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STATISTICAL EVALUATION OF CHICKEN FEATHER WASTES AS SUBSTRATE FOR BIOSURFACTANT PRODUCTION BY BACILLUS VALLISMORTIS RMS25

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Abstract
Biosurfactant (BS) production by Bacillus vallismortis RMS25 is investigated using keratin rich wastes as medium components. Biosurfactant production was observed to be higher under submerged fermentation conditions (1.12 g/L) when chicken feathers were used as medium ingredient. Plackett–Burman statistical design (PBD) and response surface methodology (RSM) are used to screen different keratinous materials and to optimize BS production. With a correlation coefficient (R^2) of 97%, a maximum BS production of 1124 mgL^{-1} (3-fold increase) was validated under optimized conditions (chicken feather 4 %, glucose 4 %, inoculum size 2 %, and 48-hour incubation period). The findings point to an environmentally friendly method for biodegrading chicken feather waste and the use of currently studied substrate minimizes the production cost significantly.
Key words: Biosurfactant, Plackett–Burman design, correlation coefficient, Response surface methodology, chicken feather

Introduction

BS are a type of surface active, secondary metabolites produced by a range of microorganisms. BS are the green surfactants which are produced by bacteria, yeasts, and fungi. BS have higher efficiency and stability than chemical surfactants, lower toxicity, and greater environmental compatibility at extreme conditions, and can be synthesized using renewable feedstock (Romero et al., 2007). BS have recently gained popularity as a possible substitute for chemically synthesized surfactants or as a current development for industrial and environmental sustainability. BS has the potential to be used in the cosmetics, pharmaceutical, food, and agricultural sectors (Muthusamy et al., 2008; Das and Chandran, 2011; Obayori et al., 2009; Kumar et al., 2015). Despite their multiple benefits, BS are not commonly used in industry due to their relatively high manufacturing costs. Banat et al. (2014) has presented the BS development problems and strategies for increasing production yield very well. The required quantities of renewable substrate media, the slow rate of organism growth on the substrate, final product purification from substrate impurities and the low yield are all issues that restrict BS industrial production.

BS, in spite of having a wide range of applications pose a serious problem in meeting the demand to supply, with high production costs and tedious purification process. Nowadays, to reduce the production cost, more emphasis is being placed on the

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production of high-yielding strains, the use of low-cost medium ingredients, improved purification methods, and cost-effective engineering processes (Al-Bahry et al., 2013). The BS development by Corynebacterium aquaticum in submerged fermentation using fish residues and sugarcane bagasse was documented by Paola et al (2018). SOC was used as a carbon and nitrogen source for iturin A processing, according to Narender Kumar et al. (2017). Since the cost of growth medium, which is mainly added by carbon and nitrogen sources, is estimated to account for 30–40% of the overall production cost when microbial products are produced for industrial purposes, composition of medium is one of the key parameters (Gnaneshwar et al, 2013; Kirk et al., 2002).

Keratinous waste generated by slaughterhouses, poultry, fur and leather processing industries around the world is a major concern for environmental pollution (R.Gupta et al., 2013). Feathers, horn, fur, hoof, paws, nails, bristles and wool all contain keratin. Feathers, for example, lead to environmental waste to a larger extent (Ismail et al., 2012). Feather waste, mostly from poultry, accounts for several million tons around the world (Gopinath et al., 2015). Keratin is the third most prevalent polymer in nature, being an insoluble, fibrous, and recalcitrant structural protein (Lange et al., 2016). Keratin protein is highly stable and are resistant to proteolytic hydrolysis (papain, pepsin and trypsin) as compared to soluble proteins (Sahoo et al., 2012). Usage of keratinolytic microbes to produce value-added products is an alternative approach to valorizing keratin-rich wastes. The majority of the available literature focuses on the development of keratinase enzymes, with little attention paid to the production of value-added products.

However, a review of the literature shows that there is little knowledge available on the development of BS using keratin-rich substrates, which prompted the current study. Furthermore, using feather (keratin) waste as a fermentation substrate provides a practical microbial solution for removing recalcitrant keratin wastes from the atmosphere while also developing a cost-effective medium formulation for BS development. PBD is a statistical approach for screening significant factors from a broad data set and RSM is a collection of mathematical tools for optimizing process conditions, interpreting interactions among process variables, and maximizing production. As a result, the current study attempted to screen various keratinous waste materials as a nutrient and energy source for BS production by PBD, optimize the process, and establish a cost-effective medium formulation by RSM for maximum BS production.

Materials and methods

Microorganism and culture conditions

In the present study, Bacillus vallismortis RMS25 strain developed in our lab, was used. Inoculum of about 10^8 cells per mL was obtained by growing the culture in mineral salts medium (MSM) [$NaNO_3$ (0.5), $MgSO_4$ (0.5), KCl (0.1), $FeSO_4$ (0.01), K_2HPO_4 (0.5), KH_2PO_4 (0.5) g/L], pH 7 and temperature of 37 °C for 18 h at 200 rpm.

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Biosurfactant production and extraction

Bacillus vallismortis RMS25 was grown in 100 mL MSM in a 250 mL Erlenmeyer flask for 48 hours at 200 rpm, then centrifuged for 15 minutes at 10,000 rpm. The supernatant was precipitated by changing the pH to 2.0 with 6 N HCl (Dubey and Juwarkar, 2001) and deposited overnight at 4 ºC. The precipitate was obtained by centrifugation at 12,000 rpm for 20 minutes, followed by methanol extraction twice (Kim et al., 2004).

Substrates

The following keratin rich materials were tested for their potency as medium components in the present study: White and colored chicken feathers, human hair collected from local saloons, horn, hoof, and chicken claws. All of these materials were gathered from local market yards and farms, washed in hot de-ionized water to remove any bloodstains or adhering particles, and dried in an incubator for two days at 50 degrees Celsius. To reduce the size of the dried substrates, they were ground and stored at room temperature in a moisture-free jar. All the above materials were used as medium ingredients, directly without any pretreatment in fermentation media.

Screening of most suitable substrates by Plackett-burman method

Six different keratin rich materials: goat horns (GH), goat hooves (GHo), White chicken feathers (WCF), pigmented chicken feathers (PCF), human hair (HH) and chicken claws (CC) were screened for BS production by Bacillus *vallismortis* RMS25, (Table-1) using Plackett-Burman design to identify the most influencing variable. In 100mL sterile medium (MSM broth) in 250mL Erlenmeyer flasks with different concentrations of substrates as per the design (Table 1), inoculated with 1% of 18 h culture (1 x 10^8 CFU mL^{-1}) and incubated for 48 hours at 200 rpm and 37 °C for screening the best substrate for BS production, a set of 12 experiments were performed at combinations of '+' (high-0.5%) and '-' (low-0.05%) values of the process variables.

In the results, the effects of variables on BS were investigated. In the experimental design, each column represents an independent variable, while each row represents an experiment (Table 2). The key effect was determined by comparing the mean of measurements taken at each factor's high (+1) and low level (-1) settings. PBD was based on the 1st order model experiments (Plackett and Burmann, 1946):

$$Y = \beta o + \sum \beta i Xi \quad (1)$$

Where Y represents the response (BS productivity), βo represents the model intercept, and βi represents the variable estimates. The findings were analyzed using MINITAB-15 statistical tools and a Plackett–Burman architecture matrix (Table 2).

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Optimization by response surface method

RSM was used to optimise substrate and glucose concentrations, inoculum size, and incubation time for optimal BS production after selecting the critical factors affecting BS production by PBD. For optimization, four variables were chosen: white chicken feather (WCF), glucose, inoculum size, and incubation duration. The central composite rotatable system (CCRD) (Montgomery, 1997) was used in a 31-experiment design with seven replicates at the central stage (Table 4) with five coded levels (-2, -1, 0, +1, +2). The real experimental values of variables were coded using the following equation (Neter et al., 1996)

$$x_i = (Xi - X0)/\Delta X \qquad (2)$$

Where xi is the variable's dimensionless coded level, Xi is the variable's actual value, X0 is the variable's average of maximum and minimum level values, and X is the variable's maximum value minus minimum value.

The data was fitted into the equation using a multiple regression method, and the BS production (mg L^{-1}) was measured using 2^{nd} order polynomial equation. The 3d graphical representation of the model equation depicts the individual and interactive effects of test variables on the response. The optimum levels of variables for maximum BS output were calculated by solving the regression equation and analyzing response surface plots. In terms of the independent variables (X1, X2, X3, and X4), the expected response (Y) is defined by the second-order polynomial equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X^2_1 + \beta_{22}X^2_2 + \beta_{33}X^2_3 + \beta_{44}X^2_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 \qquad (3)$$

Where, β_0 is intercept term, β_1, β_2, β_3, β_4, linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} quadratic coefficients and β_{12}, β_{13}, interactive coefficient estimates. The optimum levels of variables (within the experimental range) for maximum BS yield were calculated, and the BS output was measured, by running an experiment with the optimum values for variables determined by response optimization for validation of the predicted value.

Reproducibility of results:

The findings were based on the average of three separate studies that yielded consistent results, all of which were done in triplicate.

Results and Discussion

Screening of most suitable substrates by Plackett-burman method

Six different keratin rich materials were assessed as substrates (Table 1) for BS production using the Plackett-Burman statistical design. MINITAB-15 statistical software

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was used to measure the P & F values, and regression coefficient of the variables for BS production. The input data is analyzed, and the factors are ranked by magnitude of influence.

With varying influence of variables on BS production by *Bacillus vallismortis* RMS25 has shown varied BS production from 225 mg/L to 1014 mg/L in the 12 PB design experiments (Table-1). All six variables had positive effects on BS production and were found to be significantly affecting BS production with p values >0.05 in the regression co-efficient study (Table 2). The t coefficient was used in the statistical research, and analysis of variance was performed using MINITAB-15 statistical methods, with p-values of 0.001 and 0.000, respectively, for the main effects and curvature (Table 2).WCF which showed maximum BS production, with a p-value of 0.00 (Table 3) was chosen as the substrate for further research due to its ease of availability and priceless.

Optimization of various fermentation parameters by response surface method

When white chicken feather (WCF) and glucose were used as medium ingredients sources in statistical screening experiments, encouraging results were observed for BS production. RSM was used to decide the optimal operating conditions of fermentation process to optimize BS production by Bacillus vallismortis RMS25 under submerged fermentation using WCF and glucose as medium ingredients. The effects of four variables, including glucose and WCF as carbon and nitrogen source as well as inoculum size (IS) and incubation time (IP), on BS development were investigated at five levels using CCRD. Table 4 shows results of thirty-one trials that were carried out to determine optimal conditions for high BS production. The highest BS output was observed at run 16.

The significance of each coefficient is determined using the Student's t-test and p-values, as shown in Table 5. The constants X1, X2, X4, X22, X1X2, and X1X4 were found to be important (P 0.05) based on the p-value of each model expression. The positive coefficients of X_1, X_2, X_3, X_4, X_1^2, X_2^2, X_3^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4 had a direct effect on the response Y, while the negative ones indicated an inverse effect on BS production. The findings show that WCF, GLU, and IP, among the independent variables, have a major impact on BS production (Table 5). According to this theory, increasing their concentrations will boost the BS production. Among square interactions glucose concentration was found significant and in terms of adjacent interactions WCF-GLU and WCF-IP were found significant.

Multiple regression analysis was used to describe the concurrent effect of variables and to adapt the response function to the experimental results, and the following polynomial equation was derived from regression analysis:

$$Y=1451.57+268.92X_1+287.67X_2+4.50X_3+264.33X_4+32.52X_1^2+58.27X_2^2+36.27X_3^2-37.73X_4^2+95.62\,X_1X_2+26.63X_1X_3+61.88X_1X_4+36.75X_2X_3+25.75X_2X_4+18.25X_3X_4 \quad (4)$$

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Where, Y was the yeild of BS (predicted response), and X1, X2, X3, and X4 were the coded values of the test variables WCF, GLU, IS, and IP respectively. The 14-term quadratic model in equation (4) has four linear terms, four quadratic terms, and six two-factor interactions. The words that are not meaningful (based on P-values < 0.05) are excluded (Table 5). The model equation was modified to reduced fitted model (Eq-5).

$$Y=1451.57+268.92X_1+287.67X_2+264.33X_4+58.27X_2^2+95.62X_1X_2+61.88X_1X_4 \text{ -- (5)}$$

The data matches the reduced fitted model almost as well as the model (Eq.4) with all terms. As a result, it can be used for further research and validation. The regression-based determination coefficient R^2 was used to assess the model equation's fit. In order to perform ANOVA for BS production, MINITAB-15 was used. Table-6 shows the ANOVA results of the second-order response surface model fitting. The regression and linear effects are very important for BS output, according to ANOVA (Table-6). The regression-based determination coefficient R^2 was used to assess the model equation's fit. The model will better explain the difference between experimental and expected values if the R^2 values are close to 1. (Dhanya, et al., 2008). Just 3% of the total variations were not explained by the model, which had a high determination coefficient (R^2= 0.97) and explained 97 percent of the variability in the response. The modified determination coefficient (Adj R^2 = 0.945) was also very high, indicating that the observed and predicted responses were well-matched. According to current results, the response equation provided a suitable model for process optimization of BS production.

As shown in Table 5, the significance of each coefficient is calculated using p-values and the Student's t-test. A higher t-value and a lower p-value mean that the associated coefficient is more important. The constant, X1, X2, X4, X2X2, X1X2, and X1X4 were found to be important (P 0.05) based on p-value of each model expression. All positive coefficients had a direct effect on the response Y, while negative coefficients (IP*IP) indicated an inverse effect on BS production.

Figure 1(A-F) shows a response surface plot depicting the major interactive effect of the studied parameters. At the "+1" stage of WCF (4%), GLU (4%), IS (2%) and IP (48h), high BS output was observed. As the concentration of WCF and glucose increased, an increase in BS production was observed up to +1 level for both the factors and beyond which no significant increase was observed, suggesting a positive interaction between the two variables (Fig.1A). BS production was improved by increasing the concentrations of WCF powder, IS, and IP (Fig.1B and 1C). Up to 48 hours of incubation, an increase in BS production was observed with an increase in WCF concentration, after which no substantial change in BS production was observed (Fig.1C). An increase in BS production was observed as the concentrations of glucose and IS increased up to +1 level for both factors, after which no substantial increase was observed (Fig.1D). An increase in BS production was observed with an increase in glucose concentration up to 48h of incubation and beyond which no significant improvement in BS production was observed (Fig.1E). The response surface plot in Figure 1F depicts the impact of IP and IS on BS

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growth. The characteristics and rate of growth of the culture decide the duration of the incubation period. Increased IP resulted in an improvement in BS production up to 48 hours, but further incubation did not boost BS production. There was a large increase in BS production when the IS was set to 2%; but, as the IS was increased, the BS production steadily decreased. This may be due to bacterial metabolism depleting basic nutrients and accumulating toxic secondary metabolites in the fermentation medium. Bacillus spp. produced the most BS after 48 hours of incubation, according to Narendra Kumar et al. (2017).

Under ideal conditions, the maximum BS yield was 1124 mg L^{-1}, according to the data. Although the exact reason for WCF and glucose producing more BS is unknown, free amino acids and soluble protein released from substrates due to keratinolytic activity of the strain may play a role. In this analysis, the substrate WCF and glucose were found to be extremely important in the production of BS. As a result, the aim of this study was to use locally available, white chicken feather, which could otherwise get accumulated in the environment and cause pollution as substrates for BS production using Bacillus vallismortis RMS25 in submerged fermentation, and to optimize various parameters for improved BS production.

Conclusion

In the present research efforts were made to establish a low-cost medium formulation using keratin rich wastes as substrates for BS production. Plackett–Burman design was used to pick a few better ingredients for statistical screening, and the optimization of substrate concentrations was done by RSM. The use of keratin-rich white chicken feather instead of commercial sucrose, peptone, yeast extract, and malt extract resulted in a 3-fold increase in BS production (1124 mg L-1). This is the first study to look at the use of white chicken feather without any treatment for BS production by Bacillus vallismortis RMS25.

Conflict of interest The authors declare that they have no conflict of interest for this study.

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Table 1. The Plackett–Burman experimental design matrix: Screening keratin rich wastes as substrates for BS production by Bacillus vallismortis KM15.

Run	GH	GHo	WCF	PCF	GH	WL	BS (mg/L)
1	+	-	+	-	-	-	565
2	+	+	-	+	-	-	628
3	-	+	+	-	+	-	767
4	+	-	+	+	-	+	952
5	+	+	-	+	+	-	802
6	+	+	+	-	+	+	1014
7	-	+	+	+	-	+	891
8	-	-	+	+	+	-	723
9	-	-	-	+	+	+	657
10	+	-	-	-	+	+	645
11	-	+	-	-	-	+	516
12	-	-	-	-	-	-	225

Number of runs-12, variables-06 and center points-0

Table 2. Estimated Effects and Coefficients for BS production (coded units): Plackett-Burmann design.

Term	Effect	Coefficient	SE Coefficient	t	P	Significance
Constant		699.06	10.01	69.83	0.000	Significant
SfOC	138.11	69.06	10.01	6.90	0.001	Significant
SBOC	141.94	70.97	10.01	7.09	0.001	Significant
WCF	239.78	119.89	10.01	11.98	0.000	Significant
CSOC	153.50	76.75	10.01	7.67	0.001	Significant

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COC	138.39	69.19	10.01	6.91	0.001 Significant
WL	160.89	80.44	10.01	8.04	0.000 Significant

SE, Standard error; t, Student t value; p, Probability, *Insignificant.

Table 3. Analysis of Variance for BS production (coded units): Plackett-Burmann design

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	6	495946	495946	82658	68.73	0.000
Residual Error	5	6013	6013	1203		
Total	11	501959				

DF, degrees of freedom; Seq.SS, Sum of squares; Adj. SS, adjusted sum of squares; Adj. MS, adjusted sum of squares; F, Variance ratio; p,Probability.

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Table 4: CCRD: Medium optimization for BS production by Bacillus vallismortis RMS25

Run	WCF	GLU	IS	IP	BS production(U/mL)	
					Exp. Value	Pred. Value
1	-1 (2%)	-1 (2%)	-1 (1%)	-1 (24h)	374	422
2	+1 (4%)	-1 (2%)	-1 (1%)	-1 (24h)	566	495
3	-1 (2%)	+1 (4%)	-1 (1%)	-1 (24h)	573	533
4	+1 (4%)	+1 (4%)	-1 (1%)	-1 (24h)	782	771
5	-1 (2%)	-1 (2%)	+1 (2%)	-1 (24h)	410	355
6	+1 (4%)	-1 (2%)	+1 (2%)	-1 (24h)	425	474
7	-1 (2%)	+1 (4%)	+1 (2%)	-1 (24h)	524	530
8	+1 (4%)	+1 (4%)	+1 (2%)	-1 (24h)	805	813
9	-1 (2%)	-1 (2%)	-1 (1%)	+1 (48h)	626	558
10	+1 (4%)	-1 (2%)	-1 (1%)	+1 (48h)	687	737
11	-1 (2%)	+1 (4%)	-1 (1%)	+1 (48h)	707	714
12	+1 (4%)	+1 (4%)	-1 (1%)	+1 (48h)	1063	1058
13	-1 (2%)	-1 (2%)	+1 (2%)	+1 (48h)	456	523
14	+1 (4%)	-1 (2%)	+1 (2%)	+1 (48h)	768	748
15	-1 (2%)	+1 (4%)	+1 (2%)	+1 (48h)	731	742
16	+1 (4%)	+1 (4%)	+1 (2%)	+1 (48h)	1124	1132
17	-2 (1%)	0 (3%)	0 (1.5%)	0 (36h)	439	449
18	+2 (5%)	0 (3%)	0 (1.5%)	0 (36h)	917	912
19	0 (3%)	-2 (1%)	0 (1.5%)	0 (36h)	479	477
20	0 (3%)	+2 (5%)	0 (1.5%)	0 (36h)	966	972
21	0 (3%)	0 (3%)	-2 (0.5%)	0 (36h)	639	683
22	0 (3%)	0 (3%)	+2 (2.5%)	0 (36h)	730	691
23	0 (3%)	0 (3%)	0 (1.5%)	-2 (1%)	300	332
24	0 (3%)	0 (3%)	0 (1.5%)	+2 (5%)	814	787
25	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	633	624
26	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	619	624
27	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	622	624
28	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	619	624
29	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	626	624
30	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	629	624
31	0 (3%)	0 (3%)	0 (1.5%)	0 (36h)	623	624

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Table 5: Coefficients and t-values for BS production by Bacillus vallismortis RMS25 using CCRD

Variable	Coefficients	Standard error coefficient	t-Value	Probability
Constant	1451.57	39.67	36.595	0.000
WCF	268.92	21.42	12.553	0.000
GLU	287.67	21.42	13.429	0.000
IS	4.50	21.42	0.210	0.836
IP	264.33	21.42	12.339	0.000
WCF*WCF	32.52	19.63	1.657	0.117
GLU *GLU	58.27	19.63	2.969	0.009
IS* IS	36.27	19.63	1.848	0.083
IP*IP	-37.73	19.63	-1.922	0.073
WCFS*GLU	95.62	26.24	3.645	0.002
WCF* IS	26.63	26.24	1.015	0.325
WCF*IP	61.88	26.24	2.358	0.031
GLU* IS	36.75	26.24	1.401	0.180
GLU*IP	25.75	26.24	0.981	0.341
IS*IP	18.25	26.24	0.696	0.497

S = 104.945 R-Sq = 97.0% R-Sq(adj) = 94.53%

Table 5. Analysis of variance for BS production by Bacillus vallismortis RMS25 using CCRD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	5865073	5865073	418934	38.04	0.000
Linear	4	5399056	5399056	1349764	122.56	0.000
Square	4	209566	209566	52391	4.76	0.010
Interaction	6	256452	256452	42742	3.88	0.014
Residual error	16	176215	176215	11013		

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Lack-of-fit	10	175277	175277	17528	112.15	0.000
Pure Error	6	938	938	156		
Total	30	6041288				

DF, degrees of freedom; Seq.SS, sum of squares; Adj. SS, adjusted sum of squares; Adj. MS, adjusted sum of squares; F, Variance ratio; p, Probability.

Figure Captions:

Fig. 1. Response surface plots of BS production by *B.vallismortis* RMS25. (A)–(F) are Response surface plots of BS production, in terms of interaction between (A) White chicken feather and glucose concentrations (B) White chicken feather concentration and inoculums size, (C) White chicken feather concentration and incubation period (D) glucose concentration and inoculum size level (E) glucose concentration and incubation period (F) inoculum size level and incubation period