ORIGINAL ARTICLE
DOI: 10.4274/tjps.galenos.2023.83451

Optimization of Enterocin production from probiotic *Enterococcus faecium* using Taguchi experimental design

Short Title: Optimization Enterocin from probiotic using Taguchi

Dina Maany[1], Amr El-Waseif[2], Eslam Abd-elwahed[2]
[1]Chemistry of Natural and Microbial products Department, Pharmaceutical and Drug Industries Research Institute, National Research Centre, Dokki, Giza 12622, Egypt.
[2]Botany And Microbiology Dept., Faculty Of Science (boys), Al-azhar University, Cairo, Egypt

Corresponding Author Information
Amr El-Waseif
https://orcid.org/0000-0002-8605-9067
amrelwaseif@azhar.edu.eg
25.04.2023
11.07.2023
28.08.2023

ABSTRACT
Enterocin is a significant broad spectrum peptide antibiotic produced by *Enterococcus faecium*. Enterocin production by *Enterococcus faecium was investigated by* Taguchi experimental design. The Taguchi models were used in order to save time and effort required for optimizing the different conditions affecting its production. They were applied to optimize the conditions for enterocin production by the least number of experiments and the least amount of required materials. Seven factors viz., 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) performed. The experiment result 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 rpm, inoculum size 20 ml, Bile salt conc. Was 5% and the carbon concentration was 2.0%. all these factors combined led to the best enterocin production by *Enterococcus faecium*. This optimization of enterocin production by the Taguchi experimental models emphasized some important results about 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's intestinal tract. These bacteria are capable of producing peptides called bacteriocins which have antimicrobial properties and *Enterococci* are amongst those enterocin-producing bacteria[1].
Enterocins can be used for pathogen control purposes in laboratory experiments, in clinical trials and also in the process of food industry. Enterocins are extracellular products that are mainly produced by enterococci such as; *Enterococcus faecium*, *E. faecalis*, and *E. mondii.* Bacteriocins produced by *Enterococcus* include bacteriocin 35, enterocin A, B, L50A/B, and P, which belong to class II bacteriocins [2].
Enterocin A of *E. faecium* is a stable peptide and can be potentially used for treatment of pathogens and also in cancer treatment. It is a cyclic peptide with an isoelectric point of about 10 [3]. Enterocin works as an antimicrobial agent for Gram-positive as well as Gram negative bacteria, while most of the recorded literature say that LAB-derived bacteriocins have an antimicrobial effect only on Gram-positive bacteria [4].
It was scientifically reported that enterocins has an antibacterial effect againt 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 which have 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 to each other and it's much efficient and time saving when this aim is achieved with a less number of 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 adjust the interaction of several variables and their interaction in any given experint in one process instead of requiring a larger number of experiments that are often costly and time-consuming. Taguchi design allows us to obtain the information neede from any experiment by optimizing the variables involved in

this experiment and allowing more facility for best outcome of the system performance, using less number of experiments [10].

The aim of our research is to explore the power of Taguchi experimental design to optimize and validate the factors affecting the enterocin production from probiotic *Enterococcus faecium*.

MATERIALS AND METHODS

Enterocin biosynthesis from *Enterococcus faecium*

Enterococcus faecium was isolated and identified in the previous study [11]. It was propagated as 10 ml *E. faecium* liquid culture was inoculated in 1L MRS broth medium. Turbidity of bacterial growth was calibrated using 0.5 McFarland standard where a hundred microliter ($1x10^7$ cells/ml) of bacterial growth culture was used as standard inoculum and was incubated for 48 h at 30°C under static conditions.

Antibacterial assay

The antibacterial activity was assayed using 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 5.0mm 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 in the prepared holes using an automatic micropipette. The Petri-dishes were kept in a refrigerator for one hour to permit homogeneous diffusion of the antimicrobial agent before growth of the *Staphylococcus aureus* 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. The 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 indicated the antibacterial activity. In this experiment, an L-27 standard orthogonal array was generated for the examination of seven factors, *i.e*., pH, temperature, incubation time (h), inoculum size (ml), aeration (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 7 factors were coded as level 1, 2, and 3 (Table 1) and the layout of the L27 Taguchi's orthogonal array are 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 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 AND DISCUSSION

Optimization of Enterocin production

The optimized parameters in this experiment were: pH, temperature, incubation time, inoculum size, aeration, carbohydrate concentration and bile salt concentration which have a great effect in enterocin production [13]. This optimization process using Taguchi models enables us to decide the ideal factors in the experimental area. Using Minitab in the experiment provided us with the experimental data for enterocin production and were incorporated the quadratic polynomial prototype with ideal parameters provided.

The maximum enterocin production was achieved at pH=6.5, temperature=25°C, incubation time=48 h, inoculum size=20 ml, aeration= -1, carbohydrate concentration=20,
bile salt concentration 5.

The main effects for means of various parameters:

Each medium component was tested for the enterocin production and was investigated using Taguchi models. The provided data were used to analyze variance (ANOVA). The different values (P value, f value, coefficient of variation, and determination coefficient) obtained from ANOVA assured us with the significance of the selected model. The P value is less than 0.5, the variables were statistically significant.

The provided data revealed that 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 the inoculum size increase, while level of bacteriocin was constant at any carbohydrate concentration

Regression analysis:

The analysis of variance (ANOVA) was used to determine the model`s significance. Various values (*P* value, f value, coefficient of variation and determination coefficient) obtained from ANOVA demonstrate that the selected model was significant at $P< 0.5$, the analysis of the variance for means ratio with the degree of freedom (DF). From Table 3 we found that, the pH, incubation time, inoculum size and aeration had significant effect on

response due to (P< 0.5). The adjacent sum of squares (Adj SS), adjacent mean square (Adj MS) and probability (*P)* were shown in Table 4.
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 point at which the effect estimates were statistically significant was at *P*=0.5. Pareto chart revealed that pH was the best factor had an effect on enterocin production and carbohydrate concentration but doesn't show any effect on enterocin production.
A plotting 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 at both sides of it. Where values below the straight line were insignificant and above the straight line were significant. Where values below the line were insignificant and above the line are significant. Residuals represent the difference between true and predicted values using our final logistic regression models, whereas histogram of frequency versus residual had a dumbbell shape, it could be seen that residuals most concentrated around -5 and have right skewed distribution.

During the optimization process of the antibacterial products of *Enterococcus faecium* we took into consideration both the physical and chemical factors. The physical factors were pH, temperature, time of incubation, aeration rate, inoculum size and chemical factors included carbohydrate concentration, and bile salt concentrations and 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 need some novel research approaches in order to accelerate the speed of antimicrobial drug development [15]. Both the type of microbial strain and their growth conditions affect antibiotic biosynthesis of bacteria quantitatively and qualitatively [16]. In this study the production was optimized at different conditions by using 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 clearly demonstrating the relationship of each variable towards 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 positive or negative respectively) when that independent variable increases by one and p-value with confidence correlate both variables.
Orthodox optimization techniques proceeded by varying the single factor at one time while other factors remained constant that enabled us to measure the influence of those factors on the antimicrobial agent activity. The limitations of this process such as time loss, burdensome, need more experimental research to give a better conclusion about the interactions of these factors [18]. Taguchi design has been used form the improvement of a number of 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 the improvement of the bioreactor on an industrial for a better yield of antimicrobial metabolites. On the other hand the Taguchi enables the 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 include incubation time at 48 hours, pH at 6.5, temperature at 25°C, aeration rate at 0 rpm, inoculum size 20 ml, bile salt concentration 5% and carbon concentration at 2.0%.
The Taguchi technique was used by Venil and Lakshmanaperumalsamy to determine the importance of nutritional media components. This was done by optimizing the amount of chemical components and physical factors affecting the protease enzyme produced by *Bacillus subtilis* strain HB04 [20].
These results had a great emphasis on the impact of each parameter on metabolites synthesis depending on the other factors involved in the fermentation process. The percentage of involvement of each factor was shown in the ANOVA table. The last column of the ANOVA shows how much each factor was involved in the optimization process. The ANOVA results of all study showed that all factors were effective for the response variables.

CONCLUSION

The Taguchi experiment was used to optimize the production of enterocin by *E. faecium* to get the advantage of the lower number, time of experiments and less experimental errors. A study of standard variance procedure was then used to decide the statistically important factors. As there were 3-levels for each factor (7), therefore, L-27 Orthogonal Array (OA) was selected for the experimental design. The test outcomes were best at 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 20 ml, Bile salt conc. 5% and carbon concentration at 2.0%. The average effects of the affecting parameters and their relevant interactions at the given levels on enterocin synthesis.

Ethics Committee Approval: Not necessary.
Conflict of interests: Authors declare that there is no conflict of interests.
Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES
1. Konings WN, Kok J, Kuipers OP, Poolman B. Lactic acid bacteria: the bugs of the new millennium. Curr Opin Microbiol 2000; 3: 276-282.
2. Satish KR, Kanmani P, Yuvaraj N, Paari KA, Pattukumar V, Arul V. Purification and characterization of enterocin MC13 produced by a potential aquaculture probiont E*nterococcus faecium* MC13 isolated from the gut of Mugil cephalus. Can J Microbiol 2011; 57: 993-1001.
3. Franz CM, Stiles ME, Schleifer KH, Holzapfel WH. Enterococci in foods--a conundrum for food safety. Int J Food Microbiol 2003; 88:105-122.
4. Line JE, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP*, et al.*. Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 2008; 52: 1094-1100.
5. Martin M, Gutierrez J, Criado R, Herranz C, Cintas LM, Hernandez PE. Cloning, production, and expression of the bacteriocin enterocin A produced by *Enterococcus faecium* PLBC21 in *Lactococcus lactis*. Appl Microbiol Biotechnol 2007; 76: 667-675.
6. Gutierrez J, Larsen R, Cintas LM, Kok J, Hernandez PE. Highlevel heterologous production and functional expression of the sec-dependent enterocin P from *Enterococcus faecium* P13 in *Lactococcus lactis*. Appl Microbiol Biotechnol 2006; 72:41-51.
7. Bezerra M.A, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 2008; 76 (5): 965–977.
8. Teo´ filo RF., M.M.C Ferreira MMC. Chemometrics II: spreadsheets for calculations of experimental planning, a tutorial. Quim Nova 2006; 29 (2): 338–350.
9. Zhang, J, Fan Y, Smith E. Experimental design for the optimization of lipid nanoparticles. J. Pharm. Sci., 2009; 98(5): 1813-1819.
10. Bezerra, MA, Santelli RE, Oliveira EP, Villar LS, Escaleira, LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 2008; 76(5): 965-977.
11. Abd-Elwahed E S, El-Waseif A.A, Maany DA. Biosynthesis and FPLC purification of antibacterial peptide from the biotherapeutic agent *Enterococcus faecium*. Egyptian Pharmaceutical Journal. 2023; 22(2): 98-111.
12. El-Ghwas DE, El-Waseif AA. The Synthesis of Silver Nanoparticals from Streptomyces sp. with Antimicrobial Activity. Int J of Pharm Tech Research. 2016; 9(4): 179-186.
13. Webster JM, Chen G, Hu K and Li J. Bacterial metabolites. In Entomopathogenic Nematology ed. Gaugler, R. Wallingford: CAB International 2002; 99– 114.
14. Falagas ME and Kopterides P. Old antibiotics for infections in critically ill patients. Curr. Opin. Crit. Care 2007; 13(5):592-597.
15. Chen G, Maxwell P, Dunphy GB and Webster JM. Culture conditions for Xenorhabdus and Photorhabdus symbionts of entomopathogenic nematodes. Nematologica 1996; 42:124–127.
16. Houng JY, Liao JH, Wu JY, Shen SC and Hsu HF. Enhancement of asymmetric bioreduction of ethyl 4- chloro acetoacetate by the design of composition of culture medium and reaction conditions. Process Biochem. 2006; 42: 1-7.
17. Beg QK, Sahai V and Gupta R. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem 2003; 39: 203-209.
18. Krishna PK, Venkata MS, Sreenivas RR, Ranjan PB and Sarma PN. Laccase production by Pleurotus ostreatus 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem Eng J 2005; 24: 17-26.
19. Stone RA and Veevers A . The Taguchi influence on designed experiments. J. Chemometrics. 1994; 8: 103- 110.
20. Venil CK and Lakshmanaperumalsamy P. Taguchi experimental design for medium optimization for enhanced protease production by *Bacillus subtilis* HB04. e-JST. 2009; 4: 1-10.

Table 1: factors and levels selected for experimental use

Factors	**Level 1**	**Level 2**	**Level 3**
pH	5	6.5	8
Temp.	25	35	40

Incubation time (h)	24	48	72
Inoculum size (ml)	5	10	20
Aeration (RPM)	-1	0	100
Carbohydrate conc.	10	20	30
Bile salt conc. X10^{-1}	1	3	5

Table 2: The Taguchi experimental layout

Row	pH	Temp.	Incubation time (h)	Inoculum size (ml)	Aeration(RPM)	Carbohydrate conc.	Bile salt conc. 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

Table 3: The design of the experiment and results of response

Run	pH	Temp.	Incubation time (h)	Inoculum size (ml)	Aeration	Carbohydrate conc.	Bile salt conc. 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 : The 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
Temp.	1	19.11	19.114	0.35	0.563
Incubation time (h)	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 conc.	1	0.00	0.000	0.00	1.000
Bile salt conc. 10X	1	10.89	10.889	0.20	0.661
Error	19	1045.67	55.035		
Total	26	1421.19			

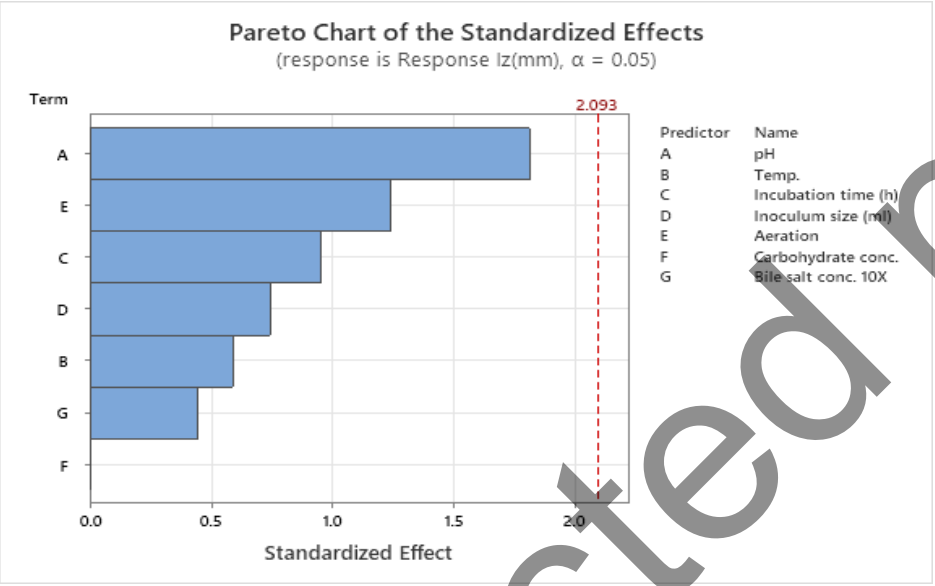

Fig 1 Pareto graph showing the different factors tested and their standardized estimates for enterocin production.

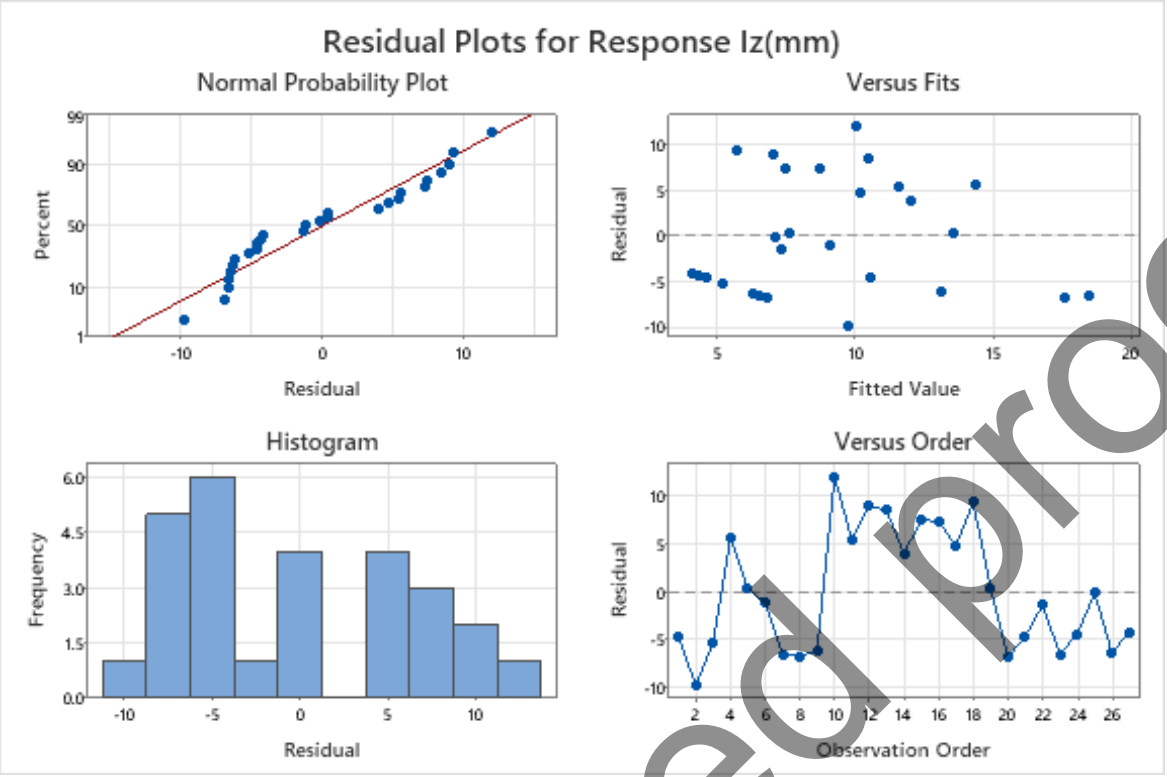

Fig 2 (a) Normal probability plot of standardized residuals for bacteriocin production, the probability showed linearity. **(b)** Residual versus fitted were randomly scattered. **(c)** The frequency of each value interval versus residual values. **(g)** Residual versus observation order had asymmetrical pattern**.**