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ORIGINAL ARTICLE

Optimization of Enterocin Production from Probiotic *Enterococcus faecium* Using Taguchi Experimental Design

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ABSTRACT

Objectives: Enterocin is a significant broad-spectrum peptide antibiotic produced by *Enterococcus faecium (E. faecium)*. Enterocin production by *E. faecium* was investigated using the Taguchi experimental design. The Taguchi models were used to save the time and effort required for optimizing the different conditions affecting its production. They were applied to optimize the conditions for enterocin production using the least number of experiments and the least number of required materials.

Materials and Methods: Seven factors i.e., pH, temperature, time of incubation, aeration rate, inoculum size, carbohydrate concentration, and bile salt concentrations, each at three levels were selected and an orthogonal array layout of $L27^3$ was performed.

Results: The experimental results indicated that the best incubation conditions were; 48 hours incubation on a nutrient medium at pH 6.5, temperature at 25 °C, aeration rate at 0 round per minute, inoculum size 20 mL, and bile salt concentration. It was 5%, and the carbon concentration was 2.0%. All these factors combined led to the best enterocin production by *E. faecium*.

Conclusion: This optimization of enterocin production by the Taguchi experimental models emphasized some important results regarding the interaction of the different driving factors leading to the best enterocin production in one experiment.

Keywords: *Enterococcus faecium*, enterocin, optimization, Taguchi design, antibacterial activity

INTRODUCTION

Probiotics are the microflora living in the human intestinal tract. These bacteria are capable of producing peptides called bacteriocins, which have antimicrobial properties. *Enterococcus faecium (E. faecium)* is among these enterocin-producing bacteria.[1]

Enterocins can be used for pathogen control purposes in laboratory experiments, clinical trials, and in the food industry. Enterocins are extracellular products mainly produced by enterococci such as; *E. faecium*, *Enterococcus faecalis*, and *Toxoplasma gondii*. Bacteriocins produced by *Enterococcus* include bacteriocin 35, enterocins A, B, L50A/B, and P, which belong to class II bacteriocins.[2]

Enterocin A of *E. faecium* is a stable peptide that can be potentially used for the treatment of pathogens and cancer. It is a cyclic peptide with an isoelectric point of approximately 10.[3]

Enterocin works as an antimicrobial agent for Gram-positive as well as Gram-negative bacteria, whereas most of the recorded literature states that lactic acid bacteria-derived bacteriocins have an antimicrobial effect only on Gram-positive bacteria.[4]

It has been scientifically reported that enterocins have an antibacterial effect against several types of bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus*.[5] *Enterococci* carry resistance genes in their genetic profile, and bacteriocin a is heat stable, so they can be used as antibacterial agents against foodborne pathogenic microorganisms. Safe bacteria with the same properties can be used to inhibit foodborne pathogens.[6]

The results of any scientific experiment depend on several chemical and physical factors. To obtain the best results from any experiment, those factors have to be optimized in reference

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to each other, and it is much more efficient and time-saving when this aim is achieved with fewer experiments, saving time, effort, and expenses.[7,8]

The Taguchi design has been successfully used to optimize the parameters involved in several experiments.[9] The design was used to adjust the interaction of several variables and their interaction in any given experiment in one process instead of requiring a more significant number of experiments that are often costly and time-consuming. The Taguchi design allows us to obtain the information needed from any experiment by optimizing the variables involved in this experiment and allowing more facility for the best outcome of the system performance, using a smaller number of experiments.[10]

The research explored the power of Taguchi experimental design to optimize and validate the factors affecting enterocin production from the probiotic *E. faecium*.

MATERIALS AND METHODS

Enterocin biosynthesis from E. faecium

E. faecium was isolated and identified in a previous study.[11] It was propagated as 10 mL of *E. faecium* liquid culture was inoculated into 1 L of MRS broth medium. The turbidity of bacterial growth was calibrated using 0.5 McFarland standard where a hundred microliter (1 x 10^7 cells/mL) of bacterial growth culture was used as the standard inoculum and incubated for 48 hours at 30 ˚C under static conditions.

Antibacterial assay

The antibacterial activity was assayed using the agar well diffusion method as follows: 40.0 mL of nutrient agar medium incubated at 55-60 ˚C was inoculated with 200.0 µL of the pathogenic bacteria cell suspensions under test separately and poured into 150.0 mm diameter Petri dishes, mixed well, and allowed to solidify. After solidification, holes of 5.0 mm in diameter were made in the agar plate with the aid of a sterile cork borer. For each sample, duplicate wells were made and then 100.0 µL of the culture filtrate was poured into the prepared holes using an automatic micropipette. The Petri dishes were kept in a refrigerator for 1 hour to permit homogeneous diffusion of the antimicrobial agent before the growth of *S. aureus* American Type Culture Collection (ATCC) 6538 and *Escherichia coli* ATCC 8739, and then the plates were incubated at 37 ˚C for 24 hours for Gram-positive and Gram-negative bacteria. Antimicrobial activities were determined by measuring the diameter of the inhibition zone.[12]

Optimization of enterocin production

Several experiments were carried out to investigate the production rate and the antibacterial activity.

In this experiment, an L-27 standard orthogonal array (OA) was generated for the examination of seven factors, *i.e.*, pH, temperature, incubation time (hour), inoculum size (mL), aeration [round per minute (RPM)] (-1; static, 0; 50 RPM, 100; 100 RPM), carbohydrate concentration (glucose g/L), and bile salt concentration $X10^{-1}$, which were selected on the basis of the results of biosynthesis. The L-27 symbolic array of the experimental matrix represents the number of runs (*i.e.*, 27 experimental trials). The three levels of the seven factors were coded as levels 1, 2, and 3 (Table 1), and the layout of L27 Taguchi's OA is shown in Table 2. The total degrees of freedom (DF) for the OA L-27 set was 26 (number of runs minus one). Runs involved a particular combination of levels to which the factors were set, and the diversity of factors was studied by crossing the factors. Experiments were conducted in duplicate, and factors were studied at three levels.

Statistical analysis

Statistical analysis and graph plotting were conducted using Design-Expert software. Analysis of variance (ANOVA) was used to evaluate the effect of each independent variable on the response, and $p < 0.05$ was considered significant. The multiple correlation coefficient (R^2) and adjusted R^2 were used to evaluate the fitness of the equation. Three-dimensional surface plots were employed to demonstrate the relationships and interactions between the variables and response.

RESULTS

Optimization of enterocin production

This optimization process using Taguchi models enables us to determine the ideal factors in the experimental area. Using Minitab in the experiment provided us with the experimental data for enterocin production and incorporated the quadratic polynomial prototype with ideal parameters.

The maximum enterocin production was achieved at pH= 6.5, temperature= 25 ˚C, incubation time= 48 hours, inoculum size= 20 mL, aeration= 1, carbohydrate concentration= 20, bile salt concentration 5. The variables tested were pH (A), temperature (B), incubation time (C), inoculum size (D), aeration (E), carbohydrate concentration (F), and bile salt concentration (G). The Pareto chart revealed that pH was the best factor that had an effect on enterocin production and carbohydrate concentration but did not show any effect on enterocin production.

A plot of the expected normal values of residuals versus residuals (Figures 1 and 2) showed that data were very close to the straight line and situated on both sides. Where values below the straight line were insignificant and those above

Table 1. Factors and levels selected for experimental use

Factors	Level 1	Level 2	Level 3
pH	5	6.5	8
Temperature	25	35	40
Incubation time (hour)	24	48	72
Inoculum size (mL)	5	10	20
Aeration (RPM)	-1	0	100
Carbohydrate concentration	10	20	30
Bile salt concentration $X10^{-1}$	1	3	5

RPM: Revolutions per minute

the straight line were significant. Where values below the line are insignificant and those above the line are significant. Residuals represent the difference between true and predicted values using our final logistic regression models, whereas the histogram of frequency versus residual had a dumbbell shape. It could be seen that residuals were most concentrated around 5 and had a right skewed distribution.

The main effects for the means of various parameters

Each medium component was tested for enterocin production and was investigated using Taguchi models.

The provided data revealed that the level of enterocin production increases as pH level, aeration, and bile salt concentration decrease. On the other hand, enterocin production increased as the temperature, incubation time, and inoculum size increased, whereas the level of bacteriocin was constant at any carbohydrate concentration.

Statistical analysis

The provided data were used for ANOVA. The different values (*p* value, *f* value, coeffcient of variation, and determination coeffcient) obtained from ANOVA confirmed the significance of the selected model. If the *p* value is less than 0.5, the variables were statistically significant.

ANOVA was used to determine the model's significance. Various values (*p* value, *f* value, coefficient of variation

Table 2. Taguchi experimental layout

Row	pH	Temperature	Incubation time (hour)	Inoculum size (mL)	Aeration (RPM)	Carbohydrate concentration	Bile salt concentration 10X
1	5.0	25	24	5	-1	10	1
2	5.0	25	24	5	0	20	3
3	5.0	25	24	5	100	30	5
4	5.0	35	48	10	-1	10	1
5	5.0	35	48	10	0	20	3
6	5.0	35	48	10	100	30	5
7	5.0	40	72	20	-1	10	1
8	5.0	40	72	20	0	20	3
9	5.0	40	72	20	100	30	5
10	6.5	25	48	20	-1	20	5
11	6.5	25	48	20	0	30	1
12	6.5	25	48	20	100	10	3
13	6.5	35	72	5	-1	20	5
14	6.5	35	72	5	0	30	1
15	6.5	35	72	5	100	10	3
16	6.5	40	24	10	-1	20	5
17	6.5	40	24	10	0	30	1
18	6.5	40	24	10	100	10	3
19	8.0	25	72	10	-1	30	3
20	8.0	25	72	10	0	10	5
21	8.0	25	72	10	100	20	1
22	8.0	35	24	20	-1	30	3
23	8.0	35	24	20	0	10	5
24	8.0	35	24	20	100	20	1
25	8.0	40	48	5	-1	30	3
26	8.0	40	48	5	0	10	5
27	8.0	40	48	5	100	20	1

RPM: Revolutions per minute

and determination coefficient) obtained from ANOVA demonstrate that the selected model was significant at $p < 0.5$, the analysis of variance for means ratio with the DF. From Table 3, we found that pH, incubation time, inoculum size, and aeration had significant effects on response due to ($p < 0.5$). The adjacent sum of squares, adjacent mean square, and probability (P) are shown in Table 4. The point at which the effect estimates were statistically significant was $p= 0.5$.

These results emphasize the impact of each parameter on metabolite synthesis depending on the other factors involved in the fermentation process. The percentage of involvement of each factor is shown in the ANOVA Table 4. The last column of the ANOVA shows how much each factor was involved in the optimization process. The ANOVA results of all studies showed that all factors were effective for the response variables.

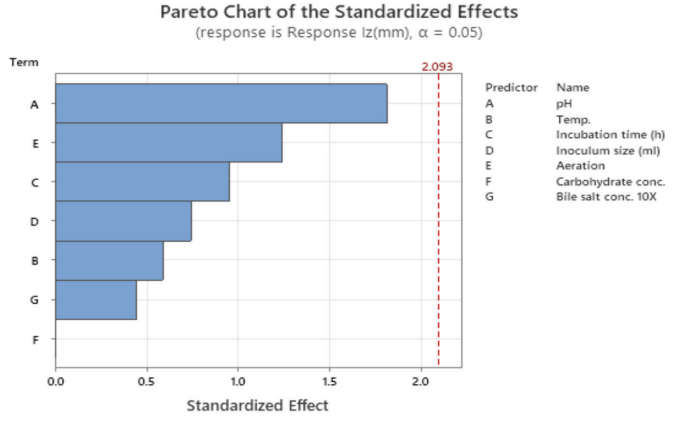

Figure 1. Pareto graph showing the different factors tested and their standardized estimates for enterocin production

DISCUSSION

The optimized parameters in this experiment were: pH, temperature, incubation time, inoculum size, aeration, carbohydrate concentration, and bile salt concentration, all of which have a great effect on enterocin production.[13]

During the optimization process of the antibacterial products of *E. faecium*, we considered both physical and chemical factors. The physical factors were pH, temperature, time of incubation, aeration rate, and inoculum size, and chemical factors included carbohydrate concentration and bile salt concentrations. These factors had a major effect on the growth and enterocin biosynthesis by *E. faecium*.[14] Optimization and enhancement of the efficiency of presently available drugs require novel research approaches to accelerate the speed of antimicrobial drug development.[15] Both the type of microbial strain and their growth conditions affect the antibiotic biosynthesis of bacteria quantitatively and qualitatively.[16] In this study, the production was optimized under different conditions using the Taguchi method, which gives us the best results for optimizing the different factors interacting to produce the maximum amount of enterocin in the fermentation process.[17]

The Taguchi design includes an analysis of results leading to a response model that clearly demonstrates the relationship of each variable toward the response, as well as the interactions between factors. In regression with a single variable, the coefficient shows how much the variable is expected to increase or decrease (if the coefficient is

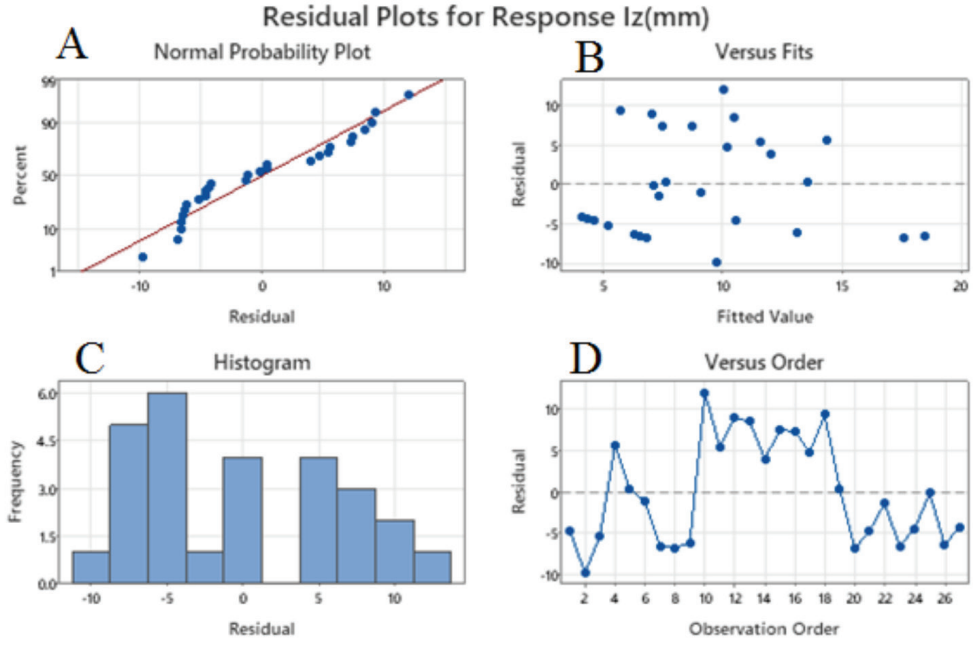

Figure 2. A) Normal probability plot of standardized residuals for bacteriocin production, the probability showed linearity. (B) Residual versus fitted samples were randomly scattered. (C) Frequency of each value interval versus residual values. (D) Residual versus observation order had an asymmetrical pattern

Table 3. Design of the experiment and results of the response

Run	pH	Temperature	Incubation time (hour)	Inoculum size (mL)	Aeration	Carbohydrate concentration	Bile salt concentration 10X	Response Iz (mm)
1	5.0	25	24	5	-1	10	1	6
2	5.0	25	24	5	0	20	3	0
3	5.0	25	24	5	100	30	5	0
4	5.0	35	48	10	-1	10	1	20
5	5.0	35	48	10	0	20	3	14
6	5.0	35	48	10	100	30	5	8
7	5.0	40	72	20	-1	10	1	12
8	5.0	40	72	20	0	20	3	11
9	5.0	40	72	20	100	30	5	7
10	6.5	25	48	20	-1	20	5	22
11	6.5	25	48	20	0	30	1	17
12	6.5	25	48	20	100	10	3	16
13	6.5	35	72	5	-1	20	5	19
14	6.5	35	72	5	0	30	1	16
15	6.5	35	72	5	100	10	3	15
16	6.5	40	24	10	-1	20	5	16
17	6.5	40	24	10	0	30	1	15
18	6.5	40	24	10	100	10	3	15
19	8.0	25	72	10	-1	30	3	8
20	8.0	25	72	10	0	10	5	0
21	8.0	25	72	10	100	20	1	0
22	8.0	35	24	20	-1	30	3	6
23	8.0	35	24	20	0	10	5	0
24	8.0	35	24	20	100	20	1	0
25	8.0	40	48	5	-1	30	3	7
26	8.0	40	48	5	0	10	5	0
27	8.0	40	48	5	100	20	1	0

Table 4. Analysis of the variance for means

Source	DF	Adj SS	Adj MS	*f* value	*p* value
Regression	7	375.51	53.645	0.97	0.477
pH	1	180.50	180.500	3.28	0.086
Temperature	1	19.11	19.114	0.35	0.563
Incubation time (hour)	1	50.00	50.000	0.91	0.352
Inoculum size (mL)	1	30.29	30.288	0.55	0.467
Aeration	1	84.72	84.724	1.54	0.230
Carbohydrate concentration	1	0.00	0.000	0.00	1.000
Bile salt concentration 10X	1	10.89	10.889	0.20	0.661
Error	19	1045.67	55.035		
Total	26	1421.19			

DF: Degrees of freedom, Adj SS: Adjacent sum of squares, Adj MS: Adjacent mean square

positive or negative, respectively) when that independent variable increases by one, and the *p* value with confidence correlates with both variables.

Orthodox optimization techniques proceeded by varying a single factor at one time while other factors remained constant, which enabled us to measure the influence of those factors on antimicrobial agent activity. The limitations of this process, such as time loss, burdensomeness, and the need more experimental research to provide a better conclusion about the interactions of these factors.[18] The Taguchi design has been used for the improvement of several factors named "orthogonal arrays" (OA) to decrease experimental errors and to increase the desired product in this experiment.[19]

The Taguchi models have been useful for improving bioreactors on an industrial scale for a better yield of antimicrobial metabolites. On the other hand, Taguchi enables researchers to investigate several factors and provides a lot of data in a limited number of experiments.[17,20] In our research, the best optimized conditions included an incubation time of 48 hours, pH of 6.5, temperature of 25 ˚C, aeration rate of 0 RPM, inoculum size of 20 mL, bile salt concentration of 5% and carbon concentration of 2.0%.

The Taguchi technique was used by Venil and Lakshmanaperumalsamy to determine the importance of nutritional media components. This was achieved by optimizing the amount of chemical components and physical factors affecting the protease enzyme produced by *Bacillus subtilis* strain HB04.[20]

CONCLUSION

The Taguchi experiment was used to optimize the production of enterocin by *E. faecium* to obtain the advantage of a lower number, shorter time of experiments, and fewer experimental errors. A standard variance procedure was then used to determine the statistically important factors. Because there were three levels for each factor (7), the L-27 OA was selected for the experimental design. The test outcomes were best under the following conditions: incubation for 48 hours, pH of the medium at 6.5, temperature at 25 ˚C, aeration rate at 0 RPM, inoculum size of 20 mL, bile salt concentration of 5%, and carbon concentration of 2.0%. Average effects of the affecting parameters and their relevant interactions at the given levels on enterocin synthesis.

Ethics

Ethics Committee Approval: This study does not require any ethical permission.

Informed Consent: Not ablicable an in vitro study.

Authorship Contributions

Concept: A.E-W., Design: D.N., A.E-W., E.A.E.-W., Data Collection or Processing: D.N., A.E-W., E.A.E.-W., Analysis or Interpretation: D.N., A.E-W., E.A.E.-W., Literature Search: D.N., A.E-W., E.A.E.-W., Writing: D.N., A.E-W., E.A.E.-W.

Conflict of Interest: Authors declare that there is no conflict of interests.

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