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ORIGINAL RESEARCH PAPER

INVESTIGATIONS ON THE CAPACITY OF LACTIC ACID BACTERIA TO PRODUCE ACE INHIBITORY PEPTIDES

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The individual and interactive effects of inoculum size, fermentation time and ratio of the freeze-dried *Lactobacillus bulgaricus* powder LB6 and the freeze-dried *Streptococcus thermophilus powder* ST78 on pH, optical density (OD) and Angiotensin converting enzyme (ACE) inhibitory activity were studied by Response Surface Methodology (RSM). As a result, the optimal starter culture formulations were obtained. It was shown that the optimal inoculum size and fermentation time were 0.3% and 4.5 h respectively, for an optimal LB6:ST78 ratio of 3:2. The experimental values of pH, OD and ACE inhibitory activity were 4.71±0.03, 0.962±0.031 and 85.16±0.05 %, which were very close to the predicted values 4.70, 0.963 and 85.10%.

Keywords: *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, functional starter culture, ACE inhibitory peptides, Response Surface Methodology

Introduction

Human blood pressure (Salampessy, *et al*., 2015) is regulated through several biochemical systems in the body. These systems include the reninangiotensin system (RAS), kinin-nitric oxide system (KNOS), renin-chymase system (RCS) and neutral endopeptidase system (NEPS) (Pihlanto, *et al*., 2000). Angiotensin converting enzyme (ACE) is a dipeptidase which is involved in the RAS and KNOS (Connolly, *et al*., 2015; Nakamura, *et al*., 1995). In the RAS, ACE cleaves angiotensin I to angiotensin II which is a potent vasoconstrictor. In the KNOS, ACE inactivates the hypotensive peptide, bradykinin (Magee, *et al*., 2008). So, the excessive ACE may result in increasing hypertension.

ACE inhibitory peptides, namely, antihypertensive ones (Pihlanto, *et al.*, 2008), which are a class of bioactive peptides (Kchaou, *et al*., 2013; Tsai, *et al*., 2008a; Tsai,

et al., 2008b) have ACE inhibitory activity and significant hypotensive effects. Therefore, it has become a hotspot in recent years.

Due to a high nutritional value and appropriated tastes, yogurt is favored among dairy products by consumers (Lourens-Hattingh & Viljoen, 2001). As a new trend, consumers pay more attention to yogurt with special health care function.

Lactobacillus bulgaricus LB6 is one of the main strains in fermented milk products, which not only has a variety of physiological health functions, but can also produce ACE inhibitory peptides in the process of fermentation (Chen, *et al.*, 2012). It was stated that *Lactobacillus bulgaricus* LB6 is the most suitable strain used as a functional starter culture (Ji, 2013).

Starter cultures are crucial for fermented products, which are the `heart' of yogurt (Thomas & Pritchard, 1987). It is an extremely important component in the fermentation of high quality yogurt. Different starter cultures influence fermentation performance and the quality of fermented milk products (Bachmann *et al.*, 2015; Speckman, *et al.*, 1974; Zhao *et al.*, 2009). Moreover, the use of a starter culture which could produce ACE inhibitory peptides would contribute to a significant enhancement of fermented milk products.

In order to optimize fermentation process in terms of functional starter culture ratio, the Box-Behnken design was used with selected points from a system arrangement, which is a three-level factorial design for three factors. Response Surface Methodology based on Box-Behnken designs of experiment is a popular statistical method (Martins, *et al.*, 2013; Piyushkumar, *et al.*, 2007; Joyce & Leung, 2013, Singh *et al.*, 2014). The Box-Behnken design can not only decrease the number of runs, but can also be used for many factors in one process (Bezerra, *et al.*, 2008). The Box–Behnken method was selected as the statistical prediction method with the aim of reducing the number of experimental runs which will directly save time and chemicals and thereby reduce the overall cost.

The aim of the present work was to optimize the fermentation process and the functional starter culture ratio by Response Surface Methodology. It will provide the technical foundation for further development of functional foods. Therefore, it is necessary to optimize starter culture ratio that can produce ACE inhibitory peptides in the process of fermentation (Narayan, 2011).

Materials and Methods

Materials

The freeze-dried powder of *Lactobacillus bulgaricus* LB6 (LB6) and *Streptococcus thermophilus* ST78 (ST78) were both provided by Shaanxi University of Science and Technology, which was found to produce high quantity of ACE inhibitory peptides (Kleekayai, *et al.*, 2015; Toopcham, *et al.*, 2015). The skim milk powder was provided by Anchor. Ethyl acetate was purchased from Tianjin Hongyan Chemical Reagent Factory. Modified Tomato Juice Agar was purchased from Qingdao Hope Bio-Technology Co., Ltd. Hippuryl-Histidyl-Leucine (HHL) and ACE from rabbit lung were purchased from Sigma Chemical Co.

Determination of pH

The 12.5% (w/w) reconstituted milk (lipids were 1.5% of the dried skim milk and proteins were the 34% of the nonfat milk solids) was inoculated with starter cultures and fermented at 42°C for 4.5, 5 and 5.5 h, and pH was measured at each interval by a pHS-3C pH-meter (Shanghai Precision Scientific Instrument Co., Ltd, China). The measurements were replicated three times.

Determination of optical density

A quantity of 1mL of fermented milk and 9 mL of 0.2% EDTA were mixed in a WH-2 micro vortex (Shanghai Analytical Instrument, China). The value of OD was tested at 640 nm according to the method of Chen *et al.* (2014), with a VIS-722 spectrophotometer (Shanghai Phenix Optical Scientific Instrument Co., Ltd, China). Control group was considered the reconstituted milk. The measurements were replicated three times.

Determination of ACE inhibitory activity

In order to determine ACE inhibitory activity of the yoghurt samples obtained, first a preliminary preparation step was performed according to the method of Pan (2005). A quantity of 5 ml of yoghurt, rested for 24 h at 4°C was taken and the pH value was adjusted to 3.4-3.6. Then, the sample was centrifuged at 7104×g for 15 min using a TG16A-WS centrifuge (Hunan Shaite Xiangyi Co., Ltd, China). The pH of the supernatant after centrifuging was adjusted to 8.3, then the supernatant was centrifuged again at 7104×g for 15 min, the supernatant was collected and used for further determinations.

ACE-inhibitory activity was determined by the method of Papadimitriou, *et al.* (2007), with some further modifications. Each assay mixture (270µL) contained the following components: 200µL of Hippuryl-L-histidyl-L-leucine (HHL) solution (5mM) in sodium borate (100mM) (pH 8.3) and 100µL of the sample as „a" group, 200µL HHL solution as „b" control group; 200µL HHL solution mixed with 250µL HCl (1M) as "c" group. "a", "b" and "c" were all incubated for 5 min at 37 °C. After 20µL ACE solution (0.1U/mL) was added to "a", "b" and "c", the "a", "b" and "c" groups were further incubated for 5 min at 37 °C. Then 250µL HCl was added to "a" group, both of 250µL HCl and 100µL sodium borate buffer were added to "b", and 100µL sodium borate buffer was added to "c" (Table 1).

After preparation procedure detailed in Table 1, the produced hippuric acid was extracted with 1.7ml of Ethyl acetate, heat-evaporated at 120°C for 30 min, re-dissolved in 3ml demonized water and cooled at room temperature. Subsequently, ACE-inhibitory activity was determined spectrophotometrically at 228 nm against deionized water as blanc and average data from triplicate tests were reported (Papadimitriou, *et al.* 2007). ACE inhibitory rate (%) for each reaction was calculated, using the formula:

$$\text{ACE inhibitory rate (\%)} = (y-x)/(y-z)\times 100\%,$$

where x is the OD_{228} of "a", y is the OD_{228} of "b" and z is the OD_{228} of "c".

Table 1. Experimental steps performed in order to determine ACE inhibitory activity in the yoghurt samples obtained

unit/µl	a	b	c
HCl	0	0	250
HHL	200	200	200
Sample	100	0	0
	Incubation 37°C 5min		
ACE	20	20	20
	Incubation 37°C 5min		
HCl	250	250	0
sodium borate buffer	0	100	100

Determination of viable bacterial count

The method for determination of viable bacterial count was applied according to the method of Feng & Sun (2013). Starter cultures was diluted to suitable concentration with NaCl (0.9% w/v) and inoculated into Tomato Juice Agar, Modified. The medium was tempered at 37°C for 48 h, the number of colony between 30 and 300 was used. Finally, the viable count per milliliter (cfu/mL) was determined.

Statistical Data Analysis

Design-Expert (Version, 8.0.6) software was used for the experiment design in order to estimate fermentation procedure of functional starter culture. The experimental values where used as averages of three replications. Analysis of variance (ANOVA) was used for evaluation of the adequacy of the fitted model and testing the significance of the coefficient.

Results

The influence of inoculum size on both pH and ACE inhibitory activity was determined with the single factor experiment. The used ratio of LB6:ST78 was 1:1. The mass ratio of inoculum size to reconstituted milk was 0.1%, 0.2%, 0.3% and 0.4%. Subsequently the obtained samples were subjected to fermentation at 42°C for 4h according to the method described by Patrignani, *et al*. (2006), after which the pH and ACE inhibitory activity was measured. As shown in Figure 1, pH decreased with the increase of inoculum size while ACE inhibitory activity first increased and then descended with inoculum size. We appreciate that the lower value of pH influenced the probiotics growth. The maximum ACE inhibitory activity was reached for 0.3% inoculum size at a registered pH value of 4.82.

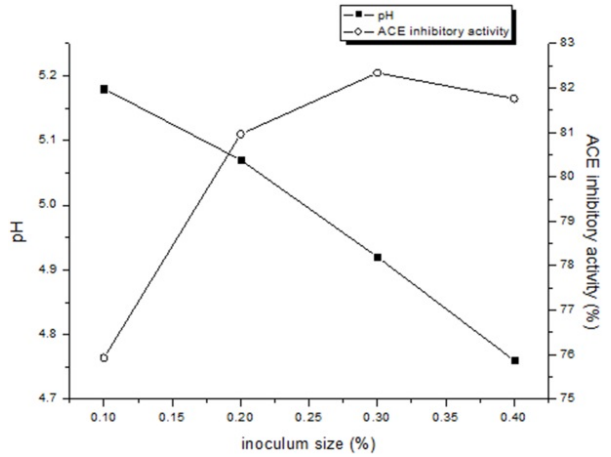

Figure 1. The influence of inoculum size on pH and ACE inhibitory activity

The influence of fermentation time on both pH and ACE inhibitory activity

According to previously obtained results, the influence of fermentation time on pH values and ACE inhibitory activity was further tested. Thus, for following investigations the inoculum size of 0.3% was considered at a LB6:ST78 ratio of 1:1, and a fermentation temperature of 42 ^{o}C. The variables were fermentation time, which were 3.5, 4, 4.5 and 5 h respectively. Finally, the pH and ACE inhibitory activity were measured. The results are shown in Figure 2. One can see that pH decreased with the increase of fermentation time, while ACE inhibitory activity first increased, and then descended with time. As we appreciate, the reason was possibly the effect of pH. After 4.5h of fermentation time, the pH dropped to 4.73, and the maximum of ACE inhibitory activity was registered (Figure 2).

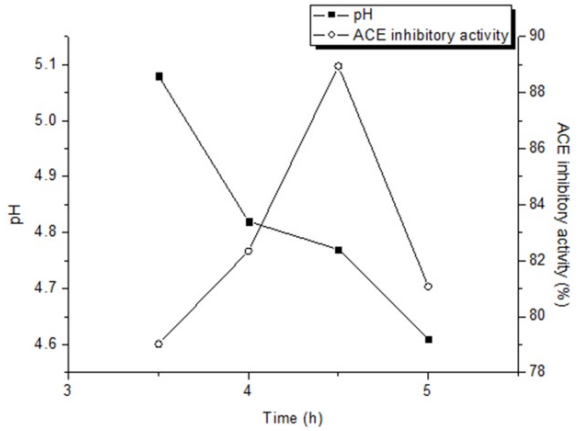

Figure 2. The effect of fermentation time on pH and ACE inhibitory activity of the yoghurt samples obtained

Optimization of the fermentation process by the Box-Behnken experiment

The optimization of the technological process was realized with the Box-Behneken experiment. The Box-Behnken experimental design (Joyce & Leung, 2013) was used to evaluate the ratio of strains and fermentation time on functional starter culture. The factor levels of the Box-Behnken are shown in Table 2. The Box-Behnken design and result are shown in Table 3.

Table 2. Levels of variables chosen

Run	-1	0	1
LB6	1	2	3
ST78	1	2	3
Time (h)	4	4.5	5

Table 3. The set-up and results of the Box-Behnken design

Run	LB6	ST78	Time (h)	pH	OD	ACE%
1.00	1.00	1.00	4.50	4.77	0.87	88.95
2.00	3.00	1.00	4.50	4.71	0.88	80.43
3.00	1.00	3.00	4.50	4.61	0.87	89.13
4.00	3.00	3.00	4.50	4.83	0.78	82.17
5.00	1.00	2.00	4.00	5.08	0.76	69.65
6.00	3.00	2.00	4.00	4.96	0.68	65.23
7.00	1.00	2.00	5.00	4.57	0.78	58.74
8.00	3.00	2.00	5.00	4.55	1.01	76.09
9.00	2.00	1.00	4.00	5.06	0.64	63.04
10.00	2.00	3.00	4.00	5.45	0.46	60.92
11.00	2.00	1.00	5.00	4.53	0.78	73.91
12.00	2.00	3.00	5.00	4.58	0.85	72.65
13.00	2.00	2.00	4.50	4.68	1.08	84.78
14.00	2.00	2.00	4.50	4.67	0.90	86.96
15.00	2.00	2.00	4.50	4.96	0.89	78.34
16.00	2.00	2.00	4.50	4.81	0.86	81.89
17.00	2.00	2.00	4.50	4.86	0.86	85.47

The Box-Behnken experimental design was applied for process optimization. Starter cultures LB6, ST78 and time were used as model variables while pH (Y1), OD (Y2) and ACE% (Y3) were considered responses.

According to Table 3, the Box-Behnken design data were analyzed by the multivariate quadratic regression model according to equations (1), (2) and (3). The

model was developed for determining the individual effects and mutual interaction effects of candidate variables:

$$Y1= +13.0815-5.00000E-004\times A+0.5770\times B-3.3820\times C+0.0700\times A\times B+0.0500\times A\times C-0.1700\times B\times C-0.0905\times A^2+0.0245\times B^2+0.3380\times C^2 \quad (1)$$

$$Y2= -9.20982-0.75885\times A-0.16885\times B+4.69960\times C-0.022250\times A\times B+0.15250\times A\times C+0.12750\times B\times C+0.031525\times A^2-0.096475\times B^2-0.55990\times C^2 \quad (2)$$

$$Y3= -1191.57550-53.04025\times A-6.66650\times B+587.81650\times C+0.39000\times A\times B+10.88500\times A\times C+0.43000\times B\times C+0.73975\times A^2+0.94225\times B^2-67.20100\times C^2 \quad (3)$$

where A, B and C are respectively the variables of LB6, ST78 and time, Y1, Y2 and Y3 are the desired optimal values of pH, OD and ACE inhibitory activity. The results obtained by analyzing pH, OD and ACE inhibitory activity data with Analysis of Variance (ANOVA) are shown in Tables 4, 5 and 6.

Table 4. ANOVA of pH

Source	SS	DF	MS	F	Pr>F	Sig.
Model	0.81	9	0.09	4.86	0.0246	*
A-LB6	5.00E-05	1	5.00E-05	2.71E-03	0.96	
B-ST78	0.02	1	0.02	1.08	0.3328	
C-Time	0.67	1	0.67	36.4	0.0005	* * *
AB	0.02	1	0.02	1.06	0.3374	
AC	2.50E-03	1	2.50E-03	0.14	0.7239	
BC	0.029	1	0.029	1.56	0.2513	
A^2	0.034	1	0.034	1.87	0.2142	
B^2	2.53E-03	1	2.53E-03	0.14	0.7225	
C^2	0.03	1	0.03	1.63	0.2429	
Residual	0.13	7	0.018			
Lack of Fit	0.069	3	0.023	1.52	0.3393	
Pure Error	0.061	4	0.015			
Cor Total	0.94	16				

Note. ***P<0.001, highly significant; ** P<0.01, very significant; * P<0.05, significant. DF refers to degrees of freedom, SS refers to sum of squares, MS refers to mean square, F and Pr>F refer to F and P values, respectively (Ba-Abbada, *et al*., 2015).

In Table 4, the model (P=0.0438<0.05) for response Y1 (pH) was significant, whereas the lack of fit (P=0.3393>0.05) was insignificant, which proved that the model could be used to analyze the response Y1. The results obtained are similar to other researches (Jofré, *et al.*, 2010; Khodadoust & Hadjmohammadi, 2011; Momenbeik *et al.*, 2010). Fermentation time resulted to be a highly significant factor (P<0.01) that could influence Y1. The low F value of the factors A (LB6) and B (ST78), A and C (time), and B and C implied that the mutual interaction between

them was weak when considering Y1. The coefficient of determination (R^2=0.8619) suggests that more than 86.19% of data variability in the Y1 response could be explained by the predicted equation (1). The adjusted R^2 coefficient (R^2_{adj}=68.44%) indicates that the final prediction is in good agreement with the experimental results.

Table 5. ANOVA of OD

Source	SS	DF	MS	F	Pr>F	Sig.
Model	0.27	9	0.03	4.7	0.0267	*
A-LB6	6.48E-04	1	6.48E-04	0.1	0.7609	
B-ST78	5.20E-03	1	5.20E-03	0.8	0.3998	
C-Time	0.097	1	0.097	15.02	0.0061	**
AB	1.98E-03	1	1.98E-03	0.31	0.5974	
AC	0.023	1	0.023	3.59	0.0999	
BC	0.016	1	0.016	2.51	0.1571	
A^2	4.19E-03	1	4.19E-03	0.65	0.4478	
B^2	0.039	1	0.039	6.05	0.0434	*
C^2	0.082	1	0.082	12.74	0.0091	**
Residual	0.045	7	6.47E-03			
Lack of Fit	0.011	3	3.74E-03	0.44	0.7377	
Pure Error	0.034	4	8.52E-03			
Cor Total	0.32	16				

In Table 5, the model P=0.0267<0.05) for Y2 response (OD) was significant while the lack of fit (P=0.7377>0.05) was insignificant, which proved the model could be used to analyze the response Y2. The fermentation time in this case also resulted to be a significant factor (P<0.01), which could influence Y2. The F value of the factors A (LB6) and B (ST78), A and C (time), and B and C were low, which implied that the effect of A and B, A and C, and B and C on Y2 was independent. The high F value of B^2 and C^2 showed that the variables and Y2 did not present a simple linear correlation. The coefficient of determination (R^2=0.8581) suggests that more than 85.81% of Y2 variability could be explained by the predicted equation (2). The adjusted R^2 coefficient (R^2_{adj}=67.57%) indicates that the final prediction is in good agreement with the experimental results, and it also confirmed that the model was significant.

In Table 6, the model (P=0.0232<0.05) for Y3 response (ACE inhibitory activity) was significant and the lack of fit (P=0.0816>0.05) was insignificant, which proved the model could be used to analyze the response Y3. The F value of the factors A (LB6) and B (ST78), A and C (time), and B and C were low, which indicated that the effect of A and B, A and C, and B and C on Y3 was independent. The high F value of C^2 showed that the variables and response Y3 had not a simple linear

correlation. The coefficient of determination (R^2=0.8644) mean that more than 86.44% of variability in Y3 could be explained by the predicted equation (3). The adjusted R^2 coefficient (R^2_{adj}=69.01%) indicates that the final prediction is in good agreement with the experimental results.

Table 6. ANOVA of ACE inhibitory activity

Source	SS	DF	MS	F	Pr>F	Sig.
Model	1372.43	9	152.49	4.96	0.0232	*
A-LB6	0.81	1	0.81	0.026	0.8754	
B-ST78	0.27	1	0.27	8.67E-03	0.9284	
C-Time	63.56	1	63.56	2.07	0.1937	
AB	0.61	1	0.61	0.02	0.8921	
AC	118.48	1	118.48	3.85	0.0904	
BC	0.18	1	0.18	6.01E-03	0.9404	
A^2	2.3	1	2.3	0.075	0.7922	
B^2	3.74	1	3.74	0.12	0.7376	
C^2	1188.41	1	1188.41	38.65	0.0004	***
Residual	215.24	7	30.75			
Lack of Fit	168.53	3	56.18	4.81	0.0816	
Pure Error	46.71	4	11.68			
Cor Total	1587.67	16				

Taking into account results presented in Tables 4, 5 and 6, Stereogram 3-D response surface and contour plot (2-D) graphs of the corresponding variables pH, OD and ACE inhibitory activity were generated in Figures 3, 4 and 5.

From Figures 2 and 3, it was observed that pH decreased following the increase of the fermentation time. The effect of LB6 and ST78, and LB6 and time on pH had no mutual interaction. Also LB6, ST78 and fermentation time had no mutual interaction.

In Figures 4-8, it can be seen that pH and ACE inhibitory activity increased first and then decreased with the increase of time. The effect of LB6 and time, and ST78 and time on OD and ACE inhibitory activity had no mutual interaction.

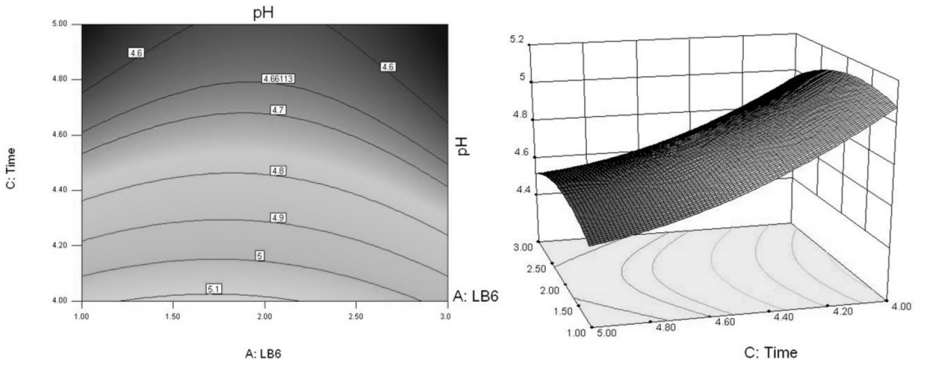

Figure 3. 2D contour plot and 3D response surface of LB6, fermentation time and pH

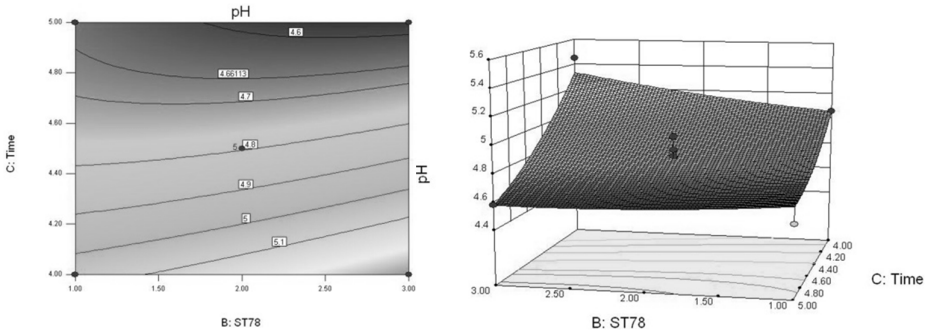

Figure 4. 2D contour plot and 3D response surface of ST78, fermentation time and pH

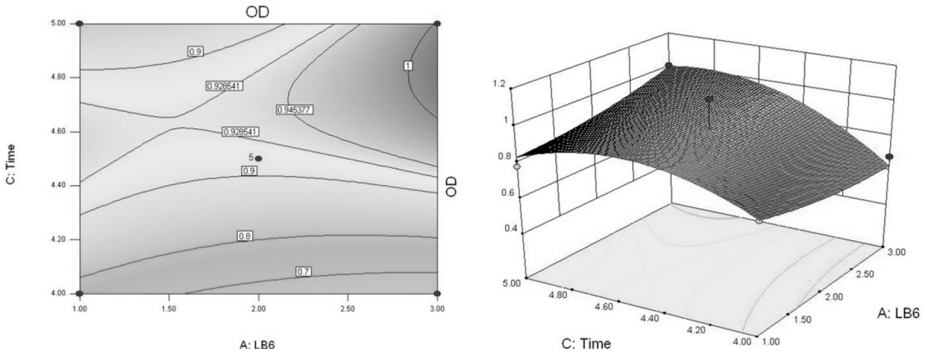

Figure 5. 2D contour plot and 3D response surface of LB6, fermentation time and OD

Discussions

The variation of ACE inhibitory activity, which first increased and then decreased with the inoculum size and fermentation time, may be due to the nutrient of reconstituted milk. At the beginning, the nutrient in the reconstituted milk is very

abundant for probiotic growth, so the ACE inhibitory activity increases with the addition of inoculum size or as fermentation time goes on. When the inoculum size or fermentation time reaches a certain point, the nutrient of the reconstituted milk has been used up, and then the probiotic begins to decompose the ACE inhibitory peptides, therefore, the ACE inhibitory activity decreases. These results are connected with most reports on ACE-inhibitory peptides (Eriksson, *et al.*, 2002; Escudero, *et al*., 2014; Korhonen, *et al.*, 1998; Norris, *et al*., 2014; Pihlanto-Leppälä, *et al*., 2000; Papadimitriou, *et al*., 2007; Pihlanto, *et al.*, 2008; Salampessy, *et al.*, 2015).

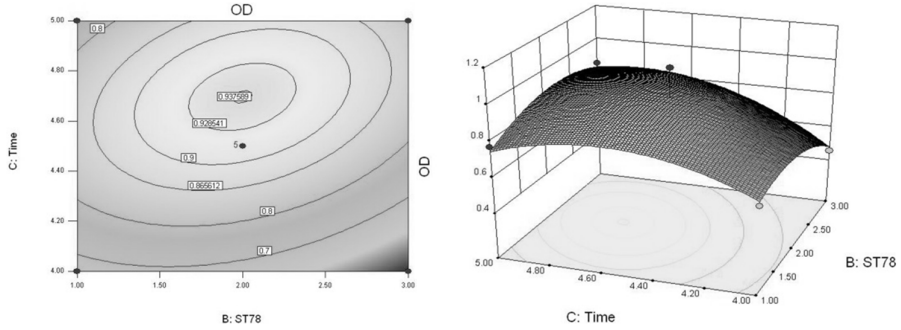

Figure 6. 2D contour plot and 3D response surface of ST78, fermentation time and OD

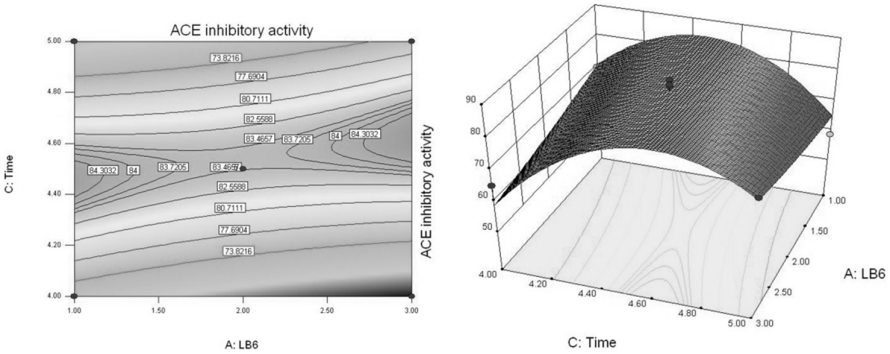

Figure 7. 2D contour plot and 3D response surface of LB6, fermentation time and ACE inhibitory activity

Another observation of the study was that the pH value followed a rapid decrease at the beginning of fermentation process (0-4 h) and then gently declined both with the inoculum size and fermentation time (from 4th to 4th.5 h), which may be also due to the nutrients found in reconstituted milk. At first, the nutrients in the reconstituted milk were very abundant for probiotic growth leading to a rapid increase of organic acids quantity and a rapid decrease of pH value respectively. At the end of the process, after nutrients` consumption from the reconstituted milk, pH value followed a slower decrease. The results accords with those presented by literature (Xiang, *et al*. 2005; Chen, *et al*. 2014; Chen, *et al.* 2015).

The coefficient (P) was used to determine the significance of factors. The smaller the value of P, the more significant the effect of variable was. The value of R-squared represents the variability of the actual response values. The value of P and F could explain the experimental factors and their interactions (Sulaiman, *et al*. 2005).

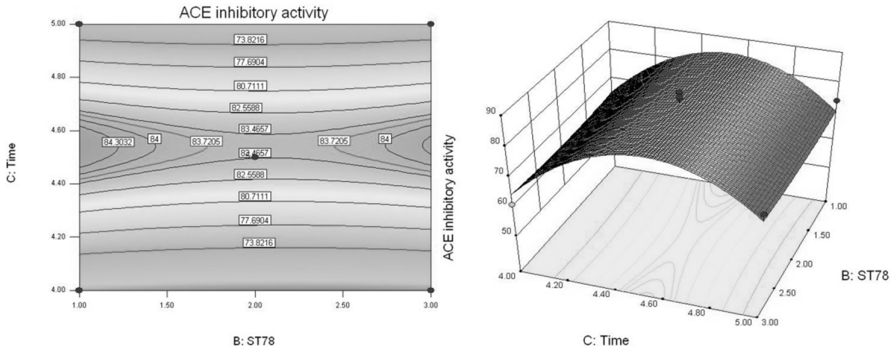

Figure 8. 2D contour plot and 3D response surface of ST78, fermentation time and ACE inhibitory activity

The fermentation time presented a significant influence on pH and OD ($P<0.05$), whereas the mutual interactions of LB6 and ST78, time and LB6, and ST78 and time did not present a significant influence on pH, OD or ACE inhibitory activity as shown previously in Tables 4, 5 and 6, indicating that two factors did not affect pH simultaneously. Similar results were obtained for OD and ACE inhibitory activity.

Results showed that for a fermentation time of 4.5 h and a LB6:ST78 ratio of 3:2, LB6 and ST78 presented a good symbiotic relationship. The predicted value of pH, OD and ACE inhibitory activity were 4.70, 0.963 and 85.10% respectively. The experimental value of pH, OD and ACE inhibitory activity resulted in 4.71±0.03, 0.962±0.031 and 85.16±0.05 %, which were very close to the predicted values. After experiment, we determined the viable bacterial count of the yogurt which was 2.93×10^9 ±0.27 cfu/mL.

Conclusions

The present paper was aimed to optimize the fermentation process and optimal ratio of two starter cultures (LB6 and ST78) by Response Surface Methodology. The results obtained by single factor experiment indicated that in order to obtain a high ACE inhibitory activity, the optimal inoculum size should be 0.3%. By applying Box-Behnken design it was shown that the optimal fermentation time and the mass ratio of LB6 and ST78 should be 4.5 h and 3:2 respectively for the same purpose.

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