Development of a novel fermented pumpkin-based beverage inoculated with water kefir grains: a response surface methodology approach

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Abstract Pumpkin (*Cucurbita pepo*) is well known for its health and nutritional benefits and is recommended for daily consumption. This is the first report on optimization and development of fermented pumpkin-based water kefir beverage. Optimum pumpkin puree and brown sugar concentrations were found at 22.28 and 9.07% w/v, respectively, were made into a pumpkin-based beverage and fermented with water kefir grains for 24 h at 32 °C. The optimized fermented pumpkin-based water kefir beverage was found to be non-alcoholic, achieved good overall acceptability and high *Lactobacillus*, acetic acid bacteria and yeast cell viability of approximately 10^{12} , 10^9 and 10^9 CFU mL⁻¹, respectively. Overall, the optimized product attained superb technological characteristics and has the potential for industrial exploitation as a refreshing water kefir drink.

Keywords Box–Behnken design · *Cucurbita pepo* · *Lactobacillus* · Water kefir · Yeast

Introduction

Pumpkin (*Cucurbita pepo*), plant of the family *Cucurbitaceae* is widely cultivated and used as an ingredient in savory culinary and pastry preparations [1]. *C. pepo* contains steroidal saponins (tetracyclic triterpenoids), proteins, fibers, polysaccharides, minerals (iron, zinc, manganese, phosphorous, etc.), antioxidants (tocopherols), carotenoids and vitamins [2, 3]. *C. pepo* has been reviewed as antioxidative, antiviral, antiulcer, lipid-lowering, hepatoprotective, anticarcinogenic, antimicrobial and antidiabetic fruit [4]. Despite superb therapeutic and nutritional benefit in *C. pepo*, its unmodified fruit pulp or puree has a very short shelf life regarding nutritional as well as microbial quality [5]. Fermentation can increase the shelf life of the food commodities and process them into value-added products which can meet the market demand [6]. The original flat taste of the pumpkin fruit could be improved by the fermentation process which contributes to a “fermented flavor” with high sensory acceptability among consumers [7]. Besides, pumpkin fruit favored the growth of friendly bacteria (*Lactobacillus*) [7, 8], suggesting that it could be used as a potential fermentation substrate.

Water kefir emerged from the Caucasus Mountains and cultured by translucent irregular granules known as water kefir grains [9]. Water kefir grains contain a variety of beneficial live microorganisms (bacteria and yeasts) depending on their source and geographic region [10]. Several of the different live microorganisms in water kefir grains have probiotic properties that exert beneficial health effects to the host when sufficiently consumed [11]. The water kefir grains are commonly utilized to ferment non-dairy substrates such as sugar, fruit or vegetable [12, 13]. Water kefir preparations must meet strict criteria related to quality, safety, and functionality. A key quality criterion is

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that kefir contains accurately defined numbers of viable cells as stated in the international food standards (Codex Alimentarius): a minimum of 10^7 CFU mL⁻¹ bacteria and 10^4 CFU mL⁻¹ yeasts [14].

Most of the investigators had reported on fermented dairy-based kefir products which contain lactose sugar and cholesterol that may pose problems to those with lactose intolerance and cholesterol issues [15–17], thus people are more likely to use lactose-free water kefir with zero cholesterol and less calorie content [18]. Due to water kefir is dairy-free, and possessed consortium of friendly bacteria and yeasts that have high adaptability to various substrates as well as its economic efficiency, water kefir could be a good alternative to the fermented dairy-based product [18].

Response surface methodology (RSM) enables us to consider the interactional effects between multiple variables and this method is more cost-effective, reliable and accurate in comparison to one-at-a-time single factor processes [19]. For instance, to the best of authors' knowledge, only one study has exclusively dealt with the optimization of fermentation condition of apple juice and whey based novel beverage fermented by milk kefir grains (dairy-based) [20]. However, no study has dealt with multilateral statistical modeling of factors affecting pumpkin-based beverage fermentation by non-dairy water kefir grains.

Therefore, this study aimed at investigating, by using RSM approach, the influences of ingredients concentration and temperature on the targeted responses of PWK beverage fermented with water kefir grains. This study also focused on the identification of the optimum condition for their fermentation time, overall acceptability, ethanol content, *Lactobacillus*, acetic acid bacteria (AAB) and yeast cell viability. The proximate, mineral, physico-chemical, antioxidants and sensory quality of the optimized PWK beverage were evaluated.

Materials and methods

Water kefir grains

The water kefir grains involved in this research were sourced from a cottage industry located in Cheras, Kuala Lumpur, Malaysia. The water kefir grains were nurtured at room temperature in a brown sugar solution. Brown sugar water was changed every 3 days to maintain the grains viability.

Production of PWK beverage

Pumpkin fruits (*C. pepo*) was collected from the commercial plantation at Balik Pulau, Penang, Malaysia. The pumpkin fruits were cleaned, deseeded, sliced and steamed

for 12 min, dried by absorbent paper and then blended into puree (Inochi Commercial Blender, USA).

In experimental runs, 17 batches containing triplicates of PWK beverage were prepared according to Table 1 experimental levels of ingredients concentrations in glass bottles. Each sample was inoculated with 5% (w/v) water kefir grains. The samples were then incubated at experimental temperature until the sample reached pH 4.5. Fermented samples were stored at 4 °C for further characterization.

pH value measurement during fermentation

The pH value of PWK beverage samples was monitored at a 10-min interval (SevenEasy pH meter; Mettler-Toledo, Griefensee, Switzerland) during the fermentation process.

Sensory evaluation

A total of fifty sensory panels were recruited from among the students in the Food Technology Department, School of Industrial Technology, Universiti Sains Malaysia for the hedonic sensory acceptance evaluation of PWK beverage samples using a 7-Point Hedonic Scale. Mean scores of overall acceptability of each sample were used as responses for optimization.

Ethanol content

Ethanol concentrations of PWK beverage samples were measured by a headspace gas chromatography-flame ionized detector (SHIMADZU, HS-GC-FID Ultra, Japan) adhering to protocol laid out by Braga et al. [21]. Data were presented as ethanol content (% v/v).

Determination of *Lactobacillus*, yeast, and acetic acid bacteria cell viability

Log₁₀ CFU mL⁻¹ (colony-forming units per mL) was determined by standard spread plate method incubated for 72 h: (1) *Lactobacillus* cell viability on acidified De Man–Rogosa–Sharpe agar (Merck, Darmstadt, Germany) at 37 °C (2) yeast cell viability on yeast–extract–peptone–glucose agar (Himedia, Mumbai, India) at 28 °C (3) AAB cell viability on *Acetobacter* agar (Himedia, Mumbai, India) at 30 °C.

Experimental design

RSM coupled with Box Behnken design (RSM-BBD) consisted of 17 experimental runs which include the central point (five replicates) was used for optimization. The ranges of the independent variables in the design were

prescribed into three levels, coded -1 , 0 , $+1$ (i.e., pumpkin puree concentration (X_1) 20, 25, 30% w/v, brown sugar concentration (X_2) 0, 5, 10% w/v and fermentation temperature (X_3) 22, 27, 32 °C) as depicted in Table 1, using the equation as shown below:

$$X_i \left(\frac{x_i - x_0}{\Delta x_i} \right) \quad i = 1, 2, 3$$

where X_i and x_i are the coded and the corresponding uncoded value of the independent variable i , respectively, while x_0 and Δx_i are the actual value of that variable i at the central point and the range of values over the variable i will vary, respectively.

Fermentation time at the endpoint pH 4.5 ($FT_{pH4.5}$), overall acceptability score (OA), ethanol content (EC), *Lactobacillus* cell viability (LCV), acetic acid bacteria viability (ACV) and yeast cell viability (YCV) were the response variables (dependent) for optimization using RSM-BBD as the second-order quadratic model equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

where Y is the predicted response whereas x_1 , x_2 and x_3 are the independent variables. The model constant (β_0), linear (β_1 , β_2 , β_3), quadratic (β_{11} , β_{22} , β_{33}) and interaction (β_{12} , β_{13} and β_{23}) regression coefficients of the model are calculated by the Design Expert® software version 7.0 (Stat-Ease Inc., Minneapolis MN, USA).

Responses obtained after each trial were analyzed by the analysis of variance *Fisher* test value (ANOVA F-test) and three-dimensional response surfaces contour maps were drawn in relation to the fitted model generated by using Design Expert® software to visualize the interactive effect of the tested independent variables on dependent variables [22].

Optimum levels of each independent variables were selected by optimizing the responses. While validation of the optimum model was performed in triplicate.

Characterization of optimized PWK beverage

The physicochemical properties, proximate and mineral composition of optimized PWK beverage was determined according to AOAC [23] methods. The phenolic acids and antioxidative properties were determined following the protocols laid out by Batista et al. [24].

Results and discussion

Model fitting

The combined effect of pumpkin puree concentration (% w/v), brown sugar concentration (% w/v) and fermentation

temperature (°C) on $FT_{pH4.5}$ (h), OA (score), EC (% v/v), LCV (Log_{10} CFU mL^{-1}), ACV (Log_{10} CFU mL^{-1}), and YCV (Log_{10} CFU mL^{-1}) were determined using RSM-BBD (Table 1). Table 2 depicted the results of analysis of variance for all the responses and regression coefficients of the mathematical models which explained the dependent variables as a function of independent variables. The eventual predictive model equations after omitting the insignificance terms were formed according to the data as depicted in Table 2. As observed in Table 2, all investigated variables demonstrated insignificant p value for lack of fit ($p > 0.05$). The six proposed mathematical models could adequately estimate the responses which were $FT_{pH4.5}$, OA, EC, LCV, ACV, and YCV. The coefficients of determination $R^2 \geq 0.95$ for all six regression models indicated that the experimental data fitted adequately to equation [2] and the models have significant statistics ($p < 0.05$) [19]. Furthermore, $\text{adj-}R^2$ and CV were estimated to check the adequacy of models. As shown in Table 2, the low CV values (3.73–9.09%) and the adequate precision value greater than 4 (16.29–45.17), implying that all six proposed models are reliable with good signal to noise ratios [19]. Therefore, these mathematical equations were appropriate to estimate the relationships between the experimental responses and independent variables in the present study. By the *Fisher* test, all independent variables (X_1 , X_2 , and X_3) illustrated a prominent linear effect on all the targeted responses, whereas the quadratic term (X_2^2) and the interaction term ($X_1 X_2$) were significant on all the targeted responses except EC (Table 2).

Effects on fermentation time ($FT_{pH4.5}$)

The following equation could explain the effect of various independent variables on the $FT_{pH4.5}$ after excluding insignificant factors ($p > 0.05$).

$$\begin{aligned} \text{Fermentation time } (FT_{pH4.5}, \text{ hours}) \\ = 42.44 - 4.01X_1 - 5.73X_2 - 22.81X_3 + 7.28X_2^2 \\ + 6.13X_3^2 + 4.66X_1X_2 \end{aligned}$$

The most dominant factor influencing the $FT_{pH4.5}$ was fermentation temperature ($p < 0.0001$, $F = 1266.19$) in the linear term, as apparently exhibited by the top-level regression coefficient. All linear, two (X_2 and X_3) quadratic variables and the interaction term of the (X_1) and (X_2) had significant effects on the $FT_{pH4.5}$ and their effects are illustrated in Fig. 1(A)–(C). ANOVA data demonstrates that the lack of fit was insignificant, the model was significant, and the R^2 values (coefficient of determination) for the developed model was 0.99 (Table 2). Concerning the combined effects exhibited by both the pumpkin puree and brown sugar concentration [Fig. 1(A)], the $FT_{pH4.5}$

Table 1 Box–Behnken design (BBD) for the observed responses under different experimental conditions

Exp. no.	X ₁ (% v/v)		X ₂ (% w/v)		X ₃ (°C)		Measured response ^e					
	C ^a	A ^b	C ^a	A ^b	C ^a	A ^b	FT _{pH4.5} ^c (h)	OA ^d (score)	EC (% v/v)	LCV (Log CFU mL ⁻¹)	ACV (Log CFU mL ⁻¹)	YCV (Log CFU mL ⁻¹)
1	-1	20.00	-1	0.00	0	27.00	65.13 ± 0.10	1.00 ± 0.10	0.45 ± 0.10	3.84 ± 0.10	2.25 ± 0.10	2.25 ± 0.10
2	1	30.00	-1	0.00	0	27.00	45.00 ± 0.15	1.00 ± 0.12	0.57 ± 0.15	5.14 ± 0.15	4.74 ± 0.15	4.88 ± 0.15
3	-1	20.00	1	10.00	0	27.00	44.73 ± 0.14	7.00 ± 0.23	0.83 ± 0.14	8.07 ± 0.14	7.32 ± 0.14	6.33 ± 0.14
4	1	30.00	1	10.00	0	27.00	43.23 ± 0.10	1.00 ± 0.09	0.99 ± 0.10	12.38 ± 0.10	7.97 ± 0.10	6.72 ± 0.10
5	-1	20.00	0	5.00	-1	22.00	74.14 ± 0.19	5.67 ± 0.17	0.50 ± 0.19	5.07 ± 0.19	5.11 ± 0.19	4.90 ± 0.19
6	1	30.00	0	5.00	-1	22.00	68.03 ± 0.16	2.67 ± 0.18	0.70 ± 0.16	7.51 ± 0.16	6.67 ± 0.16	6.94 ± 0.16
7	-1	20.00	0	5.00	1	32.00	27.83 ± 0.12	6.33 ± 0.10	0.85 ± 0.12	9.87 ± 0.12	8.84 ± 0.12	8.10 ± 0.12
8	1	30.00	0	5.00	1	32.00	23.48 ± 0.11	3.00 ± 0.08	0.79 ± 0.11	11.06 ± 0.11	10.22 ± 0.11	10.42 ± 0.11
9	0	25.00	-1	0.00	-1	22.00	83.46 ± 0.10	1.00 ± 0.09	0.26 ± 0.10	4.19 ± 0.10	3.22 ± 0.10	3.32 ± 0.10
10	0	25.00	1	10.00	-1	22.00	74.06 ± 0.12	3.67 ± 0.25	0.64 ± 0.12	8.12 ± 0.12	7.53 ± 0.12	7.28 ± 0.12
11	0	25.00	-1	0.00	1	32.00	40.06 ± 0.15	1.67 ± 0.13	0.45 ± 0.15	6.09 ± 0.15	5.89 ± 0.15	6.52 ± 0.15
12	0	25.00	1	10.00	1	32.00	25.81 ± 0.22	4.67 ± 0.10	0.80 ± 0.22	14.15 ± 0.22	9.80 ± 0.22	9.99 ± 0.22
13	0	25.00	0	5.00	0	27.00	43.00 ± 0.10	4.33 ± 0.11	0.66 ± 0.10	8.90 ± 0.10	7.70 ± 0.10	6.17 ± 0.10
14	0	25.00	0	5.00	0	27.00	43.02 ± 0.12	4.00 ± 0.12	0.57 ± 0.12	8.91 ± 0.12	7.42 ± 0.12	7.20 ± 0.12
15	0	25.00	0	5.00	0	27.00	43.06 ± 0.16	4.00 ± 0.11	0.67 ± 0.16	8.91 ± 0.16	7.42 ± 0.16	6.20 ± 0.16
16	0	25.00	0	5.00	0	27.00	40.06 ± 0.17	4.33 ± 0.16	0.60 ± 0.17	9.95 ± 0.17	7.90 ± 0.17	7.17 ± 0.17
17	0	25.00	0	5.00	0	27.00	43.05 ± 0.15	4.33 ± 0.14	0.50 ± 0.15	9.90 ± 0.15	8.09 ± 0.15	7.17 ± 0.15

^aC coded values^bA actual values^cFT_{pH4.5} fermentation completion time at pH 4.5^dOA overall acceptability score (7-point hedonic scale)^eValues of measured response are the means of three determinations ± standard error. EC, LCV, ACV and YCV are ethanol content, *Lactobacillus* cell viability, acetic acid bacteria cell viability and yeast cell viability, respectively

Table 2 Estimated coefficients of the fitted second-order polynomial models for fermentation completion time at pH 4.5 ($FT_{pH4.5}$), sensory overall acceptability (OA), ethanol content (EC), *Lactobacillus* cell viability (LCV), acetic acid bacteria cell viability (ACV) and yeast cell viability (YCV)

	$FT_{pH4.5}$ (h)	OA (score)	EC (% v/v)	LCV (Log CFU mL ⁻¹)	ACV (Log CFU mL ⁻¹)	YCV (Log CFU mL ⁻¹)
Intercept						
β_0	42.44	4.20	0.60	9.31	7.71	6.78
Linear						
β_1	- 4.01*	- 1.54*	0.053**	1.16*	0.76*	0.92*
β_2	- 5.73*	1.46*	0.19*	2.93*	2.07*	1.67*
β_3	- 22.81*	0.33*	0.099*	2.04*	1.53*	1.57*
Quadratic						
β_{11}	- 0.20	- 0.016	0.14*	- 0.86**	- 0.52**	- 0.46
β_{22}	7.28*	- 1.68*	- 0.031	- 1.10*	- 1.62*	- 1.27*
β_{33}	6.13*	0.24**	- 0.031	- 0.078	0.52**	1.27*
Interaction						
β_{12}	4.66*	- 1.50*	1.000E-002	0.75**	- 0.46**	- 0.56***
β_{13}	0.44	- 0.082	- 0.065***	- 0.31	- 0.045	0.07
β_{23}	- 1.21	0.083	- 7.500E-003	1.03*	- 0.10	- 0.12
R^{2a}	0.9955	0.9963	0.9551	0.9862	0.9857	0.9731
R^{2b}_{adj}	0.9896	0.9915	0.8974	0.9685	0.9673	0.9385
CV ^c	3.73	5.00	9.09	6.05	5.55	7.67
P_m value ^d	< 0.0001	< 0.0001	0.0006	< 0.0001	< 0.0001	0.0001
P_{ij} value ^e	0.1576	0.5276	0.8380	0.6564	0.1859	0.6211
Adequate precision	43.997	45.178	16.295	26.069	26.457	21.408

* $p < 0.01$; ** $0.01 \leq p < 0.05$; *** $0.05 \leq p < 0.1$ ^aCoefficient of multiple determination^bCoefficient (adjusted) of multiple determination^cCoefficient of variance (%)^dProbability of F value for the model^eProbability of F value for the lack of fit

presented a small but regular decreased when brown sugar concentration was elevated from 0 to 10% (w/v) at the lowest pumpkin puree concentration. This negative linear effect became more obvious as pumpkin puree concentration raised due to more substrates available for growth and activity of fermenting bacteria [25]. As can be noted, the quadratic effect of brown sugar concentration altered significantly relied on the pumpkin puree concentration. At the highest pumpkin puree concentration, the $FT_{pH4.5}$ only decreased to a level at a brown sugar concentration of approximately 5% (w/v), and after that increased to almost the same $FT_{pH4.5}$ value [Fig. 1(A)]. From a visual analysis in Fig. 1(B), for any pumpkin puree concentration tested, $FT_{pH4.5}$ values decreased with increasing fermentation temperature, with the decrement more noticeable at the highest pumpkin puree concentration. These results could be explained by the favorable mesophilic temperature and presence of pumpkin pulp (carbon source) which triggered faster rate of fermentation [8, 25, 26].

Figure 1(C) shows the effect of brown sugar concentration and fermentation temperature on $FT_{pH4.5}$. As observed, a steady increase of fermentation temperature engendered a decreased of $FT_{pH4.5}$ to a minimum at a moderate fermentation temperature (27 °C), trailed by a slight increased to almost the same $FT_{pH4.5}$ value since most kefir grains bacteria and yeasts are mesophilic [26]. As reported by Pandey and Mishra [27], higher fermentation temperature and sufficient readily available nutrients stimulated the growth and fermentation capacity of bacteria, thus resulted in shorter fermentation time.

Effects on overall sensory acceptability (OA)

The influences of all three independent variables (X_1 , X_2 , and X_3) on the overall acceptability of PWK beverage samples are shown in Fig. 1(D)–(F). All linear, two (X_2 and X_3) quadratic variable and interaction terms of the (X_1) and (X_2) had significant effects on the overall acceptability (Table 2). As observed in Fig. 1(D), a significance

Fig. 1 Response surface plots illustrating the combined effects of the ingredients concentration and fermentation temperature on fermentation time until pH reached 4.5 (A–C), overall acceptability (D–F) and ethanol content (G–I)

interaction was found between pumpkin puree and brown sugar concentrations. Depending on the pumpkin puree concentration, the quadratic effect of brown sugar concentration changed significantly, i.e., at the lowest pumpkin puree concentration, overall acceptability score steadily increased when moving from 0 to 10% w/v brown sugar concentration, whereas overall acceptability score decreased significantly for the highest pumpkin puree concentrations. As illustrated in Fig. 1(F), regardless of the fermentation temperature applied, overall acceptability score inclined, reaching a maximum at approximately 5% (w/v) brown sugar concentration and remained almost the same score, due to the squared term of the brown sugar concentration. As depicted in Fig. 1(E), a gradual and linear elevation in overall acceptability score was disclosed

when the fermentation temperature increased. This linear effect was getting more prominent as the pumpkin puree concentration decreased. At the lowest pumpkin puree concentration, overall acceptability steadily increased when moving from temperature 22–32 °C. Apparently, the overall acceptability score increased with increased in brown sugar concentration and fermentation temperature and decreased with pumpkin puree concentration which was due to readily available sucrose and favorable mesophilic temperature resulted in higher fermentation rate and desirable byproducts that contributed to higher sensory overall acceptability of the fermented beverage [9, 27]. While excess pumpkin puree might be metabolized to undesired byproducts resulted in lower acceptability of the fermented beverage [28].

The ANOVA data showed that the lack of fit was not significant (Table 2). The R^2 value of 0.99 and adjusted R^2 of 0.99 were desirable, suggesting that the model is highly significant. The polynomial model for OA was shown as below:

$$\text{OA (score)} = 4.20 - 1.54X_1 + 1.46X_2 + 0.33X_3 - 1.68X_2^2 + 0.24X_3^2 - 1.50X_1X_2$$

Effects on ethanol concentration

The PWK beverages fermented at 22–32 °C could be categorized as non-alcoholics according to the Malaysian *HALAL* legislation since the ethanol content was < 1.00% (v/v) [29]. This finding agrees with Atalar and Dervisoglu [30] who reported that ethanol values of kefir made using kefir grains was within the range between 0.02 and 0.13% (v/v). Beverage fermented by water kefir grains contains mostly lactic acid bacteria and yeasts which promote the alcoholic fermentation of the beverage and produce ethanol, which possibly further oxidize by acetic acid bacteria [31]. Non-lactose fermenting kefir yeasts (e.g. *Saccharomyces cerevisiae*) are ethanol tolerance and predominantly accountable for the high ethanol content in the beverage [32]. All linear, one (X_1) quadratic and one interaction (X_1X_3) variables possessed significant effects on the ethanol content (Table 2). The effect of the independent variables on the EC is shown in Fig. 1(G)–(I). The highest EC was exhibited when both brown sugar concentration and fermentation temperature increased [Fig. 1(I)]. The quadratic effect of the pumpkin puree concentration altered significantly corresponds to the fermentation temperature. A slow and linear increased in EC was detected when the brown sugar and pumpkin puree concentrations increased [Fig. 1(G)]. At the highest fermentation temperature, the EC gradually raised with the increased of pumpkin puree concentration [Fig. 1(H)] due to available nutrients act as a carbon source for microbial fermenting activity, leading to an elevated kefir microbial population that yield ethanol [31, 32].

The high value of determination coefficient ($R^2 = 0.96$) demonstrated that the following equation can be used to explain the effects of independent variables on the ethanol content in the PWK.

$$\text{Ethanol content (\%)} = 0.60 + 0.053X_1 + 0.19X_2 + 0.099X_3 - 0.14X_1^2 - 0.065X_1X_3$$

Effects on *Lactobacillus*, AAB and yeast cell viability

Yüksekdağ et al. [33] suggested that water kefir is a natural probiotic offering live bacteria and yeast. Muganga et al. [34] stated the recommended viable cell in fermented food

products: 10^6 to over 10^8 CFU mL⁻¹, in order to exert positive health impact on the target host. The coefficient of determination obtained for the cell viability of *Lactobacillus* ($R^2 = 0.99$), yeast ($R^2 = 0.97$) and AAB ($R^2 = 0.99$) indicated the models are appropriate for explaining up to > 97% variability (Table 2). The results concerning the effect of independent variables on LCV, ACV, and YCV of PWK beverage were found to show a similar trend although with some unique features (Fig. 2). The maximum LCV, ACV, and YCV were observed when temperature, brown sugar and pumpkin puree concentration were at its maximum point within the studied range [Fig. 2(A)–(H)]. Pandey and Mishra [27] reported that fermentation temperature, oligosaccharides, and free sugar are the important factors in ensuring sufficient viable cell in the fermented product.

Figure 2(A)–(I) depicted a gradual linear increased in LCV, ACV, and YCV when the fermentation temperature increased. As observed, when the fermentation temperature increases, all cells viability increases. This linear effect is more noticeable as both pumpkin puree and brown sugar concentration increased. Figure 2(A), (D), (G) show that LCV, ACV, and YCV increased with an increase of both brown sugar and pumpkin puree concentrations. The highest LCV was exhibited when both pumpkin puree and brown sugar concentration increased [Fig. 2(A)]. The increased in LCV was more significant than ACV and YCV as indicated by the highest coefficients. Moreover, at the highest pumpkin puree concentration, the ACV and YCV only increased to a maximum level (at brown sugar concentration < 7.5% w/v), followed by a plateau [Fig. 2(D), (G)].

An increased in LCV, ACV, and YCV upon increasing pumpkin puree and brown sugar in PWK could be ascribed to more nutrients available most probably oligosaccharides in pumpkin puree [25]. Pumpkin oligosaccharides may sustain microbial metabolic activities at compounding high concentration of organic acids, protecting the microbial cell from the high acidity of the growth media [25]. Bacteria and yeast cells could maintain high viability as essential nutrients (carbon and nitrogen sources) required for their growth are accessible. Besides, the growth environments for *Lactobacillus*, AAB, and yeasts are analogous, and they exhibit positive mutualistic interaction that stimulates high cells viability [35].

The fermentation temperature increment may result in an increase of the microbial activity [27]. The optimal temperature for water kefir fermentation is usually 25 °C; however, the mesophilic temperature of about 32 °C is optimum for the growth of mesophilic kefir bacteria and yeasts [26]. The optimized predictive model in term of coded values for LCV, ACV and YCV are presented in

Fig. 2 Response surface plots illustrating the combined effects of the ingredients concentration and fermentation temperature on *Lactobacillus* cell viability (A–C), acetic acid bacteria cell viability (D–F) and yeast cell viability (G–I)

equations below respectively after omitting insignificant factors ($p > 0.05$):

$$\begin{aligned} \text{Lactobacillus cell viability (Log}_{10} \text{ CFU mL}^{-1}) \\ = 9.31 + 1.16X_1 + 2.93X_2 + 2.04X_3 - 0.86X_1^2 \\ - 1.10X_2^2 + 0.75X_1X_2 + 1.03X_2X_3 \end{aligned}$$

$$\begin{aligned} \text{AAB cell viability (Log}_{10} \text{ CFU mL}^{-1}) \\ = 7.71 + 0.76X_1 + 2.07X_2 + 1.53X_3 \\ - 0.52X_1^2 - 1.62X_2^2 + 0.52X_3^2 - 0.46X_1X_2 \end{aligned}$$

$$\begin{aligned} \text{Yeast cell viability (Log}_{10} \text{ CFU mL}^{-1}) \\ = 6.78 + 0.92X_1 + 1.67X_2 + 1.57X_3 - 1.27X_2^2 \\ + 1.27X_3^2 - 0.56X_1X_2 \end{aligned}$$

Optimum process parameters

This research was to determine the optimal ingredients concentration and fermentation temperature for the PWK beverage with maximum LCV, YCV, ACV, OA, minimum $\text{FT}_{\text{pH}4.5}$ and EC was in range ($0 < \text{EC} < 1\%$ v/v). Less than 1% residual EC is acceptable in end products by natural ingredients, but the acceptable limit for food and beverage products differs from various countries and organizations. Most *HALAL* certifying bodies accept the small amount of inherent EC, generally less than 1%, for example, the Majlis Ulama Indonesia allows a level of 1% while Thailand, not exceeding 1.5% [29].

Table 3 Estimated predicted and observed values within their intervals

Response variable	Goal	Predicted mean ^{ab}	95% PI low ^c	Observed value ^b	95% PI high ^c
FT _{pH4.5} (h)	Minimize	24.76 ± 1.49	19.20	24.74 ± 1.20	30.31
OA (score)	Maximize	6.45 ± 0.14	5.91	6.40 ± 0.58	6.99
EC (% v/v)	In range	0.84 ± 0.05	0.66	0.80 ± 0.02	1.02
LCV (Log CFU mL ⁻¹)	Maximize	12.73 ± 0.42	11.18	12.74 ± 0.12	14.28
ACV (Log CFU mL ⁻¹)	Maximize	9.94 ± 0.32	8.76	9.90 ± 0.03	11.12
YCV (Log CFU mL ⁻¹)	Maximize	9.61 ± 0.41	8.07	9.62 ± 0.10	11.15

^aDesirability for this result was 0.92^bValues are the means of three determinations ± standard error^cPrediction interval, FT_{pH4.5}, OA, EC, LCV, ACV and YCV are fermentation completion time at pH 4.5, sensory overall acceptability, ethanol content, *Lactobacillus* cell viability, acetic acid bacteria cell viability and yeast cell viability, respectively**Table 4** Major constituents, physicochemical, sensory and antioxidant properties in optimized formula

Constituents	Values ^a	Parameters	Values ^a
Proximate composition (% wet bases)		Physicochemical properties	
Moisture (%)	78.60 ± 0.11	Total soluble solids (°Brix)	16.39 ± 0.12
Ash (%)	4.69 ± 0.02	Viscosity (mPa s)	50.09 ± 0.33
Protein (%)	3.59 ± 0.20	pH	4.56 ± 0.18
Dietary fibre (%)	2.98 ± 0.34	Colour (Instrumental)	
Fat (%)	1.20 ± 0.26	L*	44.21 ± 0.01
Carbohydrate (%) (by difference)	8.94 ± 0.08	a*	5.26 ± 0.04
		b*	13.85 ± 0.01
Minerals (mg 100 mL ⁻¹)		Phenolic acids	
Potassium	490.30 ± 0.15	Hydroxybenzoic acids (mg 100 mL ⁻¹)	
Phosphorus	83.98 ± 0.04	Syringic acid	6.49 ± 0.27
Magnesium	22.07 ± 0.06	p-Hydroxybenzoic acid	16.05 ± 0.86
Calcium	32.09 ± 0.10	Protocatechuic acid	15.03 ± 0.70
Iron	1.30 ± 0.02		
Zinc	0.69 ± 0.04	Hydroxycinnamic acids (mg 100 mL ⁻¹)	
Copper	0.26 ± 0.22	Caffeic acid	11.66 ± 0.70
Selenium	0.49 ± 0.20	Chlorogenic acid	14.21 ± 0.20
Manganese	0.20 ± 0.10	p-Coumaric acid	33.30 ± 0.11
		Ferulic acid	8.40 ± 0.58
Content of β-carotene, total polyphenols, total flavonoids and total anthocyanins			
β-Carotene (mg of β-carotene L ⁻¹)	69.45 ± 0.15	Sensory Score (7 point hedonic scale)	
TPC (mg of GAE ^b 100 mL ⁻¹)	57.03 ± 0.11	Colour	6.60 ± 0.27
TFC (mg of CE ^c 100 mL ⁻¹)	23.23 ± 0.30	Odour	6.30 ± 0.86
TAC (mg of C3GE ^d mL ⁻¹)	4.39 ± 0.23	Texture (consistency)	6.50 ± 0.70
		Sweetness	6.60 ± 0.20
Antioxidant potential		Sourness	6.30 ± 0.11
FRAP (μmol FeSO ₄ L ⁻¹)	989.12 ± 0.10	Overall acceptance	6.40 ± 0.58
DPPH (% inhibition)	192.65 ± 0.20		

^aValues are the means of three determinations ± standard error^bGAE gallic acid equivalents^cCE catechin equivalents^dC3GE cyanidin-3-glucoside equivalents^eFeSO₄ iron(II) sulphate equivalents

In the present study, the optimal condition for all responses with Derringer's desirability function of 0.92 was: 9.07% w/v brown sugar, 22.29% w/v pumpkin puree and 32 °C fermentation temperature. The obtained experimental values were confirmed by performing the estimated optimal condition to verify the predictive mathematical model of the investigated process. Table 3 showed the predicted and observed values. The experimental results were closely comparable to the predicted results and were well within the acceptable limits of prediction.

Quality of the optimized PWK beverage

Table 4 presented the quality of optimized PWK product with total soluble solids 16.39°Brix, viscosity 50.09 mPa s and pH 4.56. The prepared PWK had an orange color as indicated by the L*, a*, and b* values. The optimized product obtained a high score of 6.40 for overall acceptability. It had abundant mineral elements, satisfying antioxidant, nutritional and sensory properties. Furthermore, the optimum PWK formula displayed DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP). Reversed-phase HPLC analysis of the optimized product revealed syringic acid, ferulic acid, protocatechuic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid and chlorogenic acid as the main phenolics. The present result is supported by Puerari et al. [9] and Sabokbar et al. [20] which highlighted that the use of kefir grains as starter culture may enhance the sensory, antioxidant and nutritional quality of fermented beverages.

Therefore, this study provides significant insight to design a fermented pumpkin-based beverage using water kefir grains, which can be consumed as a delicious and refreshing health drink.

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