

*Full Length Research Paper*

# Improvement of fermentation process of bifidobacteria soybean yoghurt: A uniform design approach

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**This study aimed at producing soybean yoghurt containing bifidobacterium by temperature shifted fermentation process, and to increase the viable counts of bifidobacterium in the soybean yoghurt. First, parallel test was used to investigate the amount of carbon source of high fructose corn syrup. Secondly, using the Mathematic 7.01 software program, the relationships between the viable counts of bifidobacterium and fermentation conditions (temperature and duration) were simulated. Finally, the fermentation process was optimized through the uniform design. The optimized process conditions included 5% of soybean powder, 5.32% of high fructose corn syrup, 0.03% of lactic bacteria (*Lactobacillus bulgaricus*: *Streptococcus thermophilus* = 1:1), 0.15% of bifidobacteria, 3.5 h of the first phase fermentation process at 42°C, and 1.5 h of the second phase fermentation process at 37.2°C. The viable counts of bifidobacterium in the final product of soybean yoghurt were above  $1.1 \times 10^6$ /ml. The fermentation process proposed by this study can increase the viable counts of bifidobacterium in the soybean yoghurt.**

**Key words:** Uniform design, soybean yoghurt, lactic bacteria, bifidobacteria.

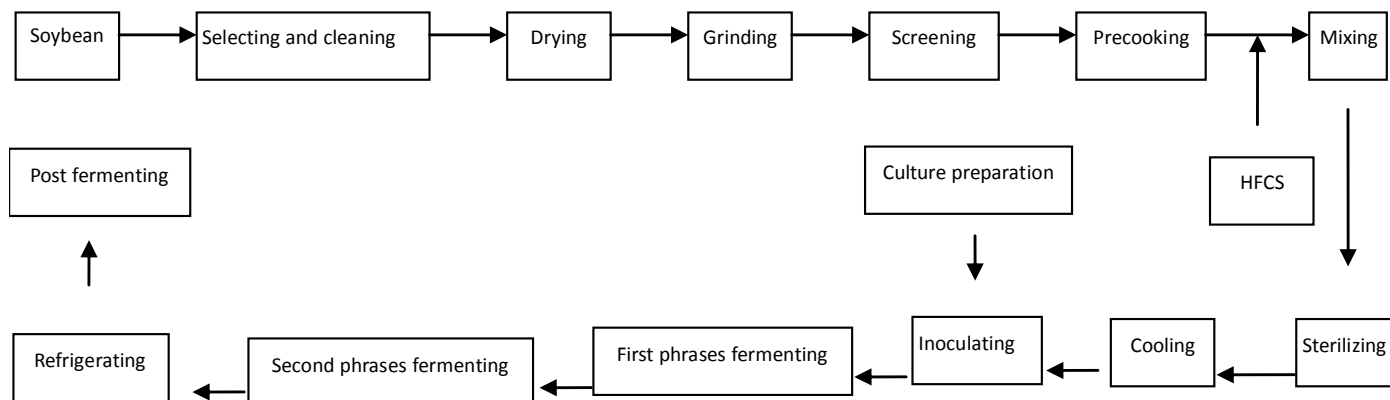
## INTRODUCTION

Using lactic bacterium in the bean product is not a recent idea. Sour mung bean milk, which is a traditional beverage in Peking (China) with hundred years of history, is yielded on the basis of the autofermentation of natural lactic bacterium in the mung bean milk (Ye, 2007). Sour mung bean milk is a pulpous state fluid food with a little obnoxious odor, which roots in the usage of mung bean as the material (Ding et al., 2010). The unpleasant odor limits the popularity of sour mung bean milk in China. However, the obnoxious odor of sour mung bean milk can be removed by exploiting the soybean as the raw material.

Soybean has been consumed by Chinese people for several thousand of years. It is a well recognized healthy food due to its high content of vegetable protein, which produces plenty of essential amino acids. Soybean products such as soybean milk are specifically suitable for individuals who have lactose intolerance. Recent research also revealed that the intake of soybean products can decrease an individual's risks of cardiopathy and hypercholesteremia. Soybean isoflavones were

generally recognized as the main active component of soybean influencing the decrease of cardiopathy and hypercholesteremia. In soybean, the majority of isoflavones exist in the form of glucosides, which compared with isoflavone aglycones, have lower bioactivity (Yamaki et al., 2002; Gao et al., 2007). The addition of lactic bacteria can hydrolyze glucosides into isoflavone aglycones and consequently facilitates the bioactivities of soybean isoflavones (Choil et al., 2002). The isoflavones are transformed due to the  $\beta$ -glucosidase produced by microorganism when the soybean milk is fermented by lactic acid bacteria and bifidobacteria (Chien et al., 2006; Wei et al., 2007).

The bifidobacteria, which is an important probiotic in intestinal tract (Hayakwa et al., 1990), can improve the gastrointestinal microflora, restrain the pathogenic bacteria and also boost the immune system of human body (Mitsuoka, 1990; Tsangalis et al., 2004). Bifidobacteria grow satisfactorily in soybean because of the presence of oligosaccharides. Incubating lactic bacteria and bifidobacteria in soybean milk can enhance



**Figure 1.** Technological process of soybean yoghurt.

the bioactivities and manufacture a yogurt-like functional food (soybean yoghurt) (Gueimonde and Delgado, 2004). In modern manufacturing, lactic bacterium and bifidobacterium are intentionally inoculated into the soybean milk to produce the soybean yoghurt. Meanwhile, one of the critical issues in the production of soybean yoghurt is the viable counts of probiotic bacterium.

Yang et al. (2011) reported that the viable counts of soybean yoghurt can reach  $10^6$  to  $10^9$  per milliliter, while they use isolated soy protein rather than soybean, and add some milk to promote the growth of probiotic bacterium. It is worth noting that there is limited amount of isoflavones in isolated soy protein. Hence, this study aimed at producing soybean yoghurt using soybean, increase the viable counts of bifidobacterium and lactic bacterium in the soybean yoghurt and optimize technological parameters for the fermentation of soybean milk.

## MATERIALS AND METHODS

The high quality soybean bought from the market was produced in Nengjiang county, Heilongjiang province. IsoClear™ high fructose corn syrup (HFCS) was a product of GBT-Cargill High Fructose (Shanghai) Co. Ltd. The soluble solid content of HFCS was 71%. YO-MIX™ Vegetal lactic bacteria and bifidobacteria were produced by Danisco Company (Suzhou, China). All culture mediums were of biochemical grade and other reagents were analytically pure.

### Culture preparation

The soybean was ground into powder after drying and soaked in water of which the volume is 20 times as the amount of soybean for 12 h. The soggy soybean powder was boiled for several minutes in order to dissolve the protein, and then the boiled mixture was filtered by the single layer cotton gauze to remove residuals and attain the soybean milk that is free of impurities. The 4% of HFCS of which amount was computed based on solid contents was mixed into the soybean milk. After sterilization and cooling, the mixture was inoculated by the lactic bacteria and bifidobacteria with the

amounts of 0.03 and 0.3%, respectively. The amounts of the bacterium were calculated based on the ratio of the dry powder of bacterium and the total amount of solids. The inoculated mixture was fermented at 37°C in anaerobic reactor until the curd was observed. The curd was the seed for the fermentation of soybean milk.

### Soybean yoghurt preparation

Figure 1 shows the technological process of soybean yoghurt production. The plump soybeans were selected and washed by tap water twice. The clean soybeans were placed into 70°C cabinet drier and dried until the hardness of them were suitable to ground. The soybean powder was obtained by a high speed disintegrator. Some soybean powder were soaked in 4 times tap water for 12 h and subsequently homogenized by the tissue homogenizer. The mixture of the homogenate and HFCS were diluted to a constant volume in a bioreactor. Hot water was pumped into the interlayer of the bioreactor to increase the temperature of bioreactor to 98°C, which should be maintained for 20 min for the purpose of sterilization. After sterilization, the temperature of mixture (soybean milk) was cooled to the fermentation temperature and the seed prepared previously was inoculated with the amount of 10%, and then the main fermentation was conducted. During the main fermentation, the soybean yogurt was extracted and examined every 20 min. The examinations included the pH test using digital acidometer and the sugar content using 3,5-dinitrosalicylic acid (DNS) method. After the main fermentation, the soybean yogurt was transferred into a 4°C refrigerator for the post fermentation. The viable counts of bacterium and bifidobacterium in the final product of soybean yogurt were measured by the methods suggested by the Chinese national standard GB/T 4789.35-2008 and GB/T 4789.34-2008.

The aim of this research was to optimize the parameters of the fermentation (the amounts of materials, the temperature-shift point and the process of fermentation), and to verify these parameters. The designs of four experiments are in line with the following logic. The first experiment measured the amounts of HFCS and soybean powder when the growth of bifidobacterium is maximized. The experiment that followed calculated the temperature shifted point and duration time for the fermentation of soybean milk, at which the growth curve of lactic bacterium reaches its peak. In the third experiment, the uniform design test was employed to optimize the process of the fermentation, in a bid to increase the viable counts of bifidobacterium. And finally, the last experiment verified and confirmed the parameters estimated by the three aforementioned

**Table 1.** Parallel experiment for HFCS amounts.

Serial	The addition of soybean powder (%)	The addition of HFCS amount to solids (%)
1	5	2
2	5	4
3	5	6
4	5	8
5	5	10

**Table 2.** Uniform design of parameters.

Section	First phrase fermentation temperature (°C)	Second phrase fermentation temperature (°C)	Duration time (h)
1	42	39	5
2	41	37	5.5
3	40	40	6
4	39	38	6.5
5	38	36	7

experiments.

#### Parallel experiment for the amounts of HFCS

According to the research of Ding et al. (2009), the texture of soybean yogurt is most palatable when the amount of dry soybean powder in soybean yogurt is 5%. Therefore, this study fixed the amount of soybean powder at 5% and HFCS ranging from 2 to 10% for the five tests (Table 1). Each test was fermented at constant temperature of 40°C for 5 h, and stored in a refrigerator at 4°C for 24 h subsequently. The viable counts of bacterium and bifidobacterium in each test were measured at the end of the process.

#### Determination of the temperature-shift point

The experiment of temperature-shift point was conducted following the technological process earlier suggested. Appropriate amounts of soybean powder and HFCS that were determined by the first parallel experiment were put into tap water. The mixture was inoculated by seed culture, and then, fermented at 40°C. It is worth mentioning that the choice of the temperature (40°C) was made considering the optimal temperature for the growth of lactic bacteria suggested by the lactic bacteria producer. The fermentation lasted 6 h and during this process, at every 20 min, the values of pH and sugar content of the mixture were measured. The data of pH and sugar content were analyzed by the Mathematic 7.01 software to estimate the point of temperature-shift.

#### Optimization of temperature-shift fermentation process

The lactic bacteria and bifidobacteria have different favorable fermentative temperatures (Gueimonde and Delgado, 2004). The fermentative temperature of the latter was lower than the former. Therefore, the main fermentation had two phrases, and the temperature was shifted across two phrases corresponding to the favorable fermentative temperatures of two bacteria. The fermentative temperatures of the first and second phrases and the fermentative duration were crucial to the fermentation. The

variations and combinations of these three parameters can form diverse conditions for the fermentation which consequently causes repeats of experiments.

The uniform design, which is a mathematic method proposed by Fang (1995) was used to reduce the amount of experiments. According to the uniform design, each parameter (fermentative temperatures of the first and second phrases and the fermentative duration) was assigned 5 different values, which can simulate  $5^3$  conditions for the fermentation. Then, the  $5^3$  conditions were reduced to 5 conditions referring to the  $U^5$  ( $5^3$ ) uniform design table suggested by software of Uniform Design Version 3.00 (Fang and Hickernell, 1995). The selected conditions (the value for each parameter in each condition) are presented in Table 2. For each selected condition, an experimental fermentation was conducted. The viable counts of bifidobacterium in the final products (soybean yogurt) of five experimental fermentations were measured. The viable counts of bifidobacterium were employed as the dependent variable, and three parameters were used as the independent variables. A four dimensional equation was formulated by Mathematic 7.01 software program, using the values of parameters shown in Table 2 and the values of viable counts of bifidobacterium attained from the experimental fermentations. The optimized value of each parameter can be estimated through the computation of the equation.

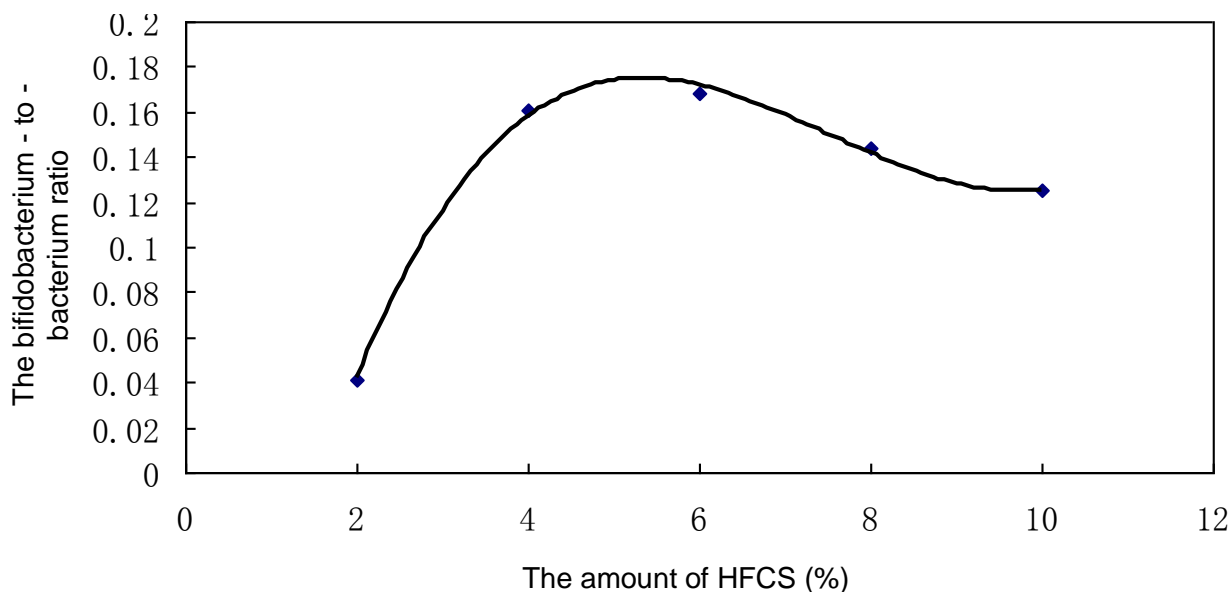
#### Verification test

The experimental fermentation was repeated five times with the optimal parameters estimated by the equation. The viable counts of bifidobacterium in the final products of the five fermentations were measured and compared with the values computed by the equation to verify the accuracy of the equation.

## RESULTS AND DISCUSSION

### The parallel test for the additions of materials

The amount of HFCS which is the main carbon source in the fermentation was determined by parallel tests. The total counts of bacterium and viable counts of



**Figure 2.** Changes in the bifidobacterium-to-bacterium ratio of soybean yoghurt with the amount of HFCS.

bifidobacterium in the final product were measured on the basis of the methods suggested by Chinese national standard methods GB/T 4789-2008. The fluctuation of ratio of viable counts of bifidobacterium in the total counts of bacterium with the variation of the amount of HFCS was described in Figure 2 in which the horizontal axis represents the amount of HFCS and the vertical axis represents the bifidobacterium-to-bacterium ratio.

Figure 2 shows that the total counts of bacteria increased corresponding to the growth of the amount of HFCS. The majority of bacteria were lactic bacteria rather than the bifidobacteria, because the bifidobacteria is insensitive to the amount of HFCS. The fermentation of bifidobacteria can utilize both the monosaccharine in HFCS, and the oligosaccharide in soybean. On the other hand, the YO-MIX™ Vegetal lactic bacteria fermented satisfactorily under the monosaccharine condition and could use the oligosaccharide. Therefore, the monosaccharine in HFCS facilitated the growth of YO-MIX™ Vegetal lactic bacteria, which metabolizes the nutrients that the bifidobacteria need. Consequently, the maximal population of bifidobacteria has a corresponding amount of HFCS. A third order equation was formulated to describe the fluctuations of microorganisms along with HFCS by Mathematic 7.01 software. The equation is (the fitting degree of regression= 0.9975):

$$/E1/ \quad B = 0.0012F^3 - 0.0272F^2 + 0.1876F - 0.234$$

Where, F is the amount of HFCS and B is the ratio of viable counts of bifidobacterium in the total counts of bacterium. The maximal ratio of bifidobacterium-to-bacterium can be calculated through derivation and was equal to 17.5%. The corresponding amount of HFCS was

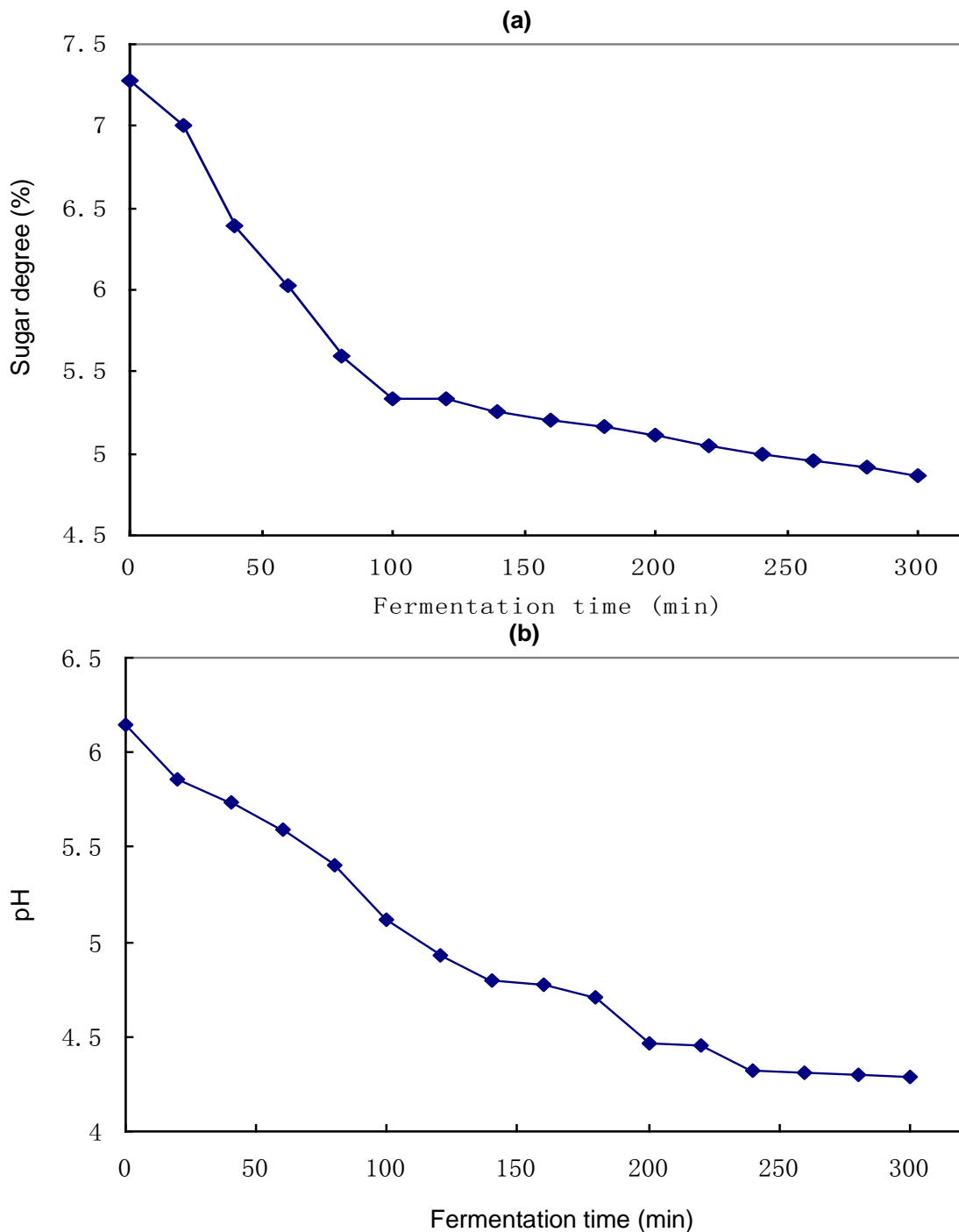
5.32%.

#### Determination of the temperature-shift point

In the fermentative experiment, the additions of HFCS and soybean powder were 5.32 and 5% respectively. The temperature for the fermentation was 40°C. The set of the temperature was determined based on the consideration of the condition that can benefit the fermentation of lactic bacteria. The pH and sugar degree of the fermentation liquor fluctuated with time are shown in Figure 3. As shown in the Figure, the pH and sugar degree decreased rapidly in initial several hours. This decrease was caused by two reasons: First, the seed culture was in the log phase at the initial couple of hours. Secondly, in this period, the condition was suitable for the proliferation of lactic bacteria, which caused the rapid increase of lactic acid and decrease of the pH value. Three more hours later, the decline of sugar content slowed down, while the decline of pH maintained the same speed. A fourth order equation was formulated to simulate the relationship between the sugar degree (C) and the fermentation duration (t). The equation was:

$$/E2/ \quad C = 7.40142 - 0.0316485t + 0.000149622t^2 - 2.56289 \times 10^{-7}t^3 + 4.61264 \times 10^{-11}t^4$$

The degree of equation fit was 0.9998, and the F value of this equation was 326.8, which indicated a satisfactory correlation between the sugar degree and fermentation duration. When  $d^2C/dt^2=0$ , the numerical value of t was 210. That means the rate of decline of sugar degree reached its inflection point at 210 min after starting the



**Figure 3.** Changes in sugar degree (a) and pH (b) of soybean yoghurt at 40°C over 300-min fermentation period.

fermentation, and the growth of lactic bacteria evolved into the stabilized phase. At this key moment (210 min after starting the fermentation) the sugar degree was continually decline, but was not caused by the proliferation of lactic bacteria. This suggested that 210 min after starting the fermentation was the right time to change the fermentation condition to benefit the growth of

bifidobacteria.

Similarly, a fourth order equation was formulated to simulate the relationship between the pH value (pH) and the fermentation duration (t). The proposed equation was:

$$\text{pH} = 6.12855 - 0.0100983t - 1.26295 \times 10^{-6} t^2 + 7.22725 \times 10^{-8} t^3 - 8.06244 \times 10^{-11} t^4$$

The degree of fit of the equation was 0.9999. The F-value of the F-test was 630.5, which was a statistical significant. The second order derivation demonstrated that the pH reached its inflexion point 442 min after starting the fermentation. At the moment of 442 min, the decline of pH value was terminated. In other words, at this moment, the lactic acid inhibited the metabolism of microorganism. Therefore, 442 min after starting the fermentation is the time to discontinue the fermentation. It is worth noting that the 442 min is the maximal duration of the fermentation rather than the time point when the quantity of bifidobacterium was largest since the metabolites of lactic bacteria, such as the lactic acid and antimicrobial peptides produced in the stationary phase might affect the growth of bifidobacteria (Chick et al., 2001).

### Optimization of temperature-shift process of fermentation

The uniform design tests were conducted to simulate the five conditions for fermentation. The values of three parameters varied in each condition as shown in Tables 2 and 3. The values obtained indicated viable counts of bifidobacteria in each condition. A quadratic multinomial stepwise regression analysis was also conducted using software package DPS 8.0 to establish the equation that can formulate the relationship between the viable counts of bifidobacteria and the three parameters (namely: the temperature of first phrase fermentation, the temperature of second phrase fermentation, and the duration time) of fermentation. The regression equation was:

$$V(T_1, T_2, t) = 5.92857 \times 10^6 + 35020.4 T_1 + 1959.74 T_1^2 + 138927 T_2 + 3719.09 T_1 T_2 - 1844.8 T_2^2 - 3.91309 \times 10^6 t - 25887 T_1 t - 31565.2 T_2 t + 512746 t^2$$

Where,  $T_1$  is the temperature of the first phrase fermentation;  $T_2$  is the temperature of the second phrase fermentation;  $t$  is the time duration;  $V$  is the viable counts of bifidobacterium in the final product.

The simulated results of the regression equation matched the experimental results satisfactorily. The derivation of this equation (within the experiment interval) showed that when  $V$  reached its maximum (the predictive value of  $V$  was  $1.22809 \times 10^6$  CFU/ml), the temperature of the first phrase fermentation ( $T_1$ ) was  $42^\circ\text{C}$ , the temperature of the second phrase ( $T_2$ ) was  $37.2^\circ\text{C}$ , and the duration time ( $t$ ) was 5 h. The derivation of  $t$  in the regression equation also indicated that the viable counts of bifidobacterium shifted from increase to decrease, as the fermentation was beyond a certain range. If the fermentation lasted a long duration, some metabolites yielded by the lactic bacteria, such as lactic acid and antibacterial peptide, accumulated and inhibited the growth of bifidobacteria in stabilization period. Hence, the duration of fermentation should avoid being prolonged.

### Verification test

The experimental fermentations were conducted 5 times with the optimal parameters (5% soybean powder, 5.32% HFCS, the first phrase fermentation temperature  $42^\circ\text{C}$ , the second phrase fermentation temperature  $37.2^\circ\text{C}$ , temperature shifted point 3.5 h and the duration time 5 h). The average of five viable counts of bifidobacteria in the final products produced by five experimental fermentations was  $1.1 \times 10^6$  CFU/ml, which deviated by 8% from the value estimated by the regression equation; the relative standard deviation was 3.9%. The total counts of bacteria were  $7.3 \times 10^6$  CFU/ml, which was double than the population of bacteria when the fermentation temperature was fixed at  $40^\circ\text{C}$ .

### Conclusion

The formula developed by this study contained 5% soybean powder and 5.32% HFCS, and the percentages were calculated based on ratios of the materials to the total amount of solid content. After inoculating 10% of the composed seeds prepared in advance, the raw materials in the formula were fermented at  $42^\circ\text{C}$  for 3.5 h initially, at  $37.2^\circ\text{C}$  for 1.5 h subsequently, and then preserved at  $4^\circ\text{C}$  refrigerator for 24 h. In the final product, the amount of living bifidobacteria was remarkable, and the total counts of living bacteria were double than the living bacteria in the soybean yogurt produced with same formula, but at a constant temperature of  $40^\circ\text{C}$ .

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