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Full Length Research Paper

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

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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 β'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).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{1\text{-}2} (x_1 \cdot x_2) + \beta_1^2 x_1^2 + \beta_2^2 x_2^2 \quad (1)$$

Predictive efficacy validations

The predictive performances of the developed models were validated using separate sets of NBS with factor 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 ($^p a_w$). 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:

$$B_f = 10\text{^} \{\Sigma (\log \text{predicted/measured})/n\} \quad (2)$$

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).

$$A_f = 10\text{^} \{\Sigma |\log \text{predicted/measured}|/n\} \quad (3)$$

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 $\geq$ 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 Sincich, 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

Table 1. Effect of different solutes and pH on the a_w of nutrient broth systems: Models 1 and 2.

CCRD Coded Combinations		Sucrose-pH Model				NaCl-pH Model			
		x_1	x_2	y		x_1	x_2	y	
x_1	x_2	%Sucrose	pH	a_w [1]		%NaCl	pH	a_w	
-1	-1	12.0	4.5	0.983	± 0.001	04.0	4.5	0.980	± 0.002
±1	-1	70.0	4.5	0.899	± 0.001	19.0	4.5	0.876	± 0.006
-1	±1	12.0	8.5	0.989	± 0.004	04.0	8.5	0.980	± 0.002
±1	±1	70.0	8.5	0.918	± 0.003	19.0	8.5	0.876	± 0.004
0	-α	41.0	3.7	0.947	± 0.005	11.5	3.7	0.934	± 0.001
0	±α	41.0	9.3	0.973	± 0.003	11.5	9.3	0.966	± 0.030
-α	0	00.0	6.5	0.989	± 0.003	00.9	6.5	0.987	± 0.002
±α	0	82.0	6.5	0.884	± 0.001	22.1	6.5	0.843	± 0.003
0	0 [2]	41.0	6.5	0.967	± 0.003	11.5	6.5	0.936	± 0.004

[1]Response values (a_w) are presented as averages of 4 trials obtained from 2 independent experiments ± standard deviation.
[2]Center points. Replicated 6 times as per the CCRD.

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 NBS 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 (β) 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.

$$a_w = 0.94 - (1.45\times10^{-4}\ \text{sucrose}) + (0.014\ \text{pH}) + (5.60\times10^{-5}\ \text{sucrose}\times\text{pH}) - (1.86\times10^{-5}\ \text{sucrose}^2) - (9.79\times10^{-4}\ \text{pH}^2) \quad (4)$$

$$a_w = 1.03 - (2.07\times10^{-3}\ \text{NaCl}) - (0.016\ \text{pH}) - (3.79\times10^{-17}\ \text{NaCl}\times\text{pH}) - (2.08\times10^{-4}\ \text{NaCl}^2) + (1.45\times10^{-3}\ \text{pH}^2) \quad (5)$$

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

Source[1]	Sucrose-pH model		NaCl-pH model	
Model	410.80	**	102.54	**
x_1	1701.35	**	476.67	**
x_2	70.48	**	5.76	*
$x_1 \cdot x_2$	6.24	*	0.00	
x_1^2	267.84	**	22.78	*
x_2^2	16.74	**	5.57	*

[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

Statistics	Sucrose-pH Model	NaCl-pH Model
Coefficient of variation (% CV)	0.2700	0.7100
Determination coefficient (r^2)	0.9966	0.9865
Adjusted r^2	0.9942	0.9769
Predicted r^2	0.9793	0.8713
Adequate precision	58.33	30.88

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

Model	n	NBS properties [1]		Model performance indices [2]			
				Bias (B_f)		Accuracy (A_f)	
		x_1	x_2	range	average	range	average
Sucrose-pH	20	10.00 - 70.00	03.78 - 08.94	0.980 - 1.049	1.005	1.000 - 1.049	1.012
NaCl-pH	20	00.50 - 20.00	03.91 - 09.18	0.984 – 1.036	0.997	1.000 - 1.036	1.009

[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 $A_f = B_f = 1.00$.
n: number of NBS used to validate each of the models

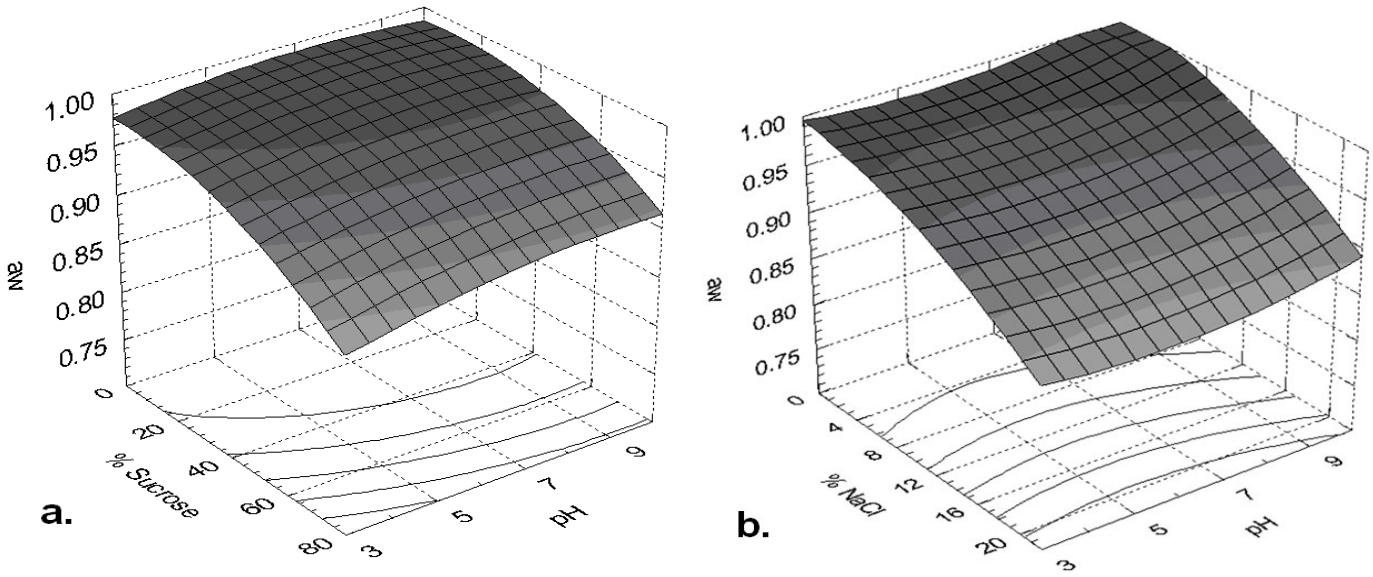

Figure 1. Response surfaces showing the influences of solutes and pH on the a_w of NBS.

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

Model	*n*	NBS Properties[1]		Model Performance Indices[2]			
				Bias (B_f)		Accuracy (A_f)	
		x_1	x_2	Range	Average	Range	Average
Sucrose-pH	20	10.00 - 70.00	03.78 - 08.94	0.980 - 1.049	1.005	1.000 - 1.049	1.012
NaCl-pH	20	00.50 - 20.00	03.91 - 09.18	0.984 - 1.036	0.997	1.000 - 1.036	1.009

[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 $A_f = B_f = 1.00$.
n: number of NBS used to validate each of the models.

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 subjection 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_f values suggest that both models over- and underestimated the actual a_w of the validating NBS.

Moreover, the calculated A_f 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_f 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_f 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_f 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.

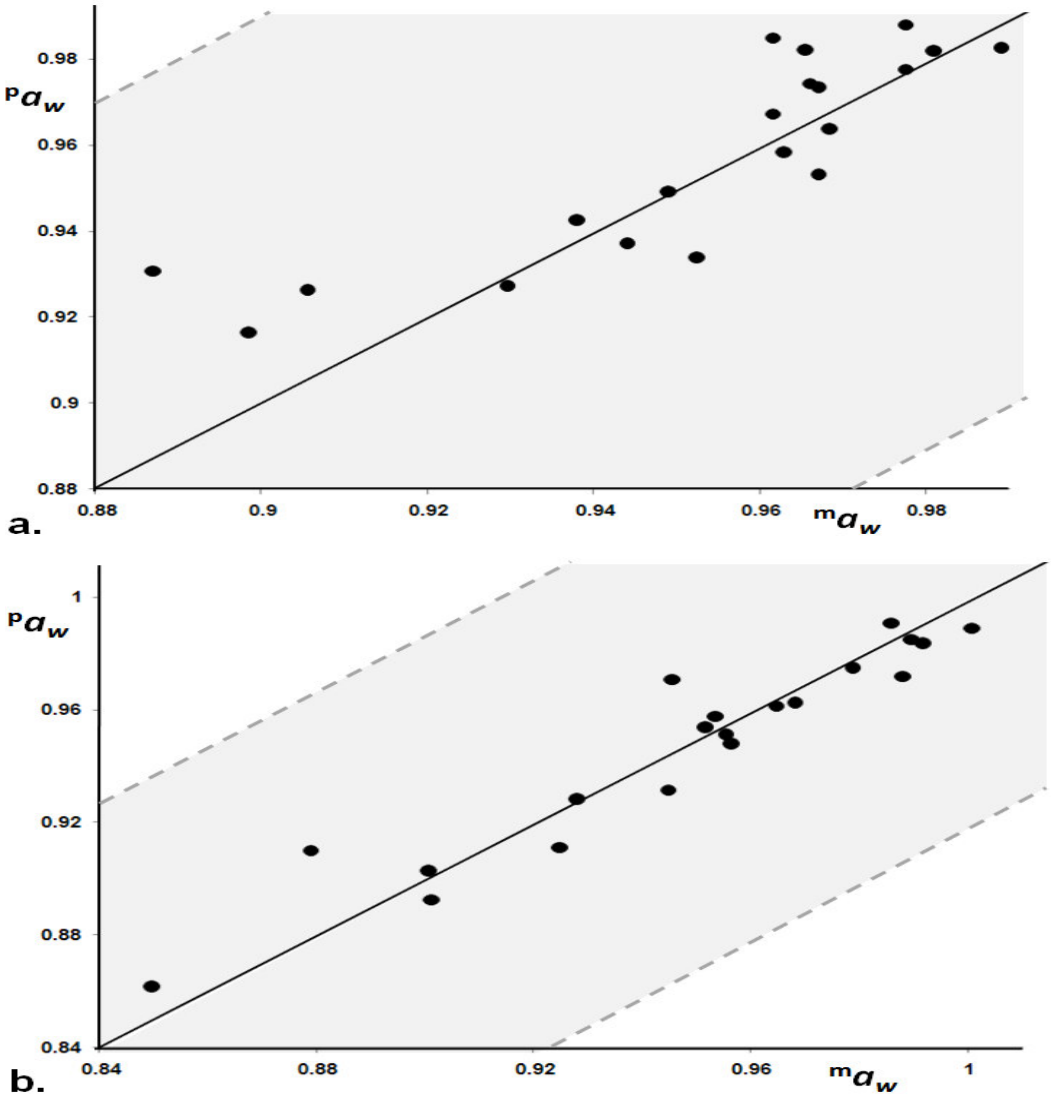

Figure 2. Graphical presentations of predictive efficacies of the sucrose-pH (a) and NaCl-pH models. The bold diagonal lines bisecting the plots are the lines of equivalence that depict the region where the predicted and measured a_w are in perfect agreement ($^{p}a_w = {}^{m}a_w$). The shaded areas represent the acceptable model prediction regions where $A_f \leq 1.10$.

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