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Optimization of fermentation conditions for sourdough by three different lactic acid bacteria using response surface methodology

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ABSTRACT. This study aimed to investigate optimal fermentation conditions for sourdough by freeze-dried *Lactobacillus curvatus* N19, *Weissella cibaria* N9 and *Lactobacillus brevis* ED25 isolated from Turkish sourdough previously. The central composite rotational design was applied to the optimization of fermentation parameters (temperature and time). The fermentation was carried out under a simulated sourdough system and biomass concentration, total acidity, and lactic and acetic acid formation were chosen as response variables. Results showed that the models developed for all variables were significant ($p < 0.05$) and there was no lack of fit in any of quantifications ($p > 0.05$), indicating the suitability for representing the relationship between variables and factors. While both of the independent parameters were effect the response, fermentation time was the most significant factor influencing the response. The validation experiments using the optimized condition showed a good agreement between the experimental and predicted values except the lactic and acetic acid formation for *W. cibaria* N9. In conclusion, freeze-dried *L. curvatus* N19 can be used as a starter culture to sourdough fermentation for bread industry due to optimum fermentation conditions (29°C temperature and 23h time).

Keywords: Biomass; fermentation conditions; lactic acid bacteria (LAB); optimization; sourdough.

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Introduction

The sourdough has been defined as a dough obtained from the mixture of flour and water, which is fermented with naturally or selected lactic acid bacteria (LAB) and yeast. It has been used in fermented bakery products due to several advantages, such as improved nutritional properties, increased bioactive compound contents, better bread flavor and extended shelf-life (Mantzourani et al., 2019; Pétel, Onno, & Prost, 2017). In addition, the use of sourdough is of great importance in terms of technology and nutrition, as it plays an important role in reducing the use of chemical preservatives in bread production (Mantzourani et al., 2019). The most of the beneficial properties attributed to sourdough are accomplished through acidification activity of LAB and a wide range of metabolites (e.g. lactic acid, acetic acid, exopolysaccharides and/or enzymes) of LAB in the sourdough (Mohsen, Aly, Attia, & Osman, 2016; Wu et al., 2012). However, the sourdough fermentation is quite difficult to control due to the spontaneous occurrence of complex phase successions of LAB communities during subculture (Oshiro, Tanaka, Zendo, & Nakayama, 2020). Besides, the metabolic properties of LAB are strain-specific (Choi, Kim, Hwang, Kim, & Yoon, 2012). The microbial ecology of the sourdough fermentation is complex, and the sourdough microbiota stability is affected by flour microbiota, flour composition (free amino acids, carbohydrates, enzymes, etc.), number of sourdough refreshment made, time between refreshments, fermentation time and temperature, storage conditions and hygienic conditions of the processing environment (Corona et al., 2016; Mantzourani et al., 2019; Minervini et al., 2012; Pontonio et al., 2017). Therefore, standardization of sourdough is very difficult, and this problem is important for bakery industries due to the stability and reproducibility of product quality (Mantzourani et al., 2019). Recently, the use of defined single or mixed starter cultures for sourdough production has been a breakthrough in the processing of sourdough by reducing fermentation time and risk of fermentation failure, resulting in better control of the fermentation process and standardization of the end product (Wu et al., 2012).

The selection of starter culture for sourdough preparation is of special importance to get a sourdough bread with desirable properties. Moreover, different environmental parameters, i.e. temperature, pH, acidity, level of specific substrates and oxygen availability influence the LAB activities during sourdough fermentation, so that the final quality of the fermented product has been negatively affected (Gebremariam, Hassani, Zarnkow, & Becker, 2015). Among the mentioned parameters, fermentation time and temperature, as well as starter culture have the largest effects on sourdough acidification. Thus, it is important to optimize the fermentation conditions for selected LAB to get maximum growth of culture and formation of desirable metabolites for quality attributes of food products is necessary. For this purpose, response surface methodology (RSM) is applicable to optimize the fermentation conditions, which is an appropriate method to design, improvement and formulation of new products and is used in bioprocess optimization to investigate the influences of individual process parameters and their interactions on the response variables (Baş & Boyaci, 2007; Gebremariam et al., 2015).

Lactobacillus curvatus N19, *Weissella cibaria* N9 and *L. brevis* ED25 were previously isolated from traditional wheat sourdoughs from Turkey by Dertli, Mercan, Arici, Yılmaz, and Sağdıç (2016) who stated that these strains have been good potential as a starter culture for industrial sourdough production. In our previous study, the optimum cryoprotective agent formulations to obtained high cell viability during freeze-drying process were determined as: 20% skim milk, 3.57% lactose and 10% sucrose for *L. curvatus* N19 (Gul, Con, & Gul, 2020); 5.65% skim milk, 20% lactose and 9.38% sucrose for *W. cibaria* N9 (Gul, Gul, Dertli, & Con, 2020); 17.28% skim milk, 2.12% lactose, and 10% sucrose for *L. brevis* ED25 (Gul, Gul, Yilmaz, Dertli, & Con, 2020). For all that, the optimum fermentation conditions for these LAB strains should be determined to be used in bakery industry. The objective of this study was to optimize the parameters of fermentation temperature and time during fermentation process by *L. curvatus* N19, *W. cibaria* N9 and *L. brevis* ED25, using a central composite rotational design (CCRD).

Material and methods

Microorganism and culture media

Three lactic acid bacteria (LAB) strains, *Lactobacillus curvatus* N19, *Weissella cibaria* N9 and *L. brevis* ED25, were isolated from Turkish sourdough and used for the sourdough fermentation. The cultures were activated in MRS broth (Merck, Darmstadt, Germany) at 30°C for 12h and then subcultured for two consecutive passages in MRS broth at 30°C for 16h before use. The cell cultures were harvested by centrifugation at 7500 × g for 10 min. at 4°C and the pellet was washed in 10 mL of sterile phosphate saline for three times. The obtained pellet was suspended in 5 mL of cryoprotectant solution (20% skim milk, 3.57% lactose and 10% sucrose for *L. curvatus* N19; 5.65% skim milk, 20% lactose and 9.38% sucrose for *W. cibaria* N9; 17.28% skim milk, 2.12% lactose, and 10% sucrose for *L. brevis* ED25) (Gul, Con, & Gul, 2020; Gul, Gul, Dertli, & Con, 2020; Gul, Gul, Yilmaz, Dertli, & Con, 2020), corresponding to ca. 9.5 log cfu mL^{-1}. The samples of bacteria suspension in cryoprotectant media were frozen at -80°C for 5h and then freeze-dried (0.061 mbar, -55°C) for 18h using the Alpha 1–4 LD plus freeze dryer (Marin Christ, Germany). The bacteria freeze-dried with cryoprotectant formulations were directly used as starter culture for the fermentation.

Wheat Sourdough Simulation Medium (WSSM) was used as the culture medium to perform simulated wheat sourdough fermentation and had the following compositions (g L^{-1}): 12 g of wheat peptone, 12 g of granulated yeast extract, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $MnSO_4.H_2O$, 4 g of KH_2PO_4, 0.5 g of glucose, 0.5 g of fructose, 10 g of maltose, 2 g of sucrose, 1 mL of Tween 80 and 1 mL of vitamin solution (consist of 0.2 g cobalamin, 0.2 g folic acid, 0.2 g nikotinamid, 0.2 g pantothenic acid, 0.2 g pyridoxal-phosphate and 0.2 g thiamin) (Vrancken, De Vuyst, Rimaux, Allemeersch, & Weckx, 2011).

Experimental design and fermentation procedure

The optimization of fermentation conditions for sourdough production by freeze-dried starter culture was performed by using the response surface methodology. Central Composite Rotatable Design (CCRD) was carried out for this course of experiments with fermentation temperature and time as independent factors and biomass, total acidity, the amount of lactic and acetic acid as responses. The independent parameters were examined at levels of $-\alpha$ as a low and $+\alpha$ as a high level. The values of the independent variables were established according to preliminary tests, with the fermentation temperature and time ranging from 20 to

35°C and 10 to 30h, respectively. Ten runs based on CCRD and two center points with two independent variables were used. The model proposed for each response evaluated by the following model equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 X_1^2 + \beta_2 X_2^2 + \beta_{1,2} X_1 X_2 \quad (1)$$

where, Y refers the response function, X_1 and X_2 refer the coded variables and the β values refer the estimated coefficients of each term of the response surface model.

The fermentation experiments were performed using 500 mL Erlenmeyer flasks containing 250 mL of WSSM medium. Lyophilized cultures were directly added to WSSM medium at a rate of 5%, obtaining an initial cell count of approximately 8.0 log CFU mL^{-1} in the final volume (100 mL). The flasks were incubated using a shaking incubator (Lab Companion, SIF-500R) at different fermentation temperature and time according to the experimental design. Following the fermentation process, analyzes were carried out.

Analytical methods

Determination of Biomass

To determine the biomass dry weight, the method reported by Takeshita et al. (2018) was used. Two mL of fermented samples were transferred to the centrifuge tubes, and centrifuged for 10 min. at 3.000 x g. The pellet was washed with distilled water, then dried at 105°C for 12 hours and weighted. Biomass dry weight was expressed in g L^{-1}.

Determination of pH and total titratable acidity

The pH values of fermented samples were measured with a calibrated pH meter (Eutech Cyberscan pH 2700, Ayer Rajah Crescent, Singapore). For the determination of total titratable acidity, 5 mL of fermented samples were titrated with 0.1 N NaOH until pH 8.1 was reached. The total titratable acidity result was calculated as amount of lactic acid.

Determination of lactic and acetic acid

The extracts for the quantification of lactic and acetic acids were obtained according to Lhomme, Orain, Courcoux, Onno, and Dousset (2015). About 1 mL of sample was mixed with 10 mL of 0.01 N perchloric acid and the mixture was shaken for 30 s using vortex. Subsequently, the mixture was centrifuged at 7.000 x g at 4°C for 7 min. and the supernatant was filtered through a 0.45 µm syringe filter (Chromafil GF / PET-45/25, Macherey-Nagel AG, Duren, Germany), followed injection into the high pressure liquid chromatography (HPLC).

The quantitative determination of lactic and acetic acid was performed by HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a pump (LC-20AT), an autosampler (SIL-10A), a Column oven (CTO-10AS VP) and a photodiode array detector (SPD-M20A). The analyses were performed at a flow rate of 0.65 mL min^{-1} with an ICE-COREGEL 87H3 (Transgenomic, USA) organic acid column and the column temperature was set at 35°C. The mobile phase used was 10 mM perchloric acid. The detection was performed at the wavelengths of 210 nm and the injection volume was 20 µL. The lactic and acetic acids were defined using external standards (Sigma-Aldrich). The amount of lactic and acetic acid was calculated according to the standard curve obtained with five different concentration standard solutions in the range of 125 to 5000 mL L^{-1}.

Statistical analysis

The RSM experimental design, regression and graphical analyses of the experimental data obtained were performed using the Design Expert statistical software package 7.0.0. trial version (StatEase, Inc., USA). The significant differences between the mean values of experimental and predicted data were determined by using t-test using SPSS Statistics software (Version 21.0, IBM SPSS Inc, USA).

Result and discussion

Statistical analysis and models fitting

RSM was employed to evaluate the effects of fermentation temperature and time and their interactive relationship on four dependent variables including biomass, total titratable acidity, lactic and acetic acid. The values of each dependent variable for each experimental run for three different LAB were enumerated in Table 1. The experimental data were analyzed and fitted to a full quadratic model for each response. The ANOVA was used for evaluating the statistical significance of the developed models and the results of ANOVA were presented in Table 2.

Table 1. Central Composite Rotatable Design (CCRD) matrix with experimental values for *L. curvatus* N19, *W. cibaria* N9 and *L. brewis* ED25.

			L. curvatus N19				*W. cibaria* N9				*L. brevis* ED25			
Run	X_1	X_2	Y_1 (g)	Y_2 (%)	Y_3 (g L^{-1})	Y_4 (g L^{-1})	Y_1 (g)	Y_2 (%)	Y_3 (g L^{-1})	Y_4 (g L^{-1})	Y_1 (g)	Y_2 (%)	Y_3 (g L^{-1})	Y_4 (g L^{-1})
1	34.1	20	3.96	0.76	8.40	1.44	3.93	0.75	9.34	2.87	4.25	0.77	9.14	2.69
2	27	5.9	0.60	0.40	2.99	0.63	0.72	0.41	2.61	0.51	0.87	0.39	5.61	0.67
3	22	30	3.26	0.71	5.89	1.06	2.65	0.69	9.51	2.79	3.91	0.71	7.75	1.76
4	32	30	3.64	0.74	7.10	1.40	3.85	0.79	8.09	3.15	4.21	0.74	8.46	2.55
5	20	20	1.10	0.43	3.81	0.73	1.37	0.48	7.32	2.42	1.21	0.47	5.41	1.66
6	27	34.1	3.63	0.67	6.55	1.45	2.18	0.67	9.15	2.78	3.55	0.66	7.95	2.01
7	32	10	1.79	0.52	5.22	1.02	0.94	0.50	8.96	2.15	1.97	0.56	8.09	2.03
8	27	20	3.53	0.74	7.85	1.60	3.56	0.72	9.85	2.74	4.04	0.75	8.77	1.02
9	22	10	0.58	0.38	4.07	0.78	0.64	0.42	2.95	0.73	0.82	0.41	5.30	1.30
10	27	20	3.64	0.75	8.02	1.86	3.89	0.74	9.90	2.84	4.20	0.74	8.50	1.16

X_1, Fermentation temperature (°C); X_2, fermentation time (h); Y_1, dry cell weight (g), Y_2, total titratable acidity (%), Y_3, lactic acid formation (g L^{-1}), Y_4, acetic acid formation (g L^{-1}).

Table 2. ANOVA analysis of the quadratic model for each response.

		L. curvatus N19							
Source	DF	Y_1		Y_2		Y_3		Y_4	
		Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value
Model	5	16.62	0.008*	0.2108	0.018*	30.55	0.022*	1.44	0.014*
X_1	1	3.96	0.011*	0.05	0.022*	9.76	0.016*	0.31	0.02*
X_2	1	9.74	0.002*	0.11	0.006*	9.54	0.016*	0.42	0.013*
X_1X_2	1	0.17	0.398	0.01	0.418	0.01	0.975	0.01	0.742
X_1^2	1	1.29	0.062	0.02	0.076	3.57	0.072	0.47	0.01*
X_2^2	1	2.49	0.023*	0.05	0.025*	10.97	0.013*	0.54	0.008*
Residual	4	0.78		0.02		2.41		0.09	
Lac of fit	3	0.77	0.112	0.02	0.066	2.39	0.095	0.06	0.738
Pure error	1	0.01		0.01		0.01		0.04	
Total	9	17.4		0.22		32.96		1.53	
R2		0.955		0.933		0.9269		0.9418	
Adj-R2		0.899		0.850		0.8355		0.8691	
CV		17.149		10.023		12.96		12.49	
		W. cibaria N9							
Source	DF	Y_1		Y_2		Y_3		Y_4	
		Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value
Model	5	15.69	0.036*	0.18	0.018*	72.41	0.004*	7.53	0.002*
X_1	1	3.36	0.046*	0.04	0.026*	6.94	0.025*	0.73	0.015*
X_2	1	6.09	0.018*	0.11	0.005*	27.89	0.002*	4.89	0.001*
X_1X_2	1	0.21	0.521	0.01	0.857	13.78	0.008*	0.28	0.063*
X_1^2	1	1.33	0.148	0.01	0.118	5.07	0.041*	0.02	0.573
X_2^2	1	6.03	0.019*	0.03	0.031*	23.76	0.003*	1.44	0.005*
Residual	4	1.66		0.01		2.28		0.17	
Lac of fit	3	1.6	0.231	0.01	0.142	2.12	0.328	0.17	0.215
Pure error	1	0.06		0.01		0.16		0.01	
Total	9	17.35		0.19		74.68		7.7	
R2		0.905		0.933		0.9695		0.9773	
Adj-R2		0.885		0.849		0.9314		0.9489	
CV		27.070		9.256		9.54		9.1	
		L. brevis ED25							
Source	DF	Y_1		Y_2		Y_3		Y_4	
		Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value	Sum of squares	p-Value
Model	5	18.68	0.022*	0.18	0.015*	18.91	0.006*	3.74	0.007*
X_1	1	4.09	0.029*	0.05	0.016*	9.62	0.002*	1.11	0.006*
X_2	1	10.44	0.006*	0.09	0.006*	4.72	0.007*	1.03	0.007*
X_1X_2	1	0.16	0.542	0.01	0.327	1.08	0.072	0.01	0.896
X_1^2	1	1.78	0.092	0.01	0.096	1.57	0.043*	1.55	0.003*
X_2^2	1	3.67	0.034*	0.04	0.018*	3.19	0.014*	0.12	0.154*
Residual	4	1.47		0.01		0.73		0.16	
Lac of fit	3	1.46	0.117	0.01	0.075	0.69	0.28	0.15	0.298
Pure error	1	0.01		0.01		0.04		0.09	
Total	9	20.14		0.19		19.64		2.77	
R2		0.927		0.939		0.9627		0.9588	
Adj-R2		0.836		0.864		0.9161		0.9074	
CV		20.866		8.662		5.71		11.87	

X_1, Fermentation temperature (°C); X_2, fermentation time (h); Y_1, dry cell weight (g), Y_2, total titratable acidity (%), Y_3, lactic acid formation (g L^{-1}), Y_4, acetic acid formation (g L^{-1}).

Results indicated that the models developed for each response were significant ($p < 0.05$) and there was no lack of fit in any equations ($p > 0.05$). The high R^2 values of the polynomial regression equations were observed as > 0.9 for responses, indicating that > 90% of the variations in the corresponding response variable could be explained by the model. Abraham and Ledolter (2006) stated that a regression model with R^2 higher than 0.9 is considered as a model having high correlation. The model F-values for responses was found in the range of 7.58 to 34.41, indicating that the model was significant for all responses. There were only little changes (between 0.22 - 3.61%) for responses, indicating that a "Model F-Value" this large could occur due to noise.

Biomass

The experimental results of the effect of fermentation conditions on the dry cell weight of three LAB during fermentation were shown in Table 1, and the response surface and contour plots for the dry cell weight were given in Figure 1. The values of dry cell weight varied from 0.58 to 3.96 g L^{-1} for *L. curvatus* N19, 0.64 to 3.93 g L^{-1} for *W. cibaria* N9 and 0.82 to 4.25 for *L. brevis* ED25. The dry cell weight of ED25 was found higher than that of other LAB with same fermentation temperature and time. The low cell concentration after the fermentation is probably due to the different adaptation to the fermentation medium and metabolic characteristics of each strain. For all three strains, the lowest dry cell weight after the fermentation was obtained at 22°C for 10h, while the highest value was determined after 20 hours of fermentation at 34.1°C. In general, sourdough LAB have a growth temperature optimum of 30–35°C and the ideal temperature for LAB growth is 35°C (Siepmann, Almeida, Waszczynskyj, & Spier, 2019). Statistical analysis of ANOVA in Table 2 indicated that independent factors significantly affect the dry cell weight ($p < 0.01$). Figure 1a-c shows, dry cell weight increased with the rise in both fermentation temperature and time. The change of dry cell weight depending on temperature increase for *L. brevis* ED25 was higher than that of other strains. The study was supported by Simonson, Salovaara, and Korhola (2003) and Hassani, Zarnkow, and Becker (2015) who reported that the bacterial cell concentration increased significantly with the increase of the fermentation temperature. A partial decrease of dry cell weight for *L. curvatus* N19 was observed after fermentation of 25h and at temperature after 29.5°C. Similarly, Zhou, Zeng, Han, and Liu (2015) stated that the cell growth rate was significantly influenced by fermentation temperature, but the growth rate decreased slightly when the temperature exceeded 35°C. On the other hand, although an increase in dry cell weight was observed due to the increase in fermentation time, the change of cell concentration after the 25h of fermentation period was very limited. Whereas, Zhou et al. (2015) reported that the maximum cell concentration was obtained after more than 30 h. While fermentation temperature and time were significantly effective on cell dry weight, temperature-time interaction was not found to be effective ($p > 0.05$). The dry cell weight after the fermentation by freeze-dried *L. curvatus* N19 (Equation 2), *W. cibaria* N9 (Equation 3) and *L. brevis* ED25 (Equation 4) was estimated using the following response surface polynomial models:

$$Y_1 = 3.59 + 9.7X_1 + 1.1X_2 - 0.21X_1X_2 - 0.53X_1^2 - 0.74X_2^2 \quad (2)$$

$$Y_1 = 3.73 + 0.65X_1 + 0.87X_2 + 0.23X_1X_2 - 0.54X_1^2 - 0.15X_2^2 \quad (3)$$

$$Y_1 = 4.12 + 0.71X_1 + 1.14X_2 - 0.2X_1X_2 - 0.62X_1^2 - 0.9X_2^2 \quad (4)$$

pH and total titratable acidity

The pH value is an important parameter for validation of the fermentation rate of different sourdough starters. Additionally, the total titratable acidity can be used for determining the information about differences in sourdough starters due to describe the concentration of undissociated acids. However, total titratable acidity provides better information than pH in determining flavor such as mild, sour, intensive of the final product (Jekle, Houben, Mitzscherling, & Becker, 2010). Therefore, in this study, total titratable acidity for sourdough fermentation was taken into rather than pH. Nevertheless, the pH values of samples after fermentation were measured and varied from 4.65 to 6.55 for strain N19, 4.69 to 6.59 for strain N9 and 4.62 to 6.57 for strain ED25.

The effect of independent factors on total titratable acidity was depicted in Figure 2a-c. After the fermentation, total titratable acidity values were found in the range of 0.38 to 0.76% for strain N19, 0.41 to 0.79% for strain N9 and 0.39 to 0.77% for strain ED25. The total titratable acidity values across all sourdough fermented by three different LAB was found similar. The highest total titratable acidity value was obtained

fermentation at 34.1°C for 20 h for *L. curvatus* N19 and *L. brevis* ED25, while it was obtained at 27°C for 20h for *W. cibaria* N9. As can be seen in Figure 2a-c, although the increase in fermentation temperature led to generally to an increase in the total titratable acidity, fermentation time seems to be the most significantly effective factor. Our result is consistent with those of Simonson et al. (2003) who reported that increasing fermentation temperature generally resulted in increased sourdough total titratable acidity values. Abedfar and Sadeghi (2019) reported that the total titratable acidity increased across all sourdough by increasing fermentation time. While fermentation temperature and time were significant model terms for the change in total titratable acidity during the fermentation ($p < 0.05$), their interactions did not show significant influence on the change in the total titratable acidity ($p > 0.05$). The final model equation for total titratable acidity can be represented by Equation (5), (6) and (7) for freeze-dried *L. curvatus* N19, *W. cibaria* N9 and *L. brevis* ED25, respectively.

$$Y_2 = 0.74 + 0.079X_1 + 0.92X_2 - 0.028X_1X_2 - 0.068X_1^2 - 0.0099X_2^2 \quad (5)$$

$$Y_2 = 0.73 + 0.07X_1 + 0.11X_2 + 0.0055X_1X_2 - 0.053X_1^2 - 0.087X_2^2 \quad (6)$$

$$Y_2 = 0.74 + 0.077X_1 + 0.1X_2 - 0.03X_1X_2 - 0.055X_1^2 - 0.097X_2^2 \quad (7)$$

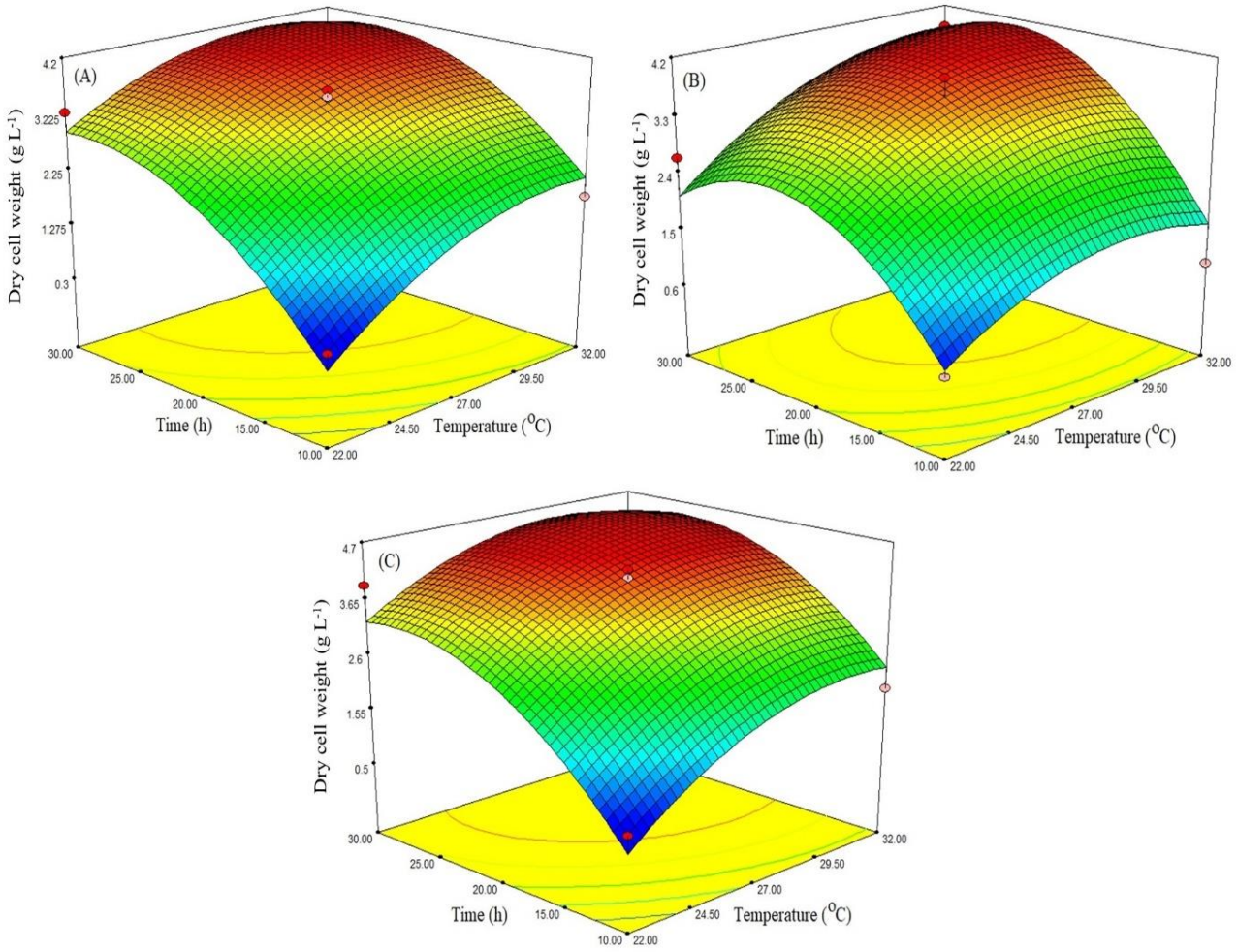

Figure 1. Response surface plots (3D) showing the effects of independent variables on dry cell weight after fermentation by freeze-dried *L. curvatus* N19 (a), *W. cibaria* N9 (b) and *L. brevis* ED25 (c).

Lactic and acetic acid formation

During the sourdough fermentation, organic acid formation is a very important factor due to strongly related to the organoleptic properties (Hassani et al., 2015). According to Gobbetti, Corsetti, and Rossi (1995), a molar ratio of 2:2.7 lactic to acetic acid is considered optimum for the sensory quality of wheat sourdough. The main metabolic product during fermentation of carbohydrates by LAB is lactic acid that has a mostly indirectly positive influence on the flavor and storability of sourdough breads. On the other hand, acetic acid

affects organoleptic properties and storability directly and more strongly because of its flavor index and antimicrobial activity (Jekle et al., 2010). Table 1 shows the lactic acid values after the fermentation process by three different LAB and the effect of fermentation temperature and time on lactic acid concentration presented in Figure 3a-c. While after 20h of fermentation at 34.1°C, lactic acid concentration reached its maximum amount as 8.4 g L^{-1} for *L. curvatus* N19 and 9.14 g L^{-1} for *L. brevis* ED25, the highest lactic acid concentration (9.9 g L^{-1}) for *W. cibaria* N9 was reached at 27°C. The lowest lactic acid formation occurred after 5 hours of fermentation at 27°C for all LAB cultures. Similar findings were observed by Siepmann et al. (2019) who stated that high concentrations of lactic acid during type II sourdough fermentation was observed at the highest fermentation temperature (35°C). As shown in Table 2, both of the independent variables had a positive second order effect on the lactic acid concentration ($p < 0.05$). Similarly, Gebremariam et al. (2015) stated that the dominant factor which controls lactic acid formation was fermentation temperature followed by fermentation time.

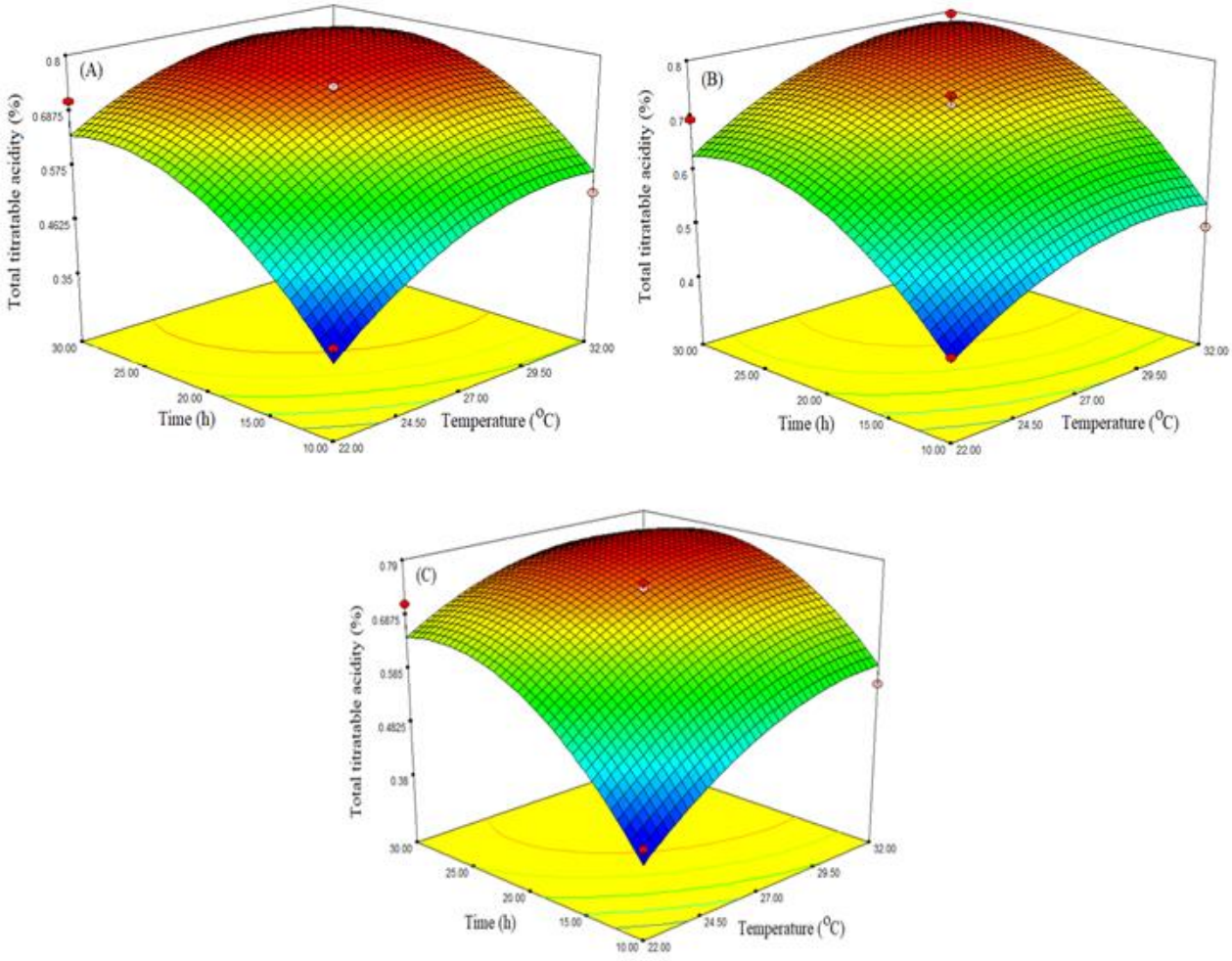

Figure 2. Response surface plots (3D) showing the effects of independent variables on total titratable acidity after fermentation by freeze-dried *L. curvatus* N19 (a), *W. cibaria* N9 (b) and *L. brevis* ED25 (c).

As observed in Figure 3a-c, the lactic acid concentration increased with increasing fermentation temperature and time, but a slight reduction in the lactic acid concentration for *L. curvatus* N19 was observed after the 25h of fermentation. These findings agree with reported by Gebremariam et al. (2015) who stated that fermentation temperature and time were found to be the parameters with the highest influence on most of the response variables including lactic acid formation and they also found that the increase, as well as an extreme decrease in temperature causes a decrease in the formation of lactic acid. In another study, Hassani et al. (2015) cited that all the fermentation parameters including the quadratic effect of temperature had significant effect ($p < 0.05$) on the lactic acid formation and temperatures outside the range considered in their study lead to low concentration.

While independent factors had a significant effect on lactic acid formation for all LAB, the interaction of these parameters was only found significant for *W. cibaria* N9 ($p < 0.05$). The final model equation for lactic acid formation can be represented by Equation (8), (9) and (10) for *L. curvatus* N19, *W. cibaria* N9 and *L. brevis* ED25, respectively.

$$Y_3 = 7.94 + 1.1X_1 + 1.09X_2 + 0.013X_1X_2 - 0.88X_1^2 - 1.55X_2^2 \quad (8)$$

$$Y_3 = 10.58 + 0.98X_1 + 1.87X_2 - 1.86X_1X_2 - 1.05X_1^2 - 2.28X_2^2 \quad (9)$$

$$Y_3 = 8.64 + 1.1X_1 + 0.77X_2 - 0.52X_1X_2 - 0.59X_1^2 - 0.84X_2^2 \quad (10)$$

The highest acetic acid concentration after the fermentation by freeze-dried *L. curvatus* N19 (1.86 g L^{-1}), *W. cibaria* N9 (3.15 g L^{-1}) and *L. brevis* ED25 (2.69 g L^{-1}) was found the interaction of temperature and time as 34.1°C for 20h, 32°C for 30h and 27°C for 20h, respectively. Increase in fermentation temperature and time significantly caused an increased acetic acid formation (Figure 4a-c). As shown in Table 2, while temperature and time were found to be significantly effective on acetic acid formation during sourdough fermentation ($p < 0.05$), temperature-time interaction did not affect the ability of microorganisms to produce acetic acid ($p > 0.05$). The acetic acid formation during the fermentation by freeze-dried *L. curvatus* N19 (Equation 11), *W. cibaria* N9 (Equation 12) and *L. brevis* ED25 (Equation 13) was estimated using the following response surface polynomial models:

$$Y_3 = 1.73 + 0.2X_1 + 0.23X_2 + 0.026X_1X_2 - 0.32X_1^2 - 0.34X_2^2 \quad (11)$$

$$Y_3 = 2.79 + 0.3X_1 + 0.78X_2 - 0.27X_1X_2 - 0.06X_1^2 - 0.56X_2^2 \quad (12)$$

$$Y_3 = 1.09 + 0.37X_1 + 0.36X_2 + 0.014X_1X_2 + 0.58X_1^2 + 0.16X_2^2 \quad (13)$$

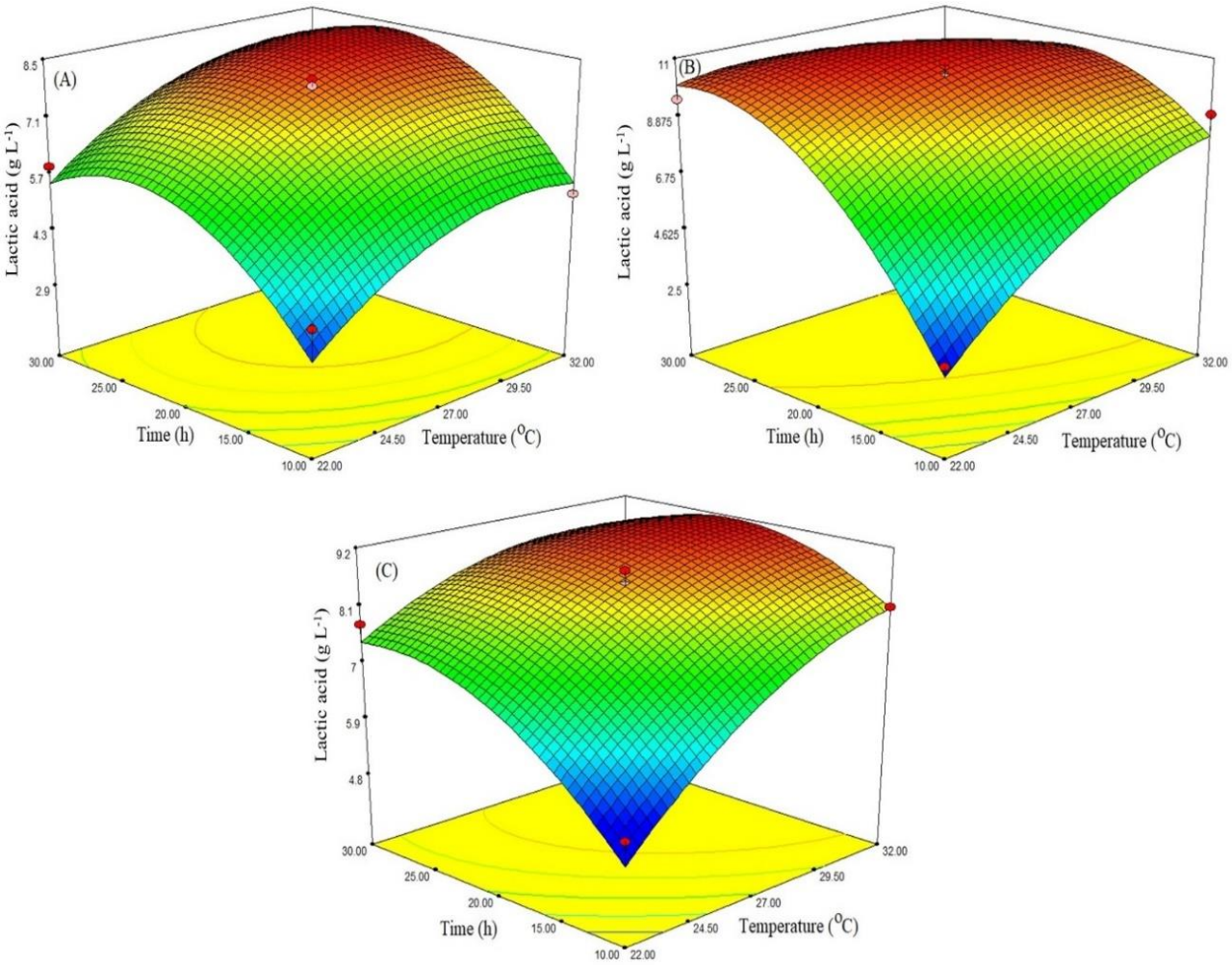

Figure 3. Response surface plots (3D) showing the effects of independent variables on lactic acid formation after fermentation by freeze-dried *L. curvatus* N19 (a), *W. cibaria* N9 (b) and *L. brevis* ED25 (c).

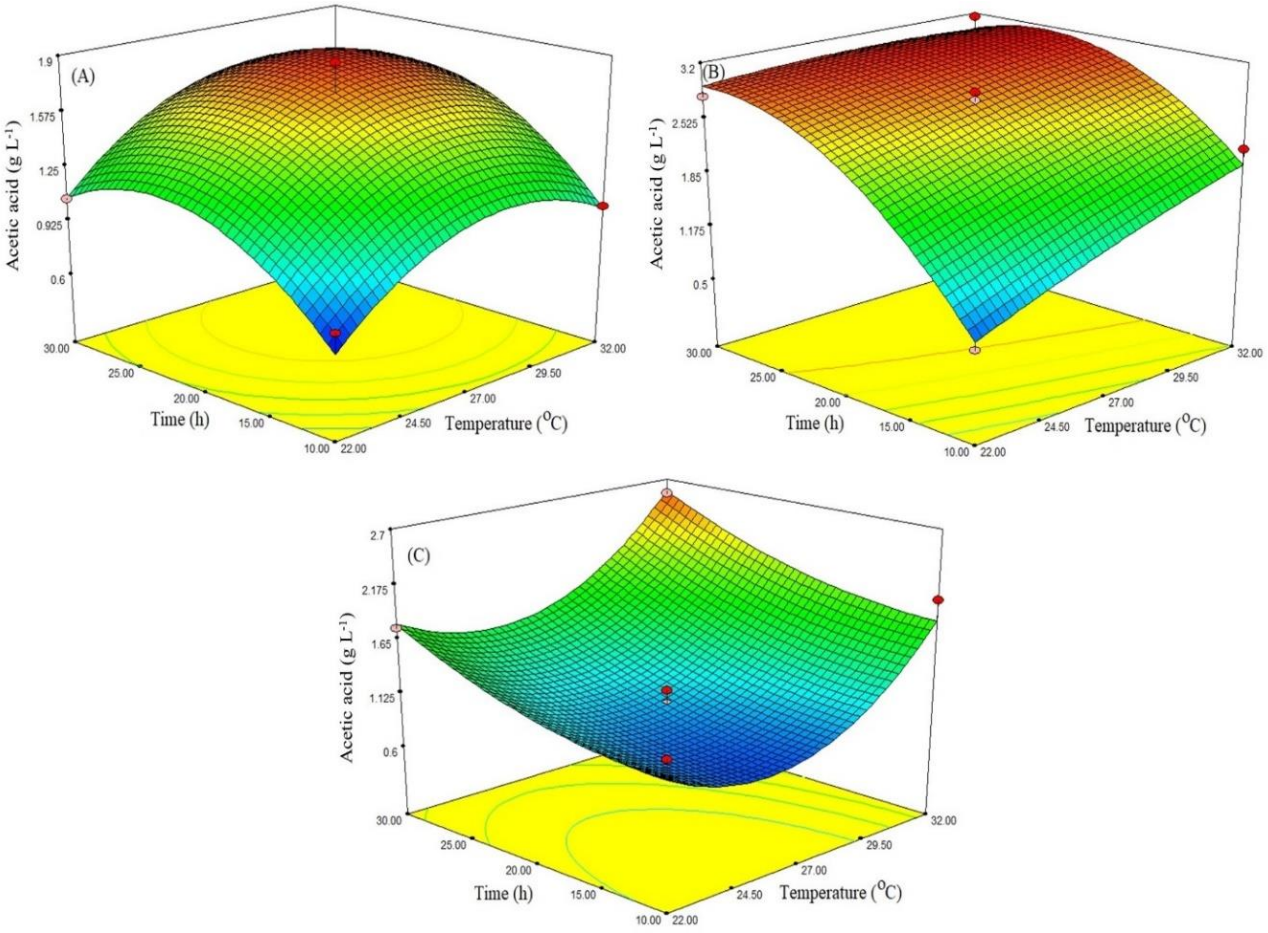

Figure 4. Response surface plots (3D) showing the effects of independent variables on acetic acid formation after fermentation by freeze-dried *L. curvatus* N19 (a), *W. cibaria* N9 (b) and *L. brevis* ED25 (c).

Optimization and verification of model

The sourdough fermentation parameters including temperature and time were optimized to maximize dependent parameters including biomass, total titratable acidity, lactic and acetic acid formation for the production of sourdough. The Derringer's desirability function was used for determining optimum conditions. According to the most desirable solution for maximization of responses as shown in Table 3, the optimum fermentation conditions were found as follows: 28.62°C temperature and 23.44h time for *L. curvatus* N19, 29.27°C temperature and 24.78h time for *W. cibaria* N9, and 32°C temperature and 26.22h time for *L. brevis* ED25. The predicted levels of responses under optimum conditions were given in Table 3. Experimental rechecking was carried out using determined optimal parameters to ensure that the predicted results were not biased toward the practical values. The experimental values for biomass, total titratable acidity and acetic acid were not significantly ($p > 0.05$) different from predicted values. However, lactic acid values obtained experimentally were found to be lower than the predicted values. The experimental results were found to be close to predicted values of dry cell weight and total titratable acidity. The deviation from the experimental and predictive value calculated from Equation (14) reported by Nahr et al. (2015) was ranged from -0.061 to -0.012 for biomass, -0.025 to 0.051 for total titratable acidity, -0.275 to -0.09 for lactic acid and -0.229 to 0.021 for acetic acid. It can be concluded that the selected model can be used to optimize the fermentation condition parameters for production sourdough by freeze-dried *L. curvatus* N19 and *L. brevis* ED25. However, the results between the predicted and experimental values of lactic and acetic acid indicated that the established model for *W. cibaria* N9 did not agree due to the high deviation.

$$Deviation = \frac{(Experimental\ value - Predicted\ optimal\ value)}{Predicted\ optimal\ value} \quad (14)$$

Table 3. Optimized conditions of fermentation according to desirability function and experimental values obtained under optimized conditions.

LAB		X_1	X_2	Y_1 (g)	Y_2 (%)	Y_3 (g L^{-1})	Y_4 (g L^{-1})	Desirability
L. curvatus N19	Optimized	28.62	23.44	4.03	0.79	8.39	1.79	0.987
	Experimental	29	23	3.98±0.18	0.77±0.08	7.62±0.23	1.38±0.1	
W. cibaria N9	Optimized	29.27	24.78	4.11	0.79	10.75	3.11	0.990
	Experimental	29	25	3.99±0.09	0.83±0.04	7.79±0.31	2.43±0.16	
L. brevis ED25	Optimized	32	26.22	4.48	0.77	8.97	2.34	0.942
	Experimental	32	26	4.21±0.21	0.79±0.06	8.16±0.19	2.39±0.26	

X_1, Fermentation temperature (°C); X_2, fermentation time (h); Y_1, dry cell weight (g), Y_2, total titratable acidity (%), Y_3, lactic acid formation (g L^{-1}), Y_4, acetic acid formation (g L^{-1})

Conclusion

In this study, the optimization of fermentation parameters for sourdough production by using freeze-dried *L. curvatus* N19, *W. cibaria* N9 and *L. brevis* ED25 was done by RSM, which is a suitable technique for fermentation optimization. Fermentation temperature and time as independent factors showed significant single effects on the studied response variables. The models obtained in this study were validated by using experimental results. According to the results between the predicted value and experimental values, established models for freeze-dried *L. curvatus* N19 and *Lb. brevis* ED25 are effective and feasible. On the other hand, the freeze-dried *L. curvatus* N19 as a potential starter culture can be preferable to sourdough fermentation, because optimum fermentation temperature and time (about 29°C and 23h, respectively) for this strain is more applicable to sourdough bread industry. Further research will be focused onto use of freeze-dried *L. curvatus* N19 for the production of sourdough under specified fermentation conditions.

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