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Development and Process Optimization of Biodegradable Films Based on Banana (*Musa sp.*) Flour

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ABSTRACT

A study was conducted to develop banana flour based edible film using Response Surface Methodology. A Central Composite Design was adopted in the optimization of banana flour and sodium alginate level for the development of edible film with 13 different runs and 5 trials of two similar centre points. Effects of banana flour (2-4% w/v) and sodium alginate (0.5-1.0% w/v) level on the thickness, moisture, solubility, Hue angle and Chroma value of banana flour based edible film were investigated. For each response, a second-order polynomial model was developed using multiple linear regression analysis. The coefficients of determination, R^2 of all the response variables were higher than 0.95 and the lack-of-fit tests were not significant. Applying desirability function method, optimum conditions were found to be 2.81% w/v banana flour and 0.80% w/v sodium alginate level for banana flour based edible film. At this optimum point, thickness, moisture, solubility, Hue angle and Chroma value were found to be 170.18µm, 17.80%, 27.23%, 83.73 and 9.27 respectively. The composite film has a good potential as secondary packaging material to enhance the functionality of meat, dairy, poultry and seafood.

Keywords: Raw banana flour, sodium alginate, composite film, central composite design

Edible films, serving as selective barriers to moisture, oxygen, lipid oxidation, and losses of volatile gases, aromas and flavors, can improve food quality and shelf-life. Although edible films can be prepared using natural polymers include polysaccharides, proteins and fats (Singh *et al*., 2015). Potential properties, applications and characterization of edible films have been extensively reviewed (Bravin *et al*., 2006; Min *et al*., 2005; Serrano *et al*., 2006; Emiroglu *et al*., 2010; Singh *et al*., 2015). In recent years, the development of fruits flour films has been investigated. Fruits flours are naturally occurring complex blends of starch, cellulose derivatives, pectin, protein, lipids and fibers that have shown intrinsic molecular interactions during film formation with excellent film characteristics as composite biodegradable film (Pelissari *et al*., 2013).

Bananas (genus *Musa*) are grown extensively in tropical and subtropical regions and are an important food crop. An interesting renewable source for the production of edible and biodegradable materials is the banana flour (Pelissari *et al*., 2013). Currently, its worldwide production is around 139.2 million tons, and India is the largest producer (Annonymous, 2016). Banana flour has 73.4% starch content (Dangaran *et al*., 2006); moreover, this fruit contains 1.0-2.5 % protein, 0.2-0.5% lipids and 1.5-2.5% fiber (Zhang *et al*., 2005). Its flour presents properties to form renewable, biodegradable and inexpensive films for the food products (Jirukkakul, 2016; Pelissari *et al*., 2013). Pelissari *et al*. (2013) reported that the protein and lipids present in the banana flour exert an important and well-known plasticizer effect, which reduces the mechanical resistance and increases the flexibility of the biopolymer films. Moreover, the polysaccharide and the protein present in the banana flour provide additional hydrogen bonding interactions between the polymer chains, accounting for the higher film strength (Sothornvit and Pitak, 2007).

Romero-Bastida *et al*. (2005) isolated starches from banana (*Musa paradisiaca*) and characterized to produce edible films. Edible film that made from banana flour is modified with adding glycerol as plasticiser. It can make film characteristic smooth, more flexible, increase film permeability toward gas, water, and volatile substance (Yan *et al*., 2012).

In this context, the objective of the present study was to determine the optimal formulation by using response surface methodology and multi-response analysis to obtain banana flour based edible films, which are relevant for food packaging applications.

MATERIALS AND METHODS

The study was conducted from December 2015 May 2016. Raw bananas (*Musa sp.*) were purchased from the local market of Bareilly, India. Food grade citric acid and stabilizer cum binder, sodium alginate was procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India) and plasticizer (glycerol) was procured from MERCK, Mumbai, India.

Preparation of banana flour

Raw banana flour was obtained by cutting peeled banana in to 5mm slices, dipping in 0.25% (w/v) citric acid solution and then drying at 45°C for 12 h in an oven and grinding. The final moisture content of flour was 8-10%. The flour was packaged and sealed and stored at 25±2°C for further use (Lalawmpuii, 2015; Agama-Acevedo *et al*., 2012). Asif-Ul-Alam *et al*. (2014) reported that the method of drying affects the physico-chemical properties and sensory quality of the banana flour.

Film preparation

The banana flour (BF) based edible film was prepared by dissolving 2.0-4.0% w/v of BF and 0.5-1.0% w/v of sodium alginate (SA) level in distilled water under continuous stirring and heating in the presence of 1.5 % w/v glycerol as plasticizer at 85°C using hot plate with magnetic stirrer (Model 11,603, Merck Specialties Pvt. Ltd., MERCK, Mumbai, India) for 30 minutes. The above levels of BF, SA and glycerol, drying temperature and time were established on the basis of preliminary trials and on consultation with literature (Jirukkakul, 2016; Pelissari *et al*., 2013; Sothornvit and Pitak, 2007). Thereafter, the solution was filtered through a muslin cloth to remove undissolved material. Then, the solution (100 ml) was poured into a glass petridish of 6 inch diameter and dried in food dryer (Ambay Biotech, India) at 45°C temperature for 12 hours. After drying, the film was carefully peeled off from the plate, sealed in a LDPE bag and stored in a

Table 1: Experimental data for the two factors (coded and un-coded) three independent level of central composite design

Runs	Variables		Responses				
	Banana flour (% w/v) X_1	Sodium alginate (% w/v) X_2	Thickness (µm)	Moisture (%)	Solubility (%)	Hue angle	Chroma value
1	4.41 (α)	0.75 (0)	187.4	16.62	23.63	82.43	9.95
2	4.00 (1)	0.50 (-1)	181.1	17.34	24.69	83.15	9.72
3	3.00 (0)	0.75 (0)	168	17.81	26.61	83.58	9.39
4	3.00 (0)	0.75 (0)	170.6	17.89	26.98	83.61	9.35
5	2.00 (-1)	1.00 (1)	160.7	18.16	28.67	83.9	8.94
6	3.00 (0)	0.75 (0)	171.9	17.77	26.78	83.59	9.32
7	1.59 (-α)	0.75 (0)	157	18.22	31.21	84.1	8.76
8	3.00 (0)	0.40 (-α)	164	18.04	27.89	83.74	9.07
9	4.00 (1)	1.00 (1)	184.3	17.02	23.99	82.77	9.86
10	2.00 (-1)	0.50 (-1)	154	18.37	32.1	84.38	8.58
11	3.00 (0)	0.75 (0)	166	17.85	27.04	83.71	9.4
12	3.00 (0)	0.75 (0)	167.5	17.83	27.01	83.78	9.42
13	3.00 (0)	1.10 (α)	175.1	17.54	25.35	83.47	9.58

humidity chamber at 50% RH and 25°C temperature. This was then analysed for different parameters in triplicate viz. thickness, moisture, solubility, Hue angle and Chroma value. Central Composite Design (CCD) was used in which, a total of 13 different trials were conducted with three different levels of BF and SA with 5 replicates of two similar centre points (3.0, 0.75) as presented in Table 1.

Experimental design and statistical analysis

Response surface methodology (RSM) is a mathematical and statistical technique for testing multiple process variables and their interactive, linear and quadratic effects, and useful in solving multivariable equations obtained from experiments simultaneously (Montgomery, 2005). In the present study, a two factor, three independent level central composite design (CCD) with five replicates at centre point was used and total thirteen number of combinations (2.0-4.0% w/v of raw banana flour (BF), 0.5-1.0% w/v sodium alginate (SA) level) (Table 1) were performed to develop predictive models for response parameters.

The following second order polynomial equation of function X_i was fitted for each factor assessed:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2_i + \sum \beta_{ij} X_i X_j \quad \ldots(1)$$

From the equation presented, Y may be defined as the response for the variables assessed, where, β_o is constant term, i is a parameter estimate for linear terms, β_{ii} is quadratic terms, β_{ij} is interactive terms. i and j are the levels of the factors with k being the number of factors assessed.

The significant terms in the model (Eq.1) were found by analysis of variance (ANOVA) for each response and the significance was judged by calculating the model F-value at the probability level of <5%. The optimal values of the process conditions were obtained by the desirability function, a multi-response analysis, proposed by Derringer and Suich (1980). Experimental results of the two factors, three independent levels CCD are shown in Table 1. The statistical software package Design-Expert (Stat-Ease Inc., Minneapolis, USA, 8.0.4 trial version) was used to conduct the regression analysis of experimental data and to plot the response surface.

Film characterization

Thickness

Film thickness was measured by using a 0-25 mm digital micrometre (Mitutoyo manufacturing, Tokyo, Japan) with an accuracy of ±0.01 mm. Measurements were taken at five different points at centre and on the sides covering the complete length of film and the mean values were represented as the film thickness in micron (µm).

Moisture

The moisture of sample was determined after drying in an oven at 90°C for 24 h as per the gravimetric method. The moisture of the sample was determined in triplicate for each film sample and expressed as a percentage based on the initial weight of film.

Table 2: F-values and effect of independent variables on responses

Source of variance	Thickness (µm) R^2=0.9558	Moisture (%) R^2=0.9861	Solubility (%) R^2=0.9916	Hue angle R^2=0.9738	Chroma value R^2=0.9669
Linear	**F value**	**F value**	**F value**	**F value**	**F value**
β_1	201.13[a]	423.39[a]	710.44[a]	228.03[a]	264.13[a]
β_2	15.01[a]	32.98[a]	81.42[a]	15.77[a]	28.12[a]
Cross Product					
β_{12}		0.52	20.35[a]	0.20	—
Quadratic					
β_{11}	—	36.99[a]	9.50[a]	15.64[a]	—
β_{22}	—	0.11	0.16	0.01	
Lack of fit	0.91	5.43	4.98	2.40	6.11
Total Model	108.07[a]	99.03[a]	164.48[a]	52.01[a]	146.12[a]

Significant = [a]$p<0.05$

Water Solubility

The water solubility of films was determined as the content of dry matter solubilized after 24 h of immersion in water (Singh *et al.,* 2015). The film was dried in a hot air oven at 90°C for 24 h. The dried film weighing 600 mg was immersed in 50 ml of water using a 100 ml flask at 23±1°C for 24 h in an orbital shaking incubator (REMI, CIS-24 Plus, Mumbai, India) with continuing shaking at 120rpm. The pieces of undissolved film were then taken out and dried in a hot air oven at 90°C for 24 h. Solubility (%) was calculated as following:

Solubility (%) = [(Initial Wt. of dried film – Wt. of undissolved dried film) / Initial Wt. of dried film] × 100

Hue and Chroma values

The colour of film was measured using a Lovibond Tintometer (Model F, Greenwich, UK). The sample colour was matched by adjusting the red (*a**) and yellow (*b**) units, keeping the blue unit fixed at 3 and the corresponding colour values displayed were noted. The Hue (relative position of colour between redness and yellowness) and Chroma (Intensity, brightness or vividness of colour) was determined by using the following formula (Little 1975).

$$\text{Hue} = (\tan^{-1})\, b/a$$

$$\text{Chroma} = [a^2 + b^2]^{0.5}$$

Where, a = red unit, b= yellow unit

RESULTS AND DISCUSSION

The effects of BF and SA level on different responses *viz*. thickness, moisture, solubility, hue angle and chroma value are shown in Table 1. Perusal of Table 2 revealed that for all the responses, F-values for the "model" were significant ($p<0.05$) and that for "lack of fit" were non-significant ($p>0.05$), which showed a good fit between the experimental data and the model. These models also adequately explained the variation of the responses with satisfactory R^2 values, which indicated that most variations were well explained by different models (Linear, 2FI or Quadratic).The independent and dependent variables were fitted to the second-order model equation and examined for the goodness of fit. Second order polynomial equation can be used to determine response for any combination by substituting values of independent variables and regression coefficients and their interaction (Table 3). The R^2 values of all the responses exceeded 0.95 indicating a good fit, as shown in Table 3. Therefore, the response surface models developed were adequate.

Thickness

The second order polynomial equation generated relating independent variables BF (X_1) and SA (X_2) and the dependent variable thickness was as:

$$\text{Thickness} = +125.0817 + 11.7115X_1 + 12.7989X_2$$

From the above equation, it was seen that thickness was found to have linear relationship with the independent variables. As software showed that the "Predected R-Squared" of 0.9260 is in reasonable agreement with the "Adjusted R-Squared" of 0.9469.

The analysis of variance showed that BF and SA level linearly significantly ($p<0.05$) affected the thickness of films (Table 2). A low value of the coefficient of variation

Table 3: Regression coefficients of second order polynomial equations showing relationships among response variables and independent variables

Term	Regression coefficients	Thickness (µm)	Moisture (%)	Solubility (%)	Hue angle	Chroma value
Constant	β_0	125.0817	18.21244	47.24119	84.66184	7.47229
Banana flour (A)	β_1	11.7115	0.58216	-7.02047	$0.32928X_1$	$0.46786X_1$
Sodium alginate (B)	β_2	12.7989	-0.51355	-10.93805	$-1.02292X_2$	$0.61062X_2$
Banana flour * Banana flour (A^2)	β_{11}	—	-0.17562	0.35363	$-0.16575X1^2$	—
Sodium alginate * Sodium alginate (B^2)	β_{22}	—	0.15000	-0.74200	$0.068000X_2^2$	—
Banana flour * Sodium alginate (AB)	β_{12}	—	-0.11000	2.73000	$0.10000X_1X_2$	—

β_0 - Intercept, β_i - regression coefficients for linear terms, β_{ii}- regression coefficients for quadratic terms, β_{ij}- regression coefficients for interactive terms, Significant at $p<0.05$

(1.38%) indicated higher precision and reliability of the experiment. The R^2 value (0.9558), being a measure of the 'goodness' of fit of the model, indicated that 4.42% of the total variation was not explained by the model (Table 2). Thickness showed positive linear relationship with the BF and SA level (Table 3) (Fig.1). Generally, ultimate thickness of the film depends on the nature, concentration, composition, drying time temperature combination (Singh *et al*., 2015).Tapia-Blacido *et al*. (2005) explained several phenomena during the drying of the film-forming solution, because of its complex composition (biopolymers and plasticizers) which influence the structure and properties of the film by evoking changes in the microstructure.

Moisture

Leceta *et al*. (2013) explained that moisture content of edible films is very important for food packaging applications. Moisture was found to have quadratic relationship with the two process variables as per the following equation:

$$\text{Moisture} = +18.21244 - 0.58216X_1 - 0.51355X_2 - 0.17562X_1^2 + 0.15000X_2^2 - 0.11000\,X_1X_2$$

Regression analysis showed that BF level (linearly and quadratic terms) and SA level (linearly) significantly ($p<0.05$) affected the moisture content of films (Table 2).The Model F-value of 99.03 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The R^2 value (0.9861) indicated that 1.39 % of the total variation was not explained by the present model (Table 2). With the increase in BF and SA level, moisture content decreased and showed curvilinear relationship with BF (Fig. 2). It might be due to the dry matter increased in film forming solution.

Water Solubility

Solubility is an important characteristic of edible films, suitable for the use in ready-to-eat food products, as these solubilize in the consumer's mouth. Moreover, these are biodegradable in nature and do not add pollution to the environment.

For the solubilty, the following equation showed a quadratic relationship between the independent and response variable:

$$\text{Solubility} = +47.24119 - 7.02047X_1 - 10.93805X_2 + 0.35363X_1^2 - 0.74200X_2^2 + 2.73000X_1X_2$$

The BF level (linearly and quadratic terms), SA level (linearly) and BF x SA interaction significantly ($p<0.05$) affected the solubility of the film (Table 2). The R^2 value (0.9916), being a measure of the goodness of fit of the model, indicated that 99.16% of the total variation was explained by the model (Table 2). Fig. 3 explained that as the concentration of BF and SA level incresed, solubility deccreased. Jirukkakul (2016) also exhibited that the solubility of the banana flour films was reduced, when banana flour content increased. Sothornvit and Pitak (2007) also explained the lower solubility of banana flour based films with glycerol due to the small sizes of glycerol molecules that give high mobility and ability to occupy the free spaces in starch matrices. The solubility in water of the banana flour film is relatively low due to the high level of cohesion within the matrix, compact structure, its

Table 4: Goals for constraints to optimize the banana flour based edible film

Name	Goal	Lower limit	Upper Limit	Importance
Independent variables				
Banana flour % w/v	is in range	2.0	4.0	3
Sodium alginate %w/v	is in range	0.5	1.0	3
Dependent variables				
Thickness (μm)	is in range	154	187.4	3
Moisture (%)	is in range	16.62	18.37	3
Solubility (%)	is in range	23.63	32.1	3
Hue angle	is in range	82.43	84.38	3
Chroma value	is in range	8.58	9.95	3

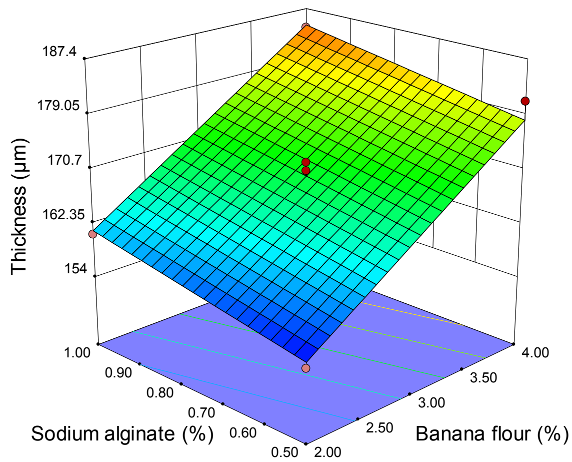

Fig. 1: Surface plot (3-D) for Thickness

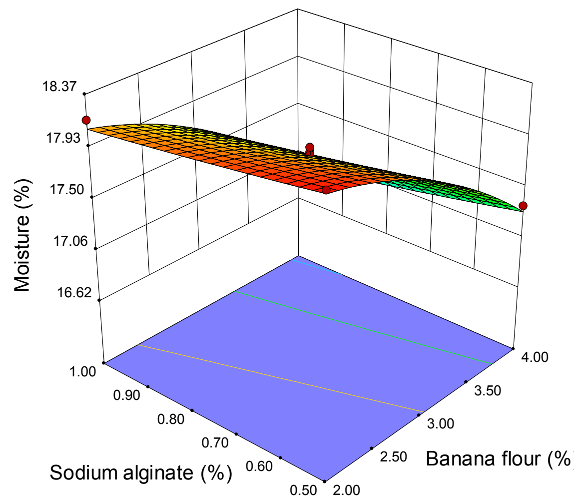

Fig. 2: Surface plot (3-D) for Moisture

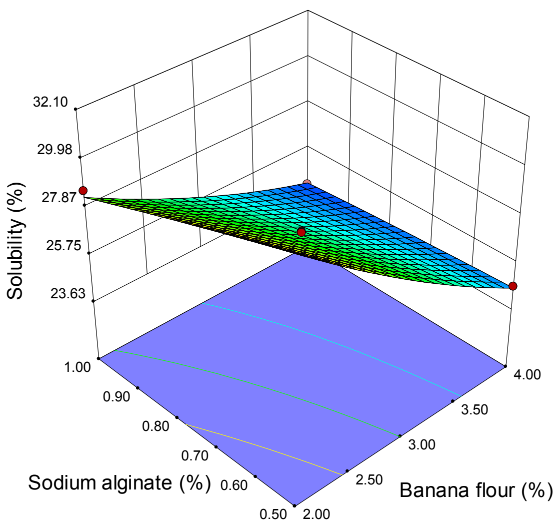

Fig 3: Surface plot (3-D) for Solubility

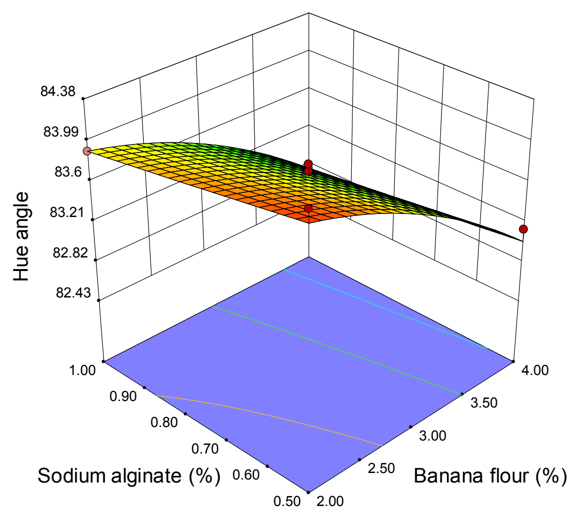

Fig. 4: Surface plot (3-D) for Hue angle

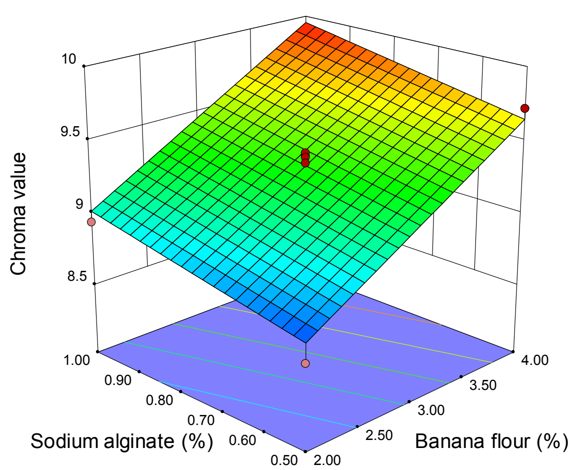

Fig. 5: Surface plot (3-D) for Chroma value

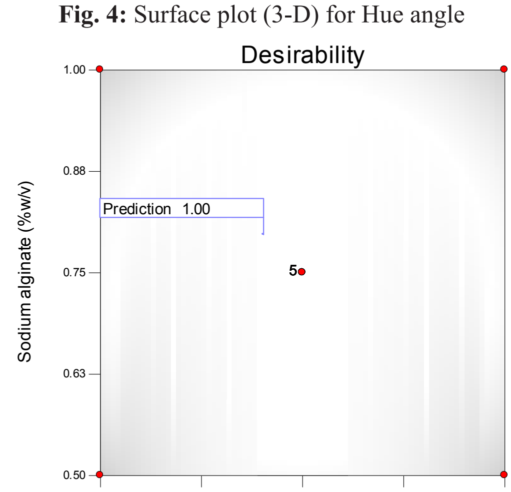

Fig. 6: Surface plot (3-D) for Desirability

composition and film-forming technique (Pelissari *et al*., 2013).

Hue angle

The second order polynomial equation generated in relation to hue angle is as following:

$$\text{Hue angle} = +84.66184 - 0.32928X_1 - 1.02292X_2 - 0.16575X_1^2 + 0.068000X_2^2 + 0.10000X_1X_2$$

As presented in Table 2, independent variables as BF level (linearly and quadratic terms) and SA level (linearly) significantly ($p<0.05$) affected the hue angle of films. The "lack of Fit F-value" of 2.40 implies the lack of Fit is not significant relative to the pure error (Table 2). Fig. 4 shown that as the concentration of BF level decreased the hue angle increased.

Chroma value

Second order polynomial equation for Chroma value is mentioned below:

$$\text{Chroma value} = +7.47229 + 0.46786X_1 + 0.61062X_2$$

From the above equation, it was seen that chroma value was found to have linear relationship with the independent variables. Regression analysis showed that linearly BF and SA level significantly ($p<0.05$) affected the chroma value of films (Table 2). The "Lack of Fit F-value" of 6.11 implies there is a 5.07% chance that a "Lack of Fit F-value" this large could occur due to noise. Chroma value showed positive linear relationship with the BF and SA level (Table 3) (Fig. 5). Jirukkakul (2016) reported that the banana flour content caused the film to have low transparency and to become more yellowish.

Optimization

Responses were optimized individually in combination using Design expert software. A series of contour plots was generated (Fig. 6) and compared visually. An area of optimum performance was located for all the response variables, in which limits of each response have been established. The criteria selected to obtain optimized solution are shown in Table 4. On the basis of ranges of different responses, the most suitable optimum point selected was having BF level at 2.81% and SA level at 0.80% with desirability of 1.0. Mean optimized values for thickness, moisture, solubility, Hue angle and Chroma value were found to be 170.18µm, 17.80%, 27.23%, 83.73 and 9.27 respectively as derived from the software.

CONCLUSION

RSM is a promising method in optimization of the basic formulation of edible film. Banana flour and sodium alginate level significantly affects the all the responses variable. The model equation developed can be used for predicting the quality of edible film. Based on the contour and superimposed plots, the basic formulation with desired quality of banana flour based edible film could be obtained by incorporating 2.81% w/v banana flour and 0.80% sodium alginate level.

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REFERENCES

Agama-Acevedo, E., Islas-Hernández, J.J., Pacheco-Vargas, G., Osorio-Díaz, P. and Bello Pérez, L.A. 2012. Starch digestibility and glycemic index of cookies partially substituted with unripe banana flour *LWT - Food Sci. Technol*., **46**: 177-182.

Asif-Ul-Alam, S. M., Islam, M. Z., Hoque, M. M. and Monalisa, K. 2014. Starch digestibility and glycaemic index of cookies partially substituted with unripe banana flour. *Am. J. Food Sci. Technol*., **2**(4): 128-133.

Bravin, B., Peressini, D. and Sensidoni, A. 2006. Development and application of polysaccharide–lipid edible coating to extend shelf life of dry bakery products. *J. Food Eng*., **76**(3): 280–290.

Dangaran, K. L., Renner-Nantz, J. and Krochta, J. M. 2006. Whey protein-Sucrose coating gloss and integrity stabilization by crystallization inhibitors. *J. Food Sci.,* **71**(3): 152-157.

Derringer, G. and Suich, R. 1980. Simultaneous optimization of several response variables. *J. Quality Technol*., **12**: 214-219.

Emiroglu, Z.K., Yemiş, G.P., Coşkun, B.K. and Candogan, K. 2010. Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. *Meat Sci*., **86**: 283–288.

Jirukkakul, N. 2016. The study of edible film production from unriped banana flour and riped banana puree. *Int. Food Res. J.*, **23**(1): 95-101.

Lalawmpuii, H. 2015. Development and quality evaluation of milk rings: A Snack. M.V.Sc. Thesis, IVRI, Bareilly.

Leceta, I., Guerrero, P. and de la Caba K. 2013. Functional properties of chitosan-based films. *Carbohydr. Polym*., **93**: 339–346.

Little, A.C. 1975. Off on a tangent. *J. Food Sci*., **40**: 1667-1671.

Min, S., Harris, L.J., Han, J.H. and Krochta, J.M. 2005. *Listeria monocytogenes* inhibition by whey protein films and coatings incorporating lysozyme. *J. Food Prot*., **68**(11): 2317–2325.

Montgomery, D.C, Wiley John and Sons. 2005. Design and Analysis of Experiments. (6^{th} Ed), pp. 201-222.

Pelissari, F.M., Andrade-Mahecha, M.M., Sobral, P.J.A. and Menegalli, F.C. 2013. Optimization of process conditions for the production of films based on the flour from plantain bananas (*Musa paradisiaca*). *LWT - Food Sci. Technol*., **52**: 1-11.

Romero-Bastida, C.A., Bello-Perez, L.A., Garcia, M.A., Martino, M.N., Solorza-Feria, J. and Zaritzky, N.E. 2005. Physicochemical and microstructural characterization of films prepared by thermal and cold gelatinization from non-conventional sources of starches. *Carbohydr. Polym*., **60**(2): 235-244.

Serrano, M., Valverde, J.M., Guillen, F., Castillo, S., Martinez-Romero, D. and Valero, D. 2006. Use of Aloe Vera gel coating preserves the functional properties of table grapes. *J. Agric. Food Chem*., **54**(11): 3882–3886.

Singh, T.P., Chatli, M.K., and Sahoo, J. 2015. Development of chitosan based edible films: Process optimization using response surface methodology. *J. Food Sci. Technol*., **52**(5): 2530-2543.

Sothornvit R and Pitak N. 2007. Oxygen permeability and mechanical properties of banana films. *Food Res. Int.*, **40**(3): 365–370.

Tapia-Blácido, D.R., Sobral, P.J.A. and Menegalli, F.C. 2005. Effects of drying temperature and relative humidity on the mechanical properties of amaranth flour films plasticized with glycerol. *Brazilian J. Chem. Eng*., **22**: 249-256.

Yan, Q., Hou, H., Guo, P. and Dong, H. 2012. Effects of extrusion and glycerol content on properties of oxidized and acetylated corn starch-based films. *Carbohydr. Polym*., **87**(1): 707–712.

Zhang, P., Whistler, R.L., BeMiller, J.N. and Hamaker, B.R. 2005. Banana starch: production, physicochemical properties, and digestibility- a review. *Carbohydr. Polym*., **59**(4): 443-458.