Application of response surface methodology in the formulation of nutrient broth systems with predetermined pH and water activities

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Polynomial models describing the individual and combined influences of solutes (sucrose and NaCl) and pH on the water activity ($a_w$) of nutrient broth systems (NBS) were established using Response Surface Methodology. For the sucrose-pH model, the linear, quadratic and interactive influences of sucrose concentration and pH significantly affected $a_w$, while only the linear and quadratic influences of NaCl and pH significantly affected $a_w$ in NaCl-pH model. The resulting models were characterized with satisfactory goodness-of-fit and efficient predictive performance. The established models may be used in the estimation of NBS components to formulate growth media with desired physicochemical property combinations and may be applied when studying microbial behaviors such as in quantitative microbial ecology.

Key words: Microbial growth media, physicochemical properties, response surface methodology.

INTRODUCTION

The emergence of predictive microbiology led food microbiologists to quantitatively elucidate influences of physicochemical food factors on the ecology of microorganisms during processing, distribution and storage (McMeekin et al., 1993). Jagannath and Tsuchido (2003a) explained that predictive microbiology is based on the premise that microbial responses to food factors are reproducible and thus by typifying microbial environments in terms of such factors, it is possible to predict the responses of microorganisms in new, similar environments. In predictive microbiology, McKellar and Lu (2003) emphasized the necessity to quantitate in terms of mathematical models. The first step in developing a model is usually the establishment of growth or death in a constant environment with a set of defined food factor combinations (Jagannath et al., 2003a; Baranyi and Roberts, 2004). Careful selection, definition and control of model-generating factors are necessary to come up with a reliable and significant model (Khuri and Cornell, 1987; Hu, 1999).

Control of pertinent food factors such as water activity ($a_w$) can however be difficult if adjustments are to be based only from gravimetric calculations. Jay et al. (2005) cited that as solutes interact with water and with each other in ways that are difficult to predict, estimations of $a_w$ of multicomponent systems based on the Raoult's law may be oversimplified and meaningless. For example, Bone (1969) mentioned that sucrose lowers the $a_w$ of food systems differently as compared to other solutes and hence does not follow the Raoult’s law. Gabriel (2008) recently reported the quadratic and interactive influences of pH and sucrose content (°Brix) on the $a_w$ of aqueous system through Response Surface Methodology (RSM). Gabriel (2008) developed and validated a highly significant polynomial model that assists formulation of sucrose solutions with specific pH and $a_w$ combinations.

Hence this study was conducted to develop and validate polynomial models that can be similarly used to facilitate the preparation of nutrient broth systems (NBS) with specific physicochemical property combinations. Preparations of NBS with specific combinations of pH, and $a_w$ may be applicable in modeling the effects of these solutes and changes in $a_w$. This study also builds on the foundation of Gabriel (2008) with the development and validation of polynomial models for the preparation of nutrient broth systems with predetermined pH and water activities.
physicochemical properties on certain microbial behaviors such as growth, inactivation and manufacture bioactive natural products.

MATERIALS AND METHODS

Experimental designs and NBS preparations

In this study, separate polynomial models that characterized the influences of various combinations of solutes and pH on NBS \( a_w \) were developed. In the development of each model, the rotatable central composite design (CCRD) of experiment was employed to determine the pH and the amounts of the solutes to be added to each of the NBS. Each CCRD was composed of 4 factorial point-, 4 axial point- and 6 center point combinations.

The pH of freshly prepared nutrient broth (NB, Eiken, Tokyo, Japan) was first adjusted with 5.0 N of HCl (Nacalai Tesque, Inc., Kyoto, Japan) or NaOH (Nakarai Chemicals, Ltd., Kyoto, Japan) before the dissolution of the respective amounts of sucrose (Nacalai Tesque, Inc., Kyoto, Japan) or NaCl (Nacalai Tesque, Inc., Kyoto, Japan) and sterilization at 121°C for 15 min. The NBS pH measurements and adjustments were conducted using a Horiba Navi pH meter (F-52, Horiba Ltd., Kyoto, Japan) calibrated with pH 7.0 and 4.0 standard solutions (Horiba Ltd., Kyoto, Japan). Minor pH adjustments were conducted prior to \( a_w \) measurements to correct for deviations in pH values that might have occurred during sterilization.

\( a_w \) measurements

The Rotronic Hygroscop DT \( a_w \) meter (Switzerland) was used in the measurement of \( a_w \) of the test NBS. Approximately 5.0 ml of the test NBS was placed inside the measuring chamber after which the head sensor was fitted to seal the chamber. The \( a_w \) values of the test NBS were recorded after equilibration which occurred within 30 - 40 min. In each of the developed model, \( a_w \) measurements were conducted in duplicates and responses were obtained from two independent experiments.

Model development and analyses

The responses obtained from each set of designed experiments were fitted in to the general form of quadratic polynomial model (Equation 1). This model incorporated the individual linear \( (x_1, x_2) \) and quadratic \( (x_1^2, x_2^2) \), and interactive \( (x_1 \cdot x_2) \) influences of the experimental variables on the measured response \( y \). The \( \beta \)’s corresponded to regression coefficients. Response fit analyses, regression coefficient estimations and model significance evaluations were conducted using the Design Expert (version 7.0.3) statistical software package (Statease, Minneapolis, MN). Surfaces showing the interactive influences of the model-generating factors on a particular response were constructed using the Statistica (version 1999) software package (Statsoft, Inc., Tulsa, OK).

\[
y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{1,2} (x_1 \cdot x_2) + \beta_1^2 x_1^2 + \beta_2^2 x_2^2 + \beta_{1,2}^2 (x_1 \cdot x_2)^2 \\
\text{(1)}
\]

Predictive efficacy validations

The predictive performances of the developed models were validated using separate sets of NBS with \( a_w \) values different from those enumerated in the model establishment phase. Freshly prepared validating NBS were subjected to pH and \( a_w \) analyses following the previously elaborated methods. For the validation of predictive performances of the models, the percentage solute and pH values of the validating NBS were factored in to the models to calculate the predicted \( a_w \). The predictive performances of the models were assessed using the performance indices namely, bias \( (B_f) \) and accuracy \( (A_f) \) factors (Ross, 1996; Baranyi et al., 1999). The \( B_f \) measured the mean difference between the predicted/ calculated and measured/actual \( a_w \) and was estimated using Equation 2:

\[
B_f = 10^{\left( \frac{\Sigma (\log \text{predicted}/\text{measured})}{n} \right)} \\
\text{(2)}
\]

Where; \( n \) corresponded to the number of replications employed during the validation processes. When the \( B_f < 1.00 \), a model underestimated the measured property (predicted < measured) while \( B_f > 1.00 \) indicates a model overestimation (measured > predicted). A \( B_f \) value of 1.00 implies that the predicted and the measured values were equal. Since the \( B_f \) does not provide a measure of the accuracy of model predictions, the \( A_f \) was also calculated (Equation 3).

\[
A_f = 10^{\left( \frac{\Sigma |\log \text{predicted}/\text{measured}|}{n} \right)} \\
\text{(3)}
\]

Take note that the only difference between Equations 2 and 3 is that the \( A_f \) value measures the mean absolute difference between the predicted and measured values. The \( A_f \) takes the values of > 1.00 where greater values indicate less predictive accuracy of a model while an \( A_f =1.00 \) is an indication of a perfect model prediction. Hence for model estimations where predicted > measured, the calculated \( A_f \) and \( B_f \) values are equal and have the same sign. On the other hand, for estimations where measured < predicted, the calculated indices shall have the same value but of opposite signs.

Graphical comparisons of the predicted and measured values were also done by plotting the predicted values against the measured results. The line of equivalence (LOE) with an equation of \( y = x \) and bisects the plot diagonally through the origin (0, 0)was traced to indicate a region where the predicted and measured values are equal. A point falling on the LOE has \( A_f = B_f = 1.00 \), hence a perfect model prediction. Points falling above \( (A_f = B_f >1.00) \) and below \( (A_f > 1.00, B_f < 1.00) \) indicate model over- and underestimations, respectively. Points that are farther from the LOE had greater \( A_f \) and \( B_f \) values. Hence, the positions of the points relative to the LOE were also used to illustrate the predictive performance of the developed models.

RESULTS AND DISCUSSION

Model fittings and analyses

Table 1 summarizes the responses obtained from each of the combinations of pH and solute concentrations per model development experiment. Each of the response values were presented as averages of 4 values obtained from 2 independent experiments. The influences of the amount of solutes and pH on the \( a_w \) of NBS were determined by fitting the responses into the second-order polynomial model (Equation 1). This general form of the equation accounted for the possible nonlinear relationships between the predictive variables and the measured responses (Mendenhall and Sinich, 1996). Results of the analyses of variance (ANOVA) that ensures fit of the developed models are presented in Table 2. The F-test results demonstrated that the models can be used to reliably predict the response variables (Adinarayana and
Ellaiah, 2002). It was also evident in both models that the predictors had nonlinear influence on the response and hence, the utilization of second-order models was deemed appropriate.

Furthermore, the statistics (Table 4) used to evaluate the goodness of fit of the responses into the model support the results obtained from the F-tests. The calculated coefficients of variation were low at 0.27 and 0.71 for the Sucrose-pH and NaCl-pH models, respectively. Such values are indicative of precision and reliability of the experiments conducted in the development of the models (Adinarayana et al., 2002). The values of the coefficients of determination ($r^2$) and adjusted $r^2$ signify that the models can predict and explain the total variations in the measured responses with high degree of accuracy. In all of the models, the values calculated for the predicted $r^2$ were in reasonable agreement with the adjusted $r^2$ and the values for adequate precision indicated desirable signal-to-noise ratios (Statease, 2008).

**Influences of solute concentration and pH on NBS $a_w$**

The predictive equations for the $a_w$ of NBS in terms of solute concentrations and pH are given in Equations 4 and 5. These equations are the Sucrose-pH and NaCl-pH models that respectively resulted from the estimated coefficients ($\beta$) presented in Table 3. In Table 2, it is also shown that while the interaction of sucrose concentration and pH significantly influenced the measured $a_w$, NaCl and pH had no significant joint influence on $a_w$. The differences in the contours of the surfaces are also indications of the varying effects of the predictive variables on the response.

\[
\begin{align*}
 a_w &= 0.94 - (1.45\times10^{-4} \text{ sucrose}) + (0.014 \text{ pH}) \\
 &\quad+ (5.60\times10^{-3} \text{ sucrose} \times \text{pH}) \\
 &\quad- (1.86\times10^{-5} \text{ sucrose}^2) - (9.79\times10^{-4} \text{ pH}^2) \\
\end{align*}
\]

(4)

\[
\begin{align*}
 a_w &= 1.03 - (2.07\times10^{-3} \text{ NaCl}) - (0.016 \text{ pH}) \\
 &\quad- (3.79\times10^{-17} \text{ NaCl} \times \text{pH}) \\
 &\quad- (2.88\times10^{-4} \text{ NaCl}^2) + (1.45\times10^{-3} \text{ pH}^2) \\
\end{align*}
\]

(5)

When charted on 3-dimensional plots, these equations resulted into response surfaces (Figure 1) that demonstrated the simultaneous influences of the solute concentration and pH on $a_w$. In Figure 1a, the curvatures along the percentage of sucrose and pH axes are indicators of the significant quadratic influences of these variables on $a_w$. The quick descent of the surface along the percent sucrose axis compared to its gradual ascent along the pH axis indicated that the solute concentration had greater influence on $a_w$. At any fixed solute level, it is also evident that the $a_w$ decreased with decreasing pH value. Such results have been similarly observed by Gabriel (2008) who established the influences of sucrose levels and pH on the $a_w$ of aqueous systems. Gabriel (2008) attributed this observation to the acid-mediated sucrose inversion that produces approximately two moles of monosaccharides, hence more water-binding solutes, from one mole of sucrose. Whistler and Daniel (1985) noted that the glycosidic linkages, such as those that link the fructose and glucose units of a sucrose molecule, are more readily cleaved in acidic than in basic environments. Bennion (1985) and McWilliams (1993) explained that organic acidulants are commonly used in sugar cookery to promote sugar inversion. In a sucrose solution, hydrolysis of sucrose into fructose and glucose hampers sugar recrystallization since the disaccharide crystallizes easier than its monosaccharide components.

The simultaneous influence on the percentage of NaCl and pH on NBS $a_w$ is demonstrated in Figure 1b. In this response surface, both the curvatures along the solute concentration and pH axes were found to be significantly associated with the response. Nonetheless, analysis of variance (ANOVA) in Table 2 showed that the quadratic
Table 2. F-values obtained from response surface model ANOVAs

<table>
<thead>
<tr>
<th>Source</th>
<th>Sucrose-pH model</th>
<th>NaCl-pH model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>410.80 **</td>
<td>102.54 **</td>
</tr>
<tr>
<td>$x_1$</td>
<td>1701.35 **</td>
<td>476.67 **</td>
</tr>
<tr>
<td>$x_2$</td>
<td>70.48 **</td>
<td>5.76 *</td>
</tr>
<tr>
<td>$x_1 \cdot x_2$</td>
<td>6.24 *</td>
<td>0.00</td>
</tr>
<tr>
<td>$x_1^2$</td>
<td>267.84 **</td>
<td>22.78 *</td>
</tr>
<tr>
<td>$x_2^2$</td>
<td>16.74 **</td>
<td>5.57 *</td>
</tr>
</tbody>
</table>

$^1 (x_1, x_2)$: Sucrose-pH model, (% sucrose, pH); NaCl-pH model, (% NaCl, pH)

** Significant at $p=0.01$ and $p=0.05$; * Significant only at $p=0.05$

Table 3. Statistics used in the evaluation of goodness-of-fit of the responses to the models

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Sucrose-pH Model</th>
<th>NaCl-pH Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient of variation (% CV)</td>
<td>0.2700</td>
<td>0.7100</td>
</tr>
<tr>
<td>Determination coefficient ($r^2$)</td>
<td>0.9966</td>
<td>0.9865</td>
</tr>
<tr>
<td>Adjusted $r^2$</td>
<td>0.9942</td>
<td>0.9769</td>
</tr>
<tr>
<td>Predicted $r^2$</td>
<td>0.9793</td>
<td>0.8713</td>
</tr>
<tr>
<td>Adequate precision</td>
<td>58.33</td>
<td>30.88</td>
</tr>
</tbody>
</table>

Table 4. Predictive efficacy of the developed polynomial models: model performance indices

<table>
<thead>
<tr>
<th>Model</th>
<th>$n$</th>
<th>NBS properties $^1$</th>
<th>Model performance indices $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$x_1$</td>
<td>$x_2$</td>
</tr>
<tr>
<td>Sucrose-pH</td>
<td>20</td>
<td>10.00</td>
<td>03.78</td>
</tr>
<tr>
<td>NaCl-pH</td>
<td>20</td>
<td>00.50</td>
<td>03.91</td>
</tr>
</tbody>
</table>

$^1 (x_1, x_2)$: Sucrose-pH model, (% sucrose, pH); NaCl-pH model, (% NaCl, pH). Values are presented as ranges.

$^2$ A predictive model that exactly predicts a particular response has $A_i = B_i = 1.00$.

$n$: number of NBS used to validate each of the models

Figure 1. Response surfaces showing the influences of solutes and pH on the $a_w$ of NBS.
influence of NaCl concentration had more significant effect on the $a_w$. NaCl dissociation and the subsequent ionic interactions with water and other chemical species present in the NBS must have been the primary mechanism of the $a_w$-lowering of the solute. Cheftel et al. (1985) discussed that protein and water interaction may be affected by pH and the presence of ions from neutral salts. Variations in $a_w$ with changing pH values may also be related to the shifting solubility of proteins in aqueous systems. Fennema (1985) defined $a_w$ to be related to the fugacity, or the escaping tendency, of water in a particular aqueous system. Hence $a_w$ determinations are commonly conducted by measuring the equilibrium relative humidity (ERH) of the sample. Dissolution of sugars, salts and other hydrophilic compounds induce dipole-dipole, ionic and hydrogen bond interactions with water that alter the system ERH (Le Maguer, 1987; Decagon Devices, Inc. 2001). In this study, the presence of other dissolved and suspended compounds in the NBS such as meat extract and peptone must have also influenced $a_w$ since hydrophobic water interactions with proteins have also been shown to affect $a_w$ (Decagon Devices, Inc., 2001). Ions introduced by the pH adjusting acid or base must have also contributed to the measured $a_w$. The production of other chemical species that resulted from chemical changes in and reactions between the NBS components must have also contributed to the measured $a_w$ values. Dehydration and thermal degradation of sugars are catalyzed in either acidic or basic environments and may produce a number of organic acids and other charged compounds (Whistler et al., 1985; Wong, 1989). Reducing sugars can also react with amino-bearing compound, usually proteins, in a process called Mallard reaction (Whistler et al., 1985; Wong, 1989). Such reaction can result to the formation of several compounds that may influence the system $a_w$.

**Validation of the models**

Predictive models may be reliably used in decision-making only after subject to validation (Jagannath and Tsuchido, 2003b). Validation is an essential step in model development because it allows for the determination of the applicable range and performance limits of models. Hence, each of the developed polynomial models was subjected to validation using NBS with sets of properties different from those used during model development (Ross, 1996; Carrasco et al., 2006). Model validations were conducted using only significant model terms listed in Table 2. Table 5 presents the bias and accuracy indices calculated in the evaluation of the predictive efficacy of the developed models. The calculated $B_i$ values suggest that both models over- and underestimated the actual $a_w$ of the validating NBS.

Moreover, the calculated $A_i$ values in both models were close to 1.00, suggesting the high level of accuracy of model predictions. The calculated predictive indices were in agreement to the results of the graphical predictive validations. In Figure 2, when the predicted and measured $a_w$ of the validating NBS were plotted, the coordinates were shown to be in close proximity to the line of equivalence indicative of highly accurate model predictions. Ross, Dalgaard and Tienungoon (2000) explained that models should ideally have $A_i$ values of 1.00 that are indicative of perfect agreement between the predicted and measured values. However, they have also explained that typically, the $A_i$ values of a model increase by 0.10 to 0.15 units for every predictive variable in the model. Hence in this study where both models estimated the $a_w$ of NBS from two factors, model predictions may be expected to have $A_i$ values that range from 1.20 to 1.30. Thus all of the developed models were evaluated to have highly acceptable predictive accuracy.

In summary, this study elucidated the influences of dissolved sucrose and NaCl on the water activity of NBS through polynomial models. The developed models were characterized and validated to have highly acceptable predictive accuracy and may be proven valuable in facilitating formulations of NBS of predetermined physico-chemical properties. Such growth media with precise pH and $a_w$ values may be used in developing models for the influences of these factors on microbial behaviors including growth, inactivation and manufacture of bioactive natural products. The developed models are however limited to be applied in the formulation of Nutrient Broth medium. The utility of the models in other microbial growth media such Trypto-Soy Broth and Brain Heart Infusion Broth should be subjected to further validation.

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**Table 5.** Predictive efficacy of the developed polynomial models: model performance indices.

<table>
<thead>
<tr>
<th>Model</th>
<th>$n$</th>
<th>NBS Properties</th>
<th>Model Performance Indices</th>
<th>$A_i$</th>
<th>$B_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$x_1$</td>
<td>$x_2$</td>
<td>Bias ($B_i$)</td>
<td>Accuracy ($A_i$)</td>
</tr>
<tr>
<td>Sucrose-pH</td>
<td>20</td>
<td>10.00 - 70.00</td>
<td>03.78 - 08.94</td>
<td>0.980 - 1.049</td>
<td>1.005</td>
</tr>
<tr>
<td>NaCl-pH</td>
<td>20</td>
<td>00.50 - 20.00</td>
<td>03.91 - 09.18</td>
<td>0.984 - 1.036</td>
<td>0.997</td>
</tr>
</tbody>
</table>

1. $(x_1, x_2)$: Sucrose-pH model, (% sucrose, pH); NaCl-pH model, (% NaCl, pH). Values are presented as ranges.
2. A predictive model that exactly predicts a particular response has $A_i = B_i = 1.00$.
3. $n$: number of NBS used to validate each of the models.
Figure 2. Graphical presentations of predictive efficacies of the sucrose-pH (a) and NaCl-pH models. The bold diagonal lines bisecting the plots are the lines of equivalence that depict the region where the predicted and measured $a_w$ are in perfect agreement ($p_{aw} = m_{aw}$). The shaded areas represent the acceptable model prediction regions where $A_f < 1.10$.

REFERENCES


Gabriel and Nakano

