Application of statistical design to the optimization of culture medium for prodigiosin production by *Serratia marcescens* SWML08

Venil, C. K.* and Lakshmanaperumalsamy, P.

Division of Microbiology, Department of Environmental Sciences, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India.

E-mail: ckvenil@gmail.com

Received 16 November 2008; received in revised form 17 April 2009; accepted 17 April 2009

**ABSTRACT**

Combination of Plackett – Burman design (PBD) and Box – Behnken design (BBD) were applied for optimization of different factors for prodigiosin production by *Serratia marcescens* SWML08. Among 11 factors, incubation temperature, and supplement of (NH₄)₂PO₄ and trace salts into the culture medium were selected due to significant positive effect on prodigiosin yield. Box - Behnken design, a response surface methodology, was used for further optimization of these selected factors for better prodigiosin output. Data were analyzed step wise and a second order polynomial model was established to identify the relationship between the prodigiosin output and the selected factors. The media formulations were optimized having the factors such as incubation temperature 30 °C, (NH₄)₂PO₄ 6 g/L and trace salts 0.6 g/L. The maximum experimental response for prodigiosin production was 1397.96 mg/L whereas the predicted value was 1394.26 mg/L. The high correlation between the predicted and observed values indicated the validity of the statistical design.

Keywords: *Serratia marcescens*, prodigiosin, Plackett - Burman design, Box - Behnken design

**INTRODUCTION**

For several decades, prodigiosin has been known to be a natural compound showing a broad range of cytotoxic activity (Furstner, 2003) and is also produced by *Vibrio psychroerythrus* (D’Aoust and Gerber, 1974), *Serratia marcescens*, *Pseudomonas magnesiorubra* and other eubacteria (Lewis and Corpe, 1964). Recently, prodigiosin has been considered effective as an immunosuppressive, antifungal and antiproliferative properties (Azuma et al., 2000; Han et al., 2001; Montaner and Perez-Thomas, 2001; Soto-Cerrato et al., 2004). Owing to these characteristics, prodigiosin may have potential for medical application, for instance, it may be used to develop antitumor drugs (Perez-Thomas et al., 2003; Furstner, 2003). In regard to its potential commercial values, there is a demand to develop high throughput and cost effective bioprocesses for prodigiosin production. However, medium components have not yet been assigned a definite role in prodigiosin production and no detailed information was reported about their optimum concentration to ensure high prodigiosin production by *S. marcescens*. Thus it is essential to carry out the research on the effect of different media components on prodigiosin accumulation in *S. marcescens*.

The growth and pigment production of the organism are strongly influenced by medium composition. Thus optimization of medium components and culture condition are the primary task in a biological process (Djekrille-Dakhmouche et al., 2006). The main strategy used is medium engineering for which the optimal operating condition of a parameter is optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). The optimization studies do not consider the interaction effects among the variables as any process is influenced by several variables (Silva and Roberto, 2001). Limitations of the single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol, 2004). Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially when interaction effects between different factors are overlooked (Wenster-Botz, 2000). But response surface methodology (RSM), consisting of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables is an ideal methodology to infer facts scientifically.

Statistical methodologies such as Plackett - Burman design (PBD) (1946) and Box - Behnken design (BBD) (1960) have shown to be efficient and effective approach to systematic investigation on the target factors. PBD is an effective screening design which considerably diminishes the number of experiment and gives information for the evaluation of the target factors as much as possible. Only the most effective factors with positive

*Corresponding author
significance are selected for further optimization. The less significance or high negative effect on response value would be omitted for further experiments (Plackett and Burman, 1946). PBD has been widely applied in many fields such as medium optimization, formulation of multi component and so on (Loukas, 2001; Naveena et al., 2005). BBD can be used to optimize target parameters within the designed scopes. The number of trials is equal to the maximum number of the designed levels of the target factor and therefore presents the advantage.

In the present work, combination of PBD and BBD was applied to select the medium components that significantly influenced the accumulation of prodigiosin and also to ascertain the optimum concentrations of those components in fermentation medium for prodigiosin production by S. marcescens SWML08.

MATERIALS AND METHODS

Microorganism and preparation of inoculum

S. marcescens SWML08 strain was grown on the nutrient agar (HI – medium, Mumbai, India) slants at 37 °C for 24 h and subcultured every two weeks. Nutrient broth was inoculated with a 24 h old culture and grown for 18 h and was used as inoculum.

Optimization of process parameters

Identification of suitable variables using Plackett - Burman design

The Plackett – Burman experimental design identifies the critical physico-chemical parameters required for elevated prodigiosin production by screening n variables in n + 1 experiments (Plackett and Burman, 1946). The variables chosen for the present study were medium type, pH, incubation temperature (°C), agitation speed (rpm), inoculum size (%), incubation time (h), the content of lactose (g/L), (NH₄)₂PO₄ (g/L), CaCl₂ (g/L), NaCl (g/L) and trace salts (g/L) in the culture medium. The experimental design for the screening of the variables was presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameters on prodigiosin production was calculated by the following equation:

\[ E = \frac{\sum M_i - \sum M_0}{N} \]

Where E is the effect of parameter under study and Mᵢ and M₀ are responses (prodigiosin activities) of trials at which the parameter was at its higher and lower levels respectively and N was the total number of trials.

Response surface methodology

The levels of the significant parameters and the interaction effects between various variables that influenced the prodigiosin production were analyzed and optimized by Box – Behnken methodology (Box and Behnken, 1960). In this study, the experiment consisted of 17 trials and the independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study was shown in Table 2. All the experiments were done in triplicate and the average of prodigiosin production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analyzed using the ‘Design Expert’ software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial equation is

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \]

Where \( Y \) is the predicted response, \( x_i \) are input variables which influence the response variable \( Y \); \( \beta_0 \) is the offset term; \( \beta_i \) is the \( i^{th} \) linear coefficient; \( \beta_{ii} \) is the \( i^{th} \) quadratic coefficient and \( \beta_{ij} \) is the \( ij^{th} \) interaction coefficient.

Extraction and determination of prodigiosin

For extraction of prodigiosin, one mL culture broth was centrifuged at 1200 x g for 10 minutes and the pellet was resuspended in 1 mL acified methanol and mixed vigorously. The solution was then centrifuged at 1200 x g for 10 minutes. Optical density of the resulting solution was determined at 535 nm (OD₅₃₅nm). The total prodigiosin (mg/L) was calculated according to the following formula (Williams et al., 1960; Chen et al., 2006):

\[ TP(\text{mg} / \text{L}) = \frac{ADV_i}{7.07 \times 10^4 V_2} \]

Where TP denotes the total pigment yield (mg/L), A the absorbance of methanol extract at 535 nm, D the dilution ratio, \( V_i \) the volume of methanol added, 7.07 x 10⁴ is extinction coefficient of prodigiosin and \( V_2 \) is the volume of fermentative liquid.

Statistical analysis

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included Fisher’s F- test (overall model significance), it’s associated probability p(F), correlation coefficient R, determination coefficient R² which measure the goodness of fit of regression model. For each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated using Design Expert software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package.
Table 1: Screening of factors using Placket – Burman design for prodigiosin production by *S. marcescens* SWML08

| Run | A: Media | B: pH | C: Temperature | D: Agitation | E: Inoculum conc | F: Incubation time | G: Lactose | H: (NH₄)₂PO₄ | J: CaCl₂ | K: NaCl | L: Trace Salts | Total Prodigiosin |
|-----|----------|-------|----------------|--------------|------------------|--------------------|-----------|----------------|---------|---------|--------------|------------------|
| 1   | {1}      | -1    | -1             | -1           | 1                | -1                 | 1         | 1              | -1      | 1       | -1           | 1                |
| 2   | {1}      | -1    | 1              | -1           | 1                | 1                  | 1         | 1              | -1      | 1       | -1           | 1                |
| 3   | {-1}     | -1    | -1             | -1           | 1                | -1                 | -1        | -1             | 1       | 1       | -1           | 1                |
| 4   | {-1}     | 1     | 1              | -1           | 1                | -1                 | 1         | 1              | -1      | 1       | -1           | 1                |
| 5   | {1}      | 1     | -1             | -1           | 1                | 1                  | 1         | 1              | 1       | 1       | 1            | 1                |
| 6   | {-1}     | -1    | -1             | 1            | 1                | -1                 | 1         | 1              | -1      | 1       | 1            | 1                |
| 7   | {-1}     | -1    | -1             | 1            | 1                | 1                  | 1         | 1              | -1      | 1       | 1            | 1                |
| 8   | {-1}     | -1    | -1             | -1           | 1                | 1                  | -1        | 1              | 1       | 1       | -1           | 1                |
| 9   | {1}      | 1     | -1             | -1           | 1                | 1                  | -1        | -1             | 1       | -1      | 1            | 1                |
| 10  | {-1}     | 1     | -1             | 1            | 1                | -1                 | 1         | 1              | 1       | 1       | 1            | 1                |
| 11  | {-1}     | 1     | -1             | 1            | 1                | -1                 | 1         | 1              | 1       | 1       | 1            | 1                |
| 12  | {1}      | 1     | -1             | 1            | 1                | 1                  | 1         | 1              | 1       | 1       | 1            | 1                |

Note: The table entries indicate the settings for each factor in the Placket – Burman design. The total prodigiosin production is measured in mg/L.
RESULTS AND DISCUSSION

Screening of suitable variables using Plackett – Burman design

The results (Table 1) indicated that there was a wide variation of total prodigiosin yield in the twelve trials (41.11 to 998.24 mg/L). These variations reflected the importance of medium optimization to obtain higher prodigiosin yield. The following first order polynomial model describes the variations of the results:

\[ Y = \beta_0 + \sum \beta x_i \]

Where \( Y \) is the responsive value, \( \beta_0 \) the model intercept, \( \beta \) the linear coefficient and \( x_i \) is the level of the independent variable.

The medium components were screened and those with a \( p \) – value of < 0.1 using 90 % confident level were accepted as significant factors affecting the production of prodigiosin. According to ANOVA for the model, the model of regression was significant (\( p < 0.0002 \)) (Table 2) which inferred that incubation temperature, (NH\(_4\))\(_2\)PO\(_4\) and trace salts as most significant variables influencing prodigiosin production. Kim et al. (2008) selected five medium components (CaCl\(_2\), Na\(_2\)SO\(_4\), Na\(_2\)SiO\(_3\), NaHCO\(_3\) and NH\(_4\)NO\(_3\)) through Plackett – Burman design for prodigiosin production by *Hahella chejuensis* KCTC 2396. In this study three medium components viz incubation temperature, (NH\(_4\))\(_2\)PO\(_4\) and trace salts were selected through Plackett – Burman design for production of prodigiosin by *S. marcescens* SWML08 and these medium components were selected for further optimization using Box – Behnken design. These results indicated that the Plackett – Burman design is a powerful tool for identification of the variables that could significantly affect prodigiosin production.

Response surface methodology

Statistical designs are effective tools that can be used to account for the main as well as the interactive influences of fermentation parameters on the process performance. Among them, response surface methodology (RSM) is a collection of certain statistical techniques for designing experiments, building models, evaluating the effect of the factors and searching for optimal conditions for desirable responses (Myers and Montgomery, 2002). Therefore, during the past decades, RSM has been extensively applied in the optimization of medium composition, fermentation conditions and food manufacturing processes (Vazquez and Martin, 1997; Ramirez et al., 2001; Park et al., 2005).

In this study, RSM (Box – Behnken design) employed to investigate the interactions among the selected factors (incubation temperature, (NH\(_4\))\(_2\)PO\(_4\) and trace salts) in the culture medium and also to determine their optimum levels for maximum prodigiosin production demonstrated markedly varied results, ranging from 411.29 to 1397.96 mg/L in prodigiosin production. The lowest prodigiosin

### Table 2: Analysis of variance for prodigiosin production using Plackett – Burman design

| Source       | Sum of square | Degree of freedom | Mean square | F-Value | p-Value |
|--------------|---------------|-------------------|-------------|---------|---------|
| Model        | 35954.28      | 7                 | 4853.6      | 59.62   | 0.0002  |
| Residual     | 1101.53       | 11                | 92.14       |         |         |
| Pure error   | 71.32         | 4                 | 17.08       |         |         |
| Total        | 37055.81      | 18                |             |         |         |

### Table 3: Experimental design and results of Box Behnken design of response surface methodology

| Run | Temperature (°C) | (NH\(_4\))\(_2\)PO\(_4\) (g/L) | Trace Salts (g/L) | Total Prodigiosin (mg/L) |
|-----|------------------|-------------------------------|-------------------|--------------------------|
|     |                  |                               |                   | Experimental | Predicted |
| 1   | 0.000            | 0.000                         | 0.000             | 834.16       | 833.64    |
| 2   | 0.000            | 0.000                         | 0.000             | 831.12       | 833.64    |
| 3   | 0.000            | 1.000                         | -1.000            | 1397.96      | 1394.26   |
| 4   | -1.000           | -1.000                        | 0.000             | 507.11       | 497.65    |
| 5   | -1.000           | 0.000                         | 1.000             | 518.59       | 520.54    |
| 6   | 0.000            | 1.000                         | 1.000             | 724.59       | 727.27    |
| 7   | 0.000            | 0.000                         | 0.000             | 827.15       | 833.64    |
| 8   | 1.000            | 0.000                         | 1.000             | 411.29       | 408.15    |
| 9   | 1.000            | 0.000                         | -1.000            | 517.28       | 515.33    |
| 10  | -1.000           | 1.000                         | 0.000             | 712.35       | 707.72    |
| 11  | 0.000            | 0.000                         | 0.000             | 829.65       | 833.64    |
| 12  | 0.000            | -1.000                        | 1.000             | 730.24       | 737.74    |
| 13  | 1.000            | 1.000                         | 0.000             | 423.59       | 433.05    |
| 14  | 1.000            | -1.000                        | -1.000            | 638.14       | 640.28    |
| 15  | -1.000           | 1.000                         | 0.000             | 521.35       | 525.98    |
| 16  | 0.000            | -1.000                        | -1.000            | 789.35       | 786.67    |
| 17  | 0.000            | 0.000                         | 0.000             | 847.11       | 833.64    |
production of 411.29 mg/L was observed when incubation temperature was at 40 °C with (NH₄)₂PO₄ (0 g/L) and trace salts (0.1 g/L) (run 8). Prodigiosin production of 1397.96 mg/L was observed at incubation temperature 30 °C, (NH₄)₂PO₄ 6 g/L and trace salts 0.6 g/L (run 3). The results were presented in Table 3.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher’s statistical analysis and the results were presented in Table 4. The model F value of 5.05 implied that the model was significant and also showed that there was 0.25% chance that the model F value could occur due to noise. The R² value (multiple correlation coefficients) closer to 1 denoted better correlation between the observed and predicted responses. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (4.12) indicated that the experiments performed were highly reliable. The p values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables.

The results obtained from the BBD were fitted to a second order polynomial equation to explain the dependence of total prodigiosin production on the medium components.

\[
Y = 833.64 - 61.58 A + 29.28 B - 58.98 C - 75.75 AB + 0.89 AC - 34.52 BC - 282.60 A^2 - 9.94 B^2 - 32.21 C^2
\]

Where Y is the predicted response (total prodigiosin production), A, B and C are the coded values of incubation temperature, (NH₄)₂PO₄ and trace salts respectively.

Kim et al. (2000) reported that, through Box - Behnken experimental design, the optimal concentrations of (NaHCO₃, Na₂SiO₃, NH₄NO₃, Na₂SO₃, and CaCl₂) were determined to be 0.45, 0.0045, 0.0045, 9.0, and 1.7115 g/L, respectively for prodigiosin production of 1.198 g/L by Hahella chejuensis KCTC 2396. In this study, the optimized values of the Box – Behnken design were found to be incubation temperature 30 °C, (NH₄)₂PO₄ 6 g/L and trace salts 0.6 g/L for production of 1.397 g/L prodigiosin by S. marcescens SWML08. Results obtained in this study are comparable to the study of Kim et al. (2000) by BBD for the production of prodigiosin and the statistical method employed was found to be a viable one for optimizing the medium factors. The optimized medium has shown the maximum prodigiosin yield by S. marcescens SWML08.

Chen and Johns (1993) and Juzlova et al. (1996) reported that ammonium chloride is a better inorganic nitrogen source for pigment production. The present study demonstrated that diammonium phosphate was a better nitrogen source for prodigiosin production in S. marcescens SWML08.

The fitted response for the above regression model was plotted in Figure 1 and 2. 3D graphs generated for the pair wise combination of the three selected factors for total prodigiosin production highlighted the roles played by these factors and also the physical constraints in the final yield of total prodigiosin.

![Figure 1: Response surface graph showing the effect of the interaction of incubation temperature and (NH₄)₂PO₄ on prodigiosin production](image-url)
The maximum experimental response for prodigiosin production was 1397.96 mg/L whereas the predicted value was 1394.26 mg/L indicating a strong agreement between them. The optimum values of the tested variables are incubation temperature 30°C, (NH₄)₂PO₄ 6 g/L and trace salts 0.6 g/L as shown in perturbation graph (Figure 3). The model was also validated by repeating the experiments under the optimized conditions, which resulted in the prodigiosin production of 1390.91 mg/L (predicted response 1394.26 mg/L), thus proving the validity of the model.

CONCLUSIONS

The combination of Plackett–Burman design with Box-Behnken design for optimizing the bioprocess variables for prodigiosin production by S. marcescens SWML08, is an effective and reliable tool to select the statistically significant factors and finding the optimal concentration of those factors in culture medium. The present work demonstrates the rewarding application of Box-Behnken design for quickly determining the conditions leading to the optimum yield of prodigiosin production. This study identified the effect of various factors in the production of prodigiosin by S. marcescens SWML08 in the culture medium and found that incubation temperature, (NH₄)₂PO₄ and trace salts are the significantly influenced factors for maximum prodigiosin production. This statistical methodology could be successfully applied to any bioprocess, where an analysis of the effects and interactions of many experimental factors are mandatory.

ACKNOWLEDGEMENT

The authors are thankful to Bharathiar University, Coimbatore, Tamil Nadu, India for providing facilities to undertake this study.

REFERENCES

Azuma, T., Watanabe, N., Yagisawa, H., Hirata, H., Iwamura, M. and Kobayashi, Y. (2000). Induction of apoptosis of activated murine splenic T cells by cycloprodigiosin hydrochloride, a novel immunosuppressant. *Immunopharmacology and immunotoxicology* 46, 29-37.

Box, G. E. P. and Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2, 455-475.

Chen, D., Han, Y. and Gu, Z. (2006). Application of statistical methodology to the optimization of fermentative medium for carotenoids production by *Rhodobacter sphaeroides*. *Process Biochemistry* 41, 1773-1778.

Chen, M. H. and Johns, M. R. (1993). The effect of pH in nitrogen source on pigment production by *Monascus purpureus*. *Applied Microbiology and Biotechnology* 40, 132-138.

D’Aoust, J. Y. and Gerber, N. N. (1974). Isolation and purification of prodigiosin from *Vibrio psychroerythrus*. *Journal of Bacteriology* 118, 756-757.

Djekrif-Dakhmouche, S., Gheribi – Aoulmi, Z., Meraih, Z. and Bennamoun, L. (2006). Application of a statistical design to the optimization of culture...
medium for α amylase production by Aspergillus niger ATCC16404 grown on orange waste powder. Journal of Food Engineering 73, 190-197.

Ellob, M. (2004). Optimization of medium composition for α-amylase production by Streptomyces coelicolor A3(2) with response surface methodology. Process Biochemistry 39, 1057-1062.

Furstner, A. (2003). Chemistry and biology of roseophilin and the prodigiosins: survey of the last 2500 years. Angewandte Chemie International Edition 42, 3582-3603.

Han, S. B., Park, S. H., Jeon, Y. J., Kim, Y. K., Kim, H. M. and Yang, K. H. (2001). Prodigiosin blocks T cell activation by inhibiting interleukin – 2Rα expression and delays progression of autoimmune diabetes and collagen-induced arthritis. Journal of Pharmacology Experimental Therapeutics 299, 415-425.

Juzlova, P., Martinkova, L. and Kren, V. (1996). Secondary metabolites of the fungus Monascus: a Review. Journal of Industrial Microbiology 16, 163-167.

Kim, S. J., Hong, K. L. and Joung, H. Y. (2008). Statistical Optimization of Medium Components for the Production of Prodigiosin by Hatsella chejuensis KCTC 2396. Journal of Microbiology and Biotechnology 18(12), 1903-1907.

Lewis, S. M. and Corpe, W. A. (1964). Prodigiosin producing bacteria from marine sources. Applied Microbiology 12, 13-17.

Liu, B. L. and Tzeng, Y. M. (1998). Optimization of growth medium for production of spores from Bacillus thuringiensis using response surface methodology. Bioprocess and Biosystems Engineering 18, 413-418.

Loukas, Y. L. (2001). A Plackett – Burman screening design directs the efficient formulation of multicomponent DRV liposomes. Journal of Pharmaceutical and Biomedical Analysis. 26, 255-263.

Montaner, B. and Perez-Thomas, R. (2001). Prodigiosin induced apoptosis in human colon cancer cells. Life Science 68, 2025-2036.

Myers R. H. and Montgomery, D. C. (2002). Response Surface Methodology. Wiley, New York.

Naveena, B. J., Altaf, Md. and Bhadriah, K. (2005). Selection of medium components by Plackett – Burman design for production of L (+) lactic acid by Lactobacillus amylophilus GV6 in SSF using wheat bran. Bioresource Technology 96, 485-490.

Park, P. K., Cho, D. H., Kim, E. Y. and Chu, K. H. (2005). Optimization of carotenoid production by Rhodotorula glutinis using statistical experimental design. World Journal of Microbiology and Biotechnology 21, 429-434.

Perez-Thomas, R., Montaner, B., Llagostera, E. and Soto-Cerrato, V. (2003). The prodigiosins, proapoptotic drugs with anticancer properties. Biochemical Pharmacology 66, 1447-1452.

Plackett, R. L. and Burman, J. P. (1946). The design of optimum multifactorial experiments. Biometrika 33, 305-325.

Ramirez, J., Gutierrez, H. and Gschaedler, A. (2001). Optimization of astaxanthin production by Phaffia rhodozyma through factorial design and response surface methodology. Journal of Biotechnology 88, 259-268.

Silva, C. J. S. M. and Roberto, I. C. (2001). Optimization of xylitol production by Candida guilliermondii FTI 20037 using response surface methodology. Process Biochemistry 361, 119-124.

Soto-Cerrato, V., Llagostera, E., Montaner, B., Scheffer, G.L. and Perez-Thomas, R. (2004). Mitochondria-mediated apoptosis operating irrespective of multidrug resistance in breast cancer cells by the anticancer agent prodigiosin. Biochemical Pharmacology 68, 1345-1352.

Vazquez, M. and Martin, A. M. (1997). Optimization of Phaffia rhodozyma continuous culture through response surface methodology. Biotechnology 57, 314-320.

Wenster-Boitz, D. (2000). Experimental design for fermentation media development: Statistical design or global random search. Journal of Bioscience and Bioengineering 90, 473-483.

Williams, R. P., Gott, C. L. and Green, J. A. (1960). Studies on pigmentation of Serratia marcescens. V. Accumulation of pigment fractions with respect to length of incubation time. Journal of Bacteriology 81, 376-379.