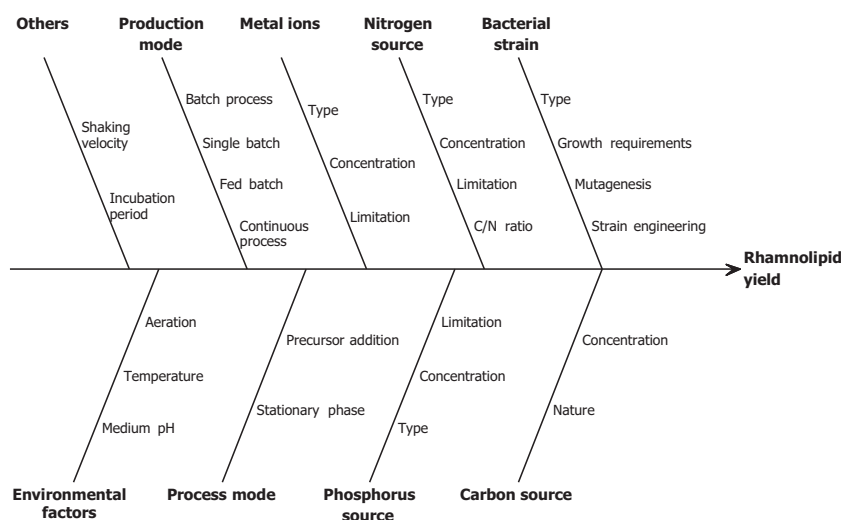

Fig. 1. Cause and effect diagram for rhamnolipid production.

medium (2% TS) was 20, means run 5 (Table 2). Pruthi and Cameotra [21] observed a likewise C/N correlation during the growth of various *Pseudomonas* spp. on *n*-dodecane. Babu et al. [1] obtained 1.60 and 1.78 g/L of cell biomass and rhamnolipids, respectively, with the $Y_{P/S}$ (g/g) and $Y_{P/X}$ (g/g) of 0.089 and 1.110, respectively, when *P. aeruginosa* BS2 was grown on whey waste as carbon source. Dubey and Juwarkar [8] observed 0.91 and 0.92 g biosurfactant/L from distillery and whey wastes, respectively, using an oily sludge isolate *P. aeruginosa* BS2. In the present study, maximum volumetric productivity was observed as 0.0167 g/L/h, under Taguchi method, in contrast to that of 0.008 and 0.012 g/L/h by *P. aeruginosa* GS3 on molasses–corn-steep [20] and *P. aeruginosa* BS2 on whey waste [1], respectively. This comparison indicated an efficient rhamnolipid production by the present molasses-adapted *P. aeruginosa* mutant strain. The maximum $Y_{P/S}$ (g/g) was observed as 4.62 for run 6 and $Y_{P/X}$ (g/g) of 1.23 for run 1 (Table 2). These observations show the rhamnolipids production kinetics improved by using Taguchi approach. The plots of normal probability and standard residuals versus fitted values for rhamnolipid yield are shown in Fig. 2. The factor effects on all the single responses are shown in Fig. 3.

3.5. Grey relational analysis of results

In the GRA, the generation of grey relations was applied to the experimental data related to quality characteristics, the results of which were used to obtain the grey relational grades hence to rank each data series. The ongoing sub-section step-by-step explains the results obtained by using the methodology discussed before.

Step 1: Calculated the S/N ratio values for a given response using one of Eqs. (1) and (2) depending upon the type of quality characteristics. The calculated S/N ratio values for reach response are shown in Table 3. The S/N ratios were expressed as higher-the-better in the case of RL, $Y_{P/S}$, $Y_{P/X}$ and P_V, whereas lower-the-better in the case of utilized TS, DCBM, ST and $Y_{X/S}$. In other words, higher rhamnolipid involving responses were required alongside less utilization of carbon source and limited biomass formation. These considerations have been made with respect to greater quality characteristics of interest.

Step 2: In order to provide the series with comparable characteristics and achieve the objectives of GRA, the normalized S/N ratio values of the multiple objective values were determined by using Eqs. (4) and (5) [7]. The normalized S/N ratio means, when the range of the series is too large or the optimal value of a quality characteristic is too enormous, this could lead to neglect some of

the factors, and the original experimental data must be normalized to eliminate such effect. This step standardizes various attributes, so that every attribute has the same extent of influence, thus the data is made dimensionless, by using upper bound effectiveness, lower bound effectiveness or moderate effectiveness, as exemplified before. The resultant normalized S/N ratios are given in Table 4. Basically, the larger normalized S/N ratio 330 corresponds to the better performance, whereas the best normalized S/N ratio is equal to unity.

Step 3: Based on the above results, the quality loss functions were calculated to measure the performance characteristics deviated from the desired value, by using the equation ($\Delta = |y_o - y_{ij}|$). The resultant values are given in Table 5.

Step 4: The grey relational coefficient was calculated to express the relationship between the ideal (best) and actual normalized S/N ratios. The grey relational co-efficient values were calculated by using Eq. (7) based on the normalized S/N ratios. The results are expressed in Table 6.

Step 5: Next step was to calculate grey relational grade by averaging the grey relational coefficients corresponding to each process response (i.e., 8 responses) (Table 6) by using the Eq. (8). The average of the derived grey relational coefficients equals the grey relational grade [33]. The overall evaluation of the multiple-responses is based on the grey relational grade. As a result, optimization of the complicated multiple process responses could be converted into optimization of a single grey relational grade. The ranking of the series based on their grey relational grades gives the grey relational order (Table 6).

Step 6: Form the values of grey relational grades, the main effects were predicted as shown in Table 7. According to the Taguchi method, the statistic delta defined as the difference between the high and the low effect of each factor was used. A classification could be done to determine the most influencing factor. When so done, the multiple objective optimization problems were transformed into a single equivalent objective optimization problem. Using the grey relational grade value, the mean of the grey relational grade for each level of different factors, and the total mean of the grey relational grade is summarized in Table 7. Then a response graph of the grey relational analysis is obtained by main effect analytic computation, as shown in Fig. 4, hence to obtain the optimal combination of parameters to satisfy multiple quality objectives. Fig. 4 shows the effect of rhamnolipid production factor levels on the grey grade. Basically, the larger the grey relational grade, the better the multiple performance characteristics. A higher grey relational grade indicates that the
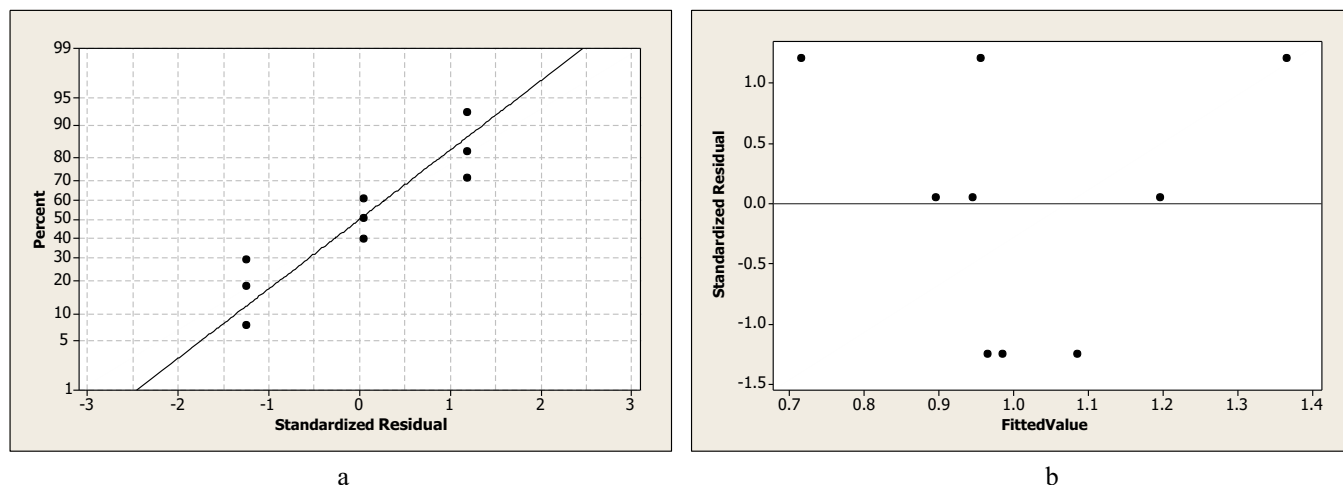

a

b

Fig. 2. Plots of normal probability (a), and standard residuals versus fitted values (b) for RL.
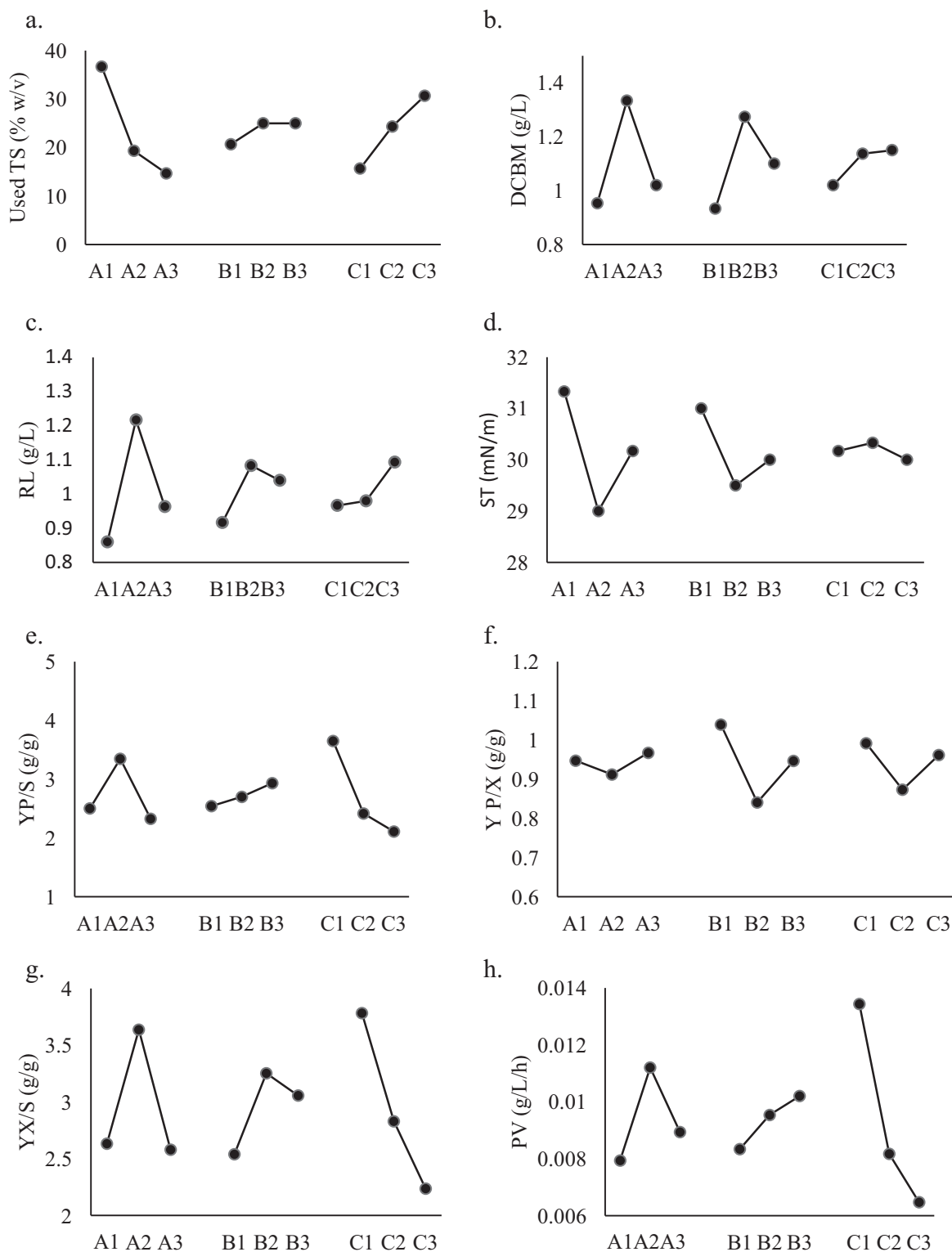
Fig. 3. Factor effects on (a) utilized TS, (b) DCBM, (c) RL, (d) ST, (e) $Y_{P/S}$, (f) $Y_{P/X}$, (g) $Y_{X/S}$ and (h) P_V.

corresponding S/N ratio is closer to the normalized S/N ratio which in return corresponds to a characteristic setup closer to optimal [22].

Step 7: Finally, by considering the maximization of grade values (by using Eq. (10)) as per shown in Table 7 and Fig. 4, we could obtain the optimal process parameter conditions as $A_2B_2C_1$ i.e., a TS of 20% (w/v), C/N ratio of 20 and incubation time of 3 days. Table 9 compares the experimental results of the optimal parameter

combinations derived using Taguchi method and grey relational analysis. As shown in the table, the improvement of 3% was exhibited in rhamnolipid yield, increasing from 1.45 (Taguchi method) to 1.50 g/L (grey relational analysis); and 142% in volumetric productivity. Also, the biomass formation was suppressed up to 33%. It is worth noting that in simple Taguchi method, the maximum rhamnolipid yield (1.45 g/L) was observed at 7 days of incubation, whereas when integrated with GRA an

Table 3
S/N ratio values at all runs.

Run	Utilized TS	DCBM	RL	ST	$Y_{P/S}$	$Y_{P/X}$	$Y_{X/S}$	P_V
1	−27.604	3.742	−1.938	−30.103	10.449	1.805	−8.659	−39.094
2	−31.821	−0.906	−0.915	−29.827	7.272	1.820	−9.097	−42.499
3	−33.44	−0.828	−1.110	−29.827	5.437	1.938	−7.384	−45.680
4	−25.575	−2.279	0.000	−29.542	8.399	2.282	−10.680	−41.618
5	−28.300	−3.522	3.227	−28.943	8.912	0.292	−9.188	−41.310
6	−22.279	−1.584	1.584	−29.248	13.293	0.000	−13.274	−35.546
7	−25.575	1.412	−0.446	−29.827	4.454	−0.969	−3.464	−45.0362
8	−20.000	−1.656	−0.915	−29.396	9.542	2.568	−12.106	−38.062
9	−23.522	0.000	0.341	−29.542	7.272	−0.341	−6.927	−41.210

Table 4
Normalized S/N ratio values at all runs.

Run	Utilized TS	DCBM	RL	ST	$Y_{P/S}$	$Y_{P/X}$	$Y_{X/S}$	P_V
1	0.566	0.000	0.000	1.000	0.678	0.784	0.530	0.650
2	0.879	0.640	0.1980	0.762	0.319	0.788	0.574	0.314
3	1.000	0.629	0.1603	0.762	0.111	0.822	0.400	0.000
4	0.415	0.829	0.375	0.517	0.446	0.919	0.736	0.401
5	0.617	1.000	1.000	0.000	0.504	0.356	0.583	0.431
6	0.1170	0.733	0.682	0.263	1.000	0.274	1.000	1.000
7	0.415	0.321	0.289	0.762	0.000	0.000	0.000	0.064
8	0.000	0.743	0.198	0.391	0.576	1.000	0.881	0.752
9	0.262	0.515	0.441	0.517	0.319	0.178	0.353	0.441

Table 5
Quality loss function values at all runs.

Run	$\Delta_{utilizedTS}$	Δ_{DCBM}	Δ_{RL}	Δ_{ST}	$\Delta.Y_{P/S}$	$\Delta.Y_{P/X}$	$\Delta.Y_{X/S}$	$\Delta.P_V$
1	0.434	1.000	1.000	0.000	0.322	0.216	0.470	0.350
2	0.1206	0.360	0.802	0.238	0.681	0.212	0.426	0.686
3	0.000	0.371	0.840	0.238	0.889	0.178	0.600	1.000
4	0.585	0.171	0.625	0.483	0.554	0.0812	0.264	0.599
5	0.383	0.000	0.000	1.000	0.496	0.644	0.416	0.569
6	0.830	0.267	0.318	0.737	0.000	0.726	0.000	0.000
7	0.585	0.679	0.711	0.238	1.000	1.000	1.000	0.936
8	1.000	0.257	0.802	0.609	0.424	0.000	0.119	0.248
9	0.738	0.485	0.559	0.483	0.681	0.822	0.647	0.559

Table 6
Grey relational co-efficient and grey grade values at all runs.

Run	GC$_{utilizedTS}$	GC$_{DCBM}$	GC$_{RL}$	GC$_{ST}$	GC.$Y_{P/S}$	GC.$Y_{P/X}$	GC.$Y_{X/S}$	GC.P_V	G_i	Grey relational order
1	0.697	0.5	0.5.00	1.000	0.757	0.822	0.680	0.741	0.712	6
2	0.892	0.735	0.555	0.808	0.595	0.825	0.701	0.593	0.713	5
3	1.000	0.729	0.544	0.808	0.529	0.849	0.625	0.500	0.698	7
4	0.631	0.854	0.615	0.674	0.644	0.925	0.791	0.625	0.720	4
5	0.723	1.000	1.000	0.500	0.669	0.608	0.706	0.637	0.730	3
6	0.546	0.789	0.759	0.576	1.000	0.579	1.000	1.000	0.781	1
7	0.631	0.596	0.584	0.808	0.500	0.500	0.500	0.516	0.580	9
8	0.500	0.796	0.555	0.621	0.702086	1.000	0.894	0.801	0.734	2
9	0.575	0.673	0.642	0.674	0.594821	0.549	0.607	0.641	0.620	8

enhanced rhamnolipid amount (1.50 g/L) was obtained just at 3 days of incubation. This reduced the process duration by 57.14%, which resulted in improved process productivity.

Table 7
Main effects on grey grades.

Factor	1	2	3	Max − Min
A	0.708	**0.744**	0.644	0.100
B	0.670	**0.726**	0.700	0.056
C	**0.742**	0.684	0.669	0.073

3.6. Analysis of variance

The ANOVA is successfully applied to investigate which rhamnolipid production parameter significantly affects the performance characteristic. The ANOVA analysis in Table 8 and percentage contributions for each term affecting grey relational grade (Fig. 5) indicate that the TS concentration and incubation time are the significant rhamnolipid production process parameters affecting the multiple performance characteristics. Furthermore, the TS concentration is the most significant process parameter due to its highest percentage contribution (of 50%)
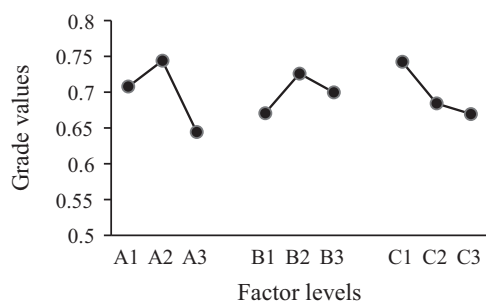
Fig. 4. Factors effects on grade values.

Table 8
Table of ANOVA.

Factor	SS	Df	MS	F-test	% Contribution
A	0.0153	2	0.0076	9.71	50.30
B	0.0046	2	0.0023	2.91	15.10
C	0.0089	2	0.0045	5.67	29.41
Error	0.0016	2	0.0008		5.18
Total	0.0304	8			
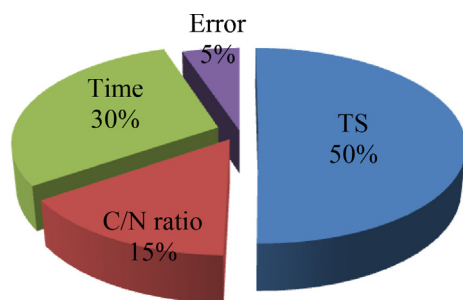
Fig. 5. Percentage contribution of factors in the grey relational grade.

among the process parameters. Based on the above discussion, the optimal rhamnolipid production process parameters were total sugars concentration (2% w/v) at level 2, C/N ratio (20) at level 2 and incubation time (3 days) at level 1.

3.7. Validation of the GRG of optimum result

After the optimal level of the different factors is selected, the final step was to predict the performance characteristic using the optimal level of factors. As none of the experiments shown in Table 2 fits the optimal process conditions, so an experiment was conducted on the basis of predicted run. Table 9 shows the results of the confirmation experiment using optimal factors. As shown in Table 9, rhamnolipid yield increased from 1.45 to 1.50 g/L, substrate utilization decreased from 26 to 14% (w/v) and lesser biomass, being a side-product, was formed. Overall, the volumetric productivity of the process improved from 0.0086 to 0.0208 g/L/h by 142%. Through this study, it is clearly

shown that the multiple performance characteristics are improved. The result of the confirmation tests yielded that the grey relational grade improved from 0.781 (GR order 1) to 0.807, after validation. Therefore, the integration of grey relational analysis and the Taguchi method could be applicable for the optimization of process parameters and help improve the process efficiency.

4. Conclusions

This study proposes an approach integrating the Taguchi method and GRA to identify optimal combination of parameters required to meet multiple quality objectives in rhamnolipid production. The ANONA shows that total sugars concentration has been the most significant factor followed by incubation time and then C/N ratio. The silent features of present study have been low number of experiments, less allocated incubation tenure and less substrate amount under Taguchi based multi-response optimization. Moreover, the use of blackstrap molasses as carbon source accompanies environmental clearance and so on. At the end of day, we find a biocompatible production via sustainable technology.

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Table 9
Comparison of Taguchi method and grey relational analysis results.

Response	Taguchi method (at $A_2B_2C_3$)	Great relational analysis (at $A_2B_2C_1$)	Variation rate (%)
Utilized TS	26.0	14.00	−46
DCBM	1.50	1.00	−33
RL	1.45	1.50	3
ST	28.00	28.00	0
$Y_{P/S}$	2.79	5.36	92
$Y_{P/X}$	0.97	1.50	55
$Y_{X/S}$	2.88	3.57	24
P_V	0.0086	0.0208	142

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