Journal of Animal and Veterinary Advances 10 (8): 993-998, 2011
ISSN: 1680-5593

Optimization of Synbiotics and Growth Factors for Calcium Conversion of *Lactobacillus rhamnosus* Based on Response Surface Methodology

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Abstract: To optimize prebiotics and growth factors for maximizing the calcium conversion of a probiotic, *Lactobacillus rhamnosus* during the fermentation of boving bone-meal and to assess the effects of these factors by using response surface methodology. After single factor experiment determined the addition of prebiotics and growth factors, conversion rate characteristic of calcium as indicators. Response Surface Methodology (RSM) was used to optimize the best for enhancing the prebiotics and growth factors of *Lactobacillus rhamnosus* for calcium conversion. It was found that prebiotics were the most effective for calcium conversion, amino acids second, vitamin minimum the last. Analyzing the response surface contour and surface plots, the optimal combination of the prebiotics and growth factors were determined as nicotinic acid/vitamin B_2 = 2.82:1, arginine/met = 1.82:1, glucose/isomaltooligosaccharide = 3.80:1. The best conversion rate of calcium was 28.32%.

Key words: *Lactobacillus rhamnosus*, probiotic, response surface methodology, synbiotics, calcium formutions, China

INTRODUCTION

Synbiotics, a combination of probiotics and prebiotics can induce additional effects (Katharina *et al*., 2007). A positive outcome of prebiotics is promoted by a high level for calcium (Rachmilewitz *et al*., 2004). A synbiotic is a product containing prebiotics and probiotics and in which the prebiotic compound selectively favors the probiotic compound (Schrezenmeir and Vrese, 2001). *Lactobacillus rhamnosus*, originally isolated from human intestinal tract has been proven as an important probiotics functioned in the human body (Zhang *et al*., 2000).

L. rhamnosus have complex nutritional requirements and are strictly fermentative. They were regarded with the capacity of intestinal cell lines, host immunostimulation (Kuisma *et al*., 2003). *L. rhamnosus* are potential L-lactate due to their homofermenting capacity (Begovic *et al*., 2009).

They lacks an enzyme to self-synthesize vitamins and amino acids (Fengjie *et al*., 2002). Therefore, productivity could be significantly influenced by growth nutrients, especially synbiotics and growth factor.

The study was undertaken bovine bone-meal as fermentation substrate used the technologies of microbial fermentation combined with optimization of important prebiotics and growth factors for *Lactobacillus rhamnosus* are needed to promote calcium transformation and to determine the optimal production of *L. rhamnosus* by response surface methodology (Azaola *et al*.,1999).

MATERIALS AND METHODS

Bovine bone was obtained from supermaker in Gui Yang. All other chemicals were of reagent grade.

Strains and culture conditions: *Lactobacillus rhamnosus* 1.2466 was provided by the Institute of Microbiology, Chinese Academy of Science. Strain of *L . rhamnosus* was activated in deMan-Rogosa-Sharpe (MRS) broth at 37±1°C for 24 h determined as viablecount after two generation microecological additives could reach up to 10^6 cfu mL^{-1} then inoculated with 3% (w/v) to the formula of fermentation: bovine bone meal 15%, glucose 5%, skim milk 10%, MRS culture medium 2%, distilled water 68% medium pH 6.2 at 37°C 100 r min^{-1} for 24 h microecological additives could reach up to 10^6 cfu mL^{-1} then inoculated to the optimum formula of the expanded fermentation: bovine bone meal 4.45%, glucose 12.26%, growth factor, distilled water, pH 6 .2 (Yin *et al*., 2009a, b).

Single factor experiment: *L. rhamnosus* have complex nutritional requirements and are strictly fermentative based on MRS broth, addtioned different kinds of prebiotics and growth factors, the controlled trials were as follows:

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- Control group: MRS medium
- Trial χ: (vitamin trials)
- Trial¯: MRS medium+0.02% vitamin B_1
- Trialα: MRS medium+0.02% vitamin B_2
- Trialβ: MRS medium+0.06% vitamin B_6
- Trialχ: MRS medium+0.06% vitamin PP
- Trialδ-ϕ: (Amino acid trials)
- Trialδ: MRS medium+0.5% arginine
- Trialε: MRS medium+0.5% methionine
- Trialϕ: MRS medium+1.0% cysteine
- Trialγ-φ: Prebiotics trials (Wei *et al*., 2007)
- Trialχ: MRS medium+2.0% inulin
- Trialη: MRS medium+2.0% isomaltooligosaccharide
- Trialι: MRS medium+2.0% fructo-oligosaccharides
- Trialφ: MRS medium+2.0% chitosan

An inoculum size of 3% (v/v) *L. rhamnosus* to MRS medium, fermented for 24 h determined as colony forming units as indicator selectived the addtion of prebiotics and growth factors.

Response surface methodology: Optimization the selected prebiotics and growth factors for *L. rhamnosus* fermenting of boving bone-meal by using response surface methodology of 3 factors 3-levels in the same time, calcium as indicators.

Statistical analyses: For statistics purpose, part of the experiments were repeated at least three times. All the data were analyzed with the aid of the statistics program Design expert 7.0.

RESULTS AND DISCUSSION

The experiments were carried out according to the experimental plan understand the influence of prebiotics and growth factors on *L. rhamnosus* shown in Table 1. It was evident that the viablecount of trial α, trial χ and trial ε were obtained at 1.2×10^9, 6.0×10^9, 3.3×10^9 cfu mL^{-1} after fermentation. The trials were significantly different ($p<0.05$) between control group that vitamin B_2, vitamin PP and methionine promoted in growth for LGG. The groups on β̄ , ϕ and φ were significantly lower than control group and significantly different ($p<0.05$).

It was evident that the vitamin B_1, vitamin B_6, cysteine and chitosan inhibiting the growth for LGG. For this study, vitamin B_1, vitamin B_6, cysteine and chitosan was not selected. The results from the 1st experiment on prebiotics about the affect of calcium conversation are shown in Fig. 1. It can be concluded that the addition of 2.0~8.0% for isomaltooligosaccharide and Fructo-oligosaccharides may benefit the conversation of calcium. When treated at 8%, the conversastion reach to maximum. With an increase in go down. Similarly with the addition

Table 1: The influence of prebiotics and growth factors for LGG on the growth

Trial group	Control group	¯	α	β	χ	δ
LGG	0.6 ± 0.05^{c}	0.2 ± 0.13^{a}	1.2 ± 0.20^{d}	0.4 ± 0.03^{b}	0.6 ± 0.03^{c}	0.8 ± 0.04^{bc}
Trial group	ε	ϕ	γ	η	ι	φ
LGG	3.3 ± 0.01^{e}	0.2 ± 0.01^{ab}	0.7 ± 0.02^{cd}	0.9 ± 0.05^{bc}	0.7 ± 0.03^{cd}	0.1 ± 0.12^{a}

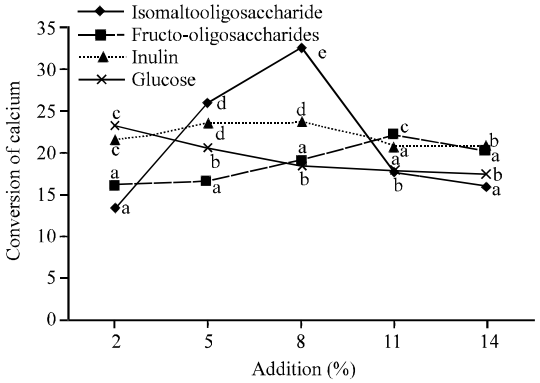

Fig. 1: Effects of different prebiotics LGG fermentation of cattle bone meal

of 2.0~11.0% for inulin, the conversation of calcium raising and the maximum for 11% with an increase in the conversation go down (Galdeano and Perdigon, 2004). This results in prebiotics of isomaltooligosaccharide, Fructo-oligosaccharides and inulin with fit concentration was promotion for *L. rhamnosus*, excessive addition could control the growth.

Increasing addition of glucose, the conversation of calcium reduced (Lee and Puong, 2002). Therefore, 8% appeared to isomaltooligosaccharide and Fructo-oligosaccharides be the optimal addition, inulin were suitable for 11%, glucose 2%. Selection of the compound of isomaltooligosaccharide abd glucose for the next step was caused by the conversation of isomaltooligo-saccharide significant higher than control. Therefore, researchers tested the conversation of calcium of standards (0, 20, 40, 60, 80 and 100%) for glucose: (glucose + isomaltooligosaccharide) wih the total addition for sugars on 11%.

The single factors experimentations shown in Fig. 1. In the range of 0-80% was detected that the conversation of calcium presented an increasing tendency with adding glucose. The highest conversationg of 36.65% was obtained at the addition of 80% for glucosein the range of 80-100%, the conversation went down quickly. Remarkably, conversation with compoud of glucose and isomaltooligosaccharide at the addition of 80% higher than other groups. The highest conversationg of glucose/ isomaltooligosaccharide = 4:1 (Fig. 2). Vitamin group was selected for vitamin PP and vitamin B_2 and the content was 0.08% of total addition. Fermentation of zymotic fluid

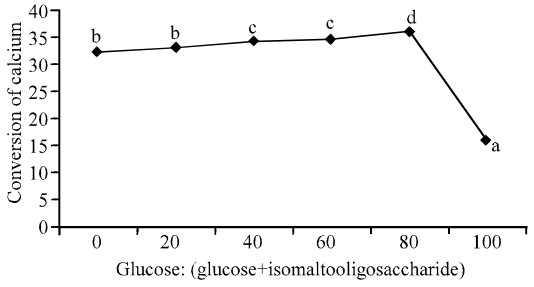

Fig. 2: LGG fermented prebiotics on the impact of cattle bone meal

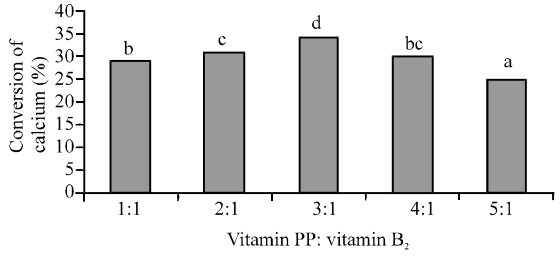

Fig. 3: Vitamin LGG fermented cattle bone meal on the effect on fermented cattle bone meal

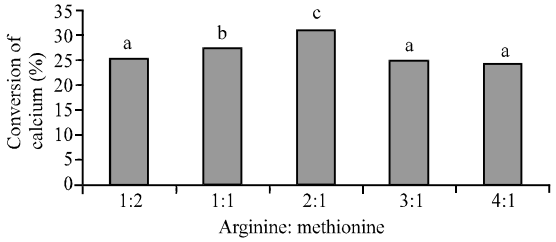

Fig. 4: Amino acids LGG fermented cattle bone meal on the effects

with an concentration boving of 3% (w/v) for 72 h was conducted at 5-level with 1:1, 2:1, 3:1, 4:1 and 5:1 of vitamin PP/vitamin B_2 (Fig. 3) (Wei *et al*., 2002). As shown in Fig. 3, the conversation of calcium reached the highest of 33.94% at vitamin PP/ vitamin B_2 = 3:1. The performance of amino acid was selected for arginine and methionine with the proportion at 5 level with 1:2, 1:1, 2:1, 3:1 and 4:1 of arginine/methionine based on the content 1.0% of total addition. Conversion rate characteristic of calcium as indicators of arginine/ methionine as shown in Fig. 4, the conversation of calcium reached the highest of 30.41% at arginine/methionine = 2:1 and significantly different ($p<0.05$).

Optimization using RSM: RSM was used to analyze the experimental data. Optimization, the selected prebiotics and growth factors for *L. rhamnosus*, fermenting of

Table 2: LGG factors and the level of response surface analysis table

		Levels		
Factors	No.	-1	0	+1
vitamin PP: vitamin B_2	X_1	2:1	3:1	4:1
Argnine: methionine	X_2	1:1	2:1	3:1
Glucose: Isomaltooligosaccharide	X_3	3:1	4:1	5:1

Table 3: LGG response surface results

	Levels			Conversion rate of calcium (%)	
Trial no.	X_1 (A)	X_2 (B)	X_3 (C)	Observed	Predicted
1	-1	-1	-1	24.4475	24.25016
2	1	-1	-1	25.8126	25.85770
3	-1	1	-1	26.9687	26.53486
4	1	1	-1	26.2174	26.66345
5	-1	-1	1	30.4810	29.92451
6	1	-1	1	28.7606	29.08400
7	-1	1	1	28.2183	28.06276
8	1	1	1	25.6564	25.74330
9	-1	0	0	28.0126	29.35582
10	1	0	0	29.9013	28.99986
11	0	-1	0	28.0247	28.41004
12	0	1	0	27.8256	27.88204
13	0	0	-1	28.8420	28.98204
14	0	0	1	31.0574	31.35914
15	0	0	0	30.2940	30.23969
16	0	0	0	30.5558	30.23969
17	0	0	0	30.7989	30.23969
18	0	0	0	30.6130	30.23969
19	0	0	0	29.3480	30.23969
20	0	0	0	30.7120	30.23969

boving bone-meal by using response surface methodology of 3 factors 3-levels in the same time calcium as indicators. An inoculum size of 3% (v/v), fermented for 24 h concentration of boving bone-meal with 5%. The objective of the present study was to find the optimum combination of vitaminPP/B_2 and argnine/methionine and glucose/isomaltooligosaccharide to maximize the cell viability of *L. rhamonosus* for calcium conversion. The experiments were carried out according to the experimental plan shown in Table 2 and 3. The experimental and predicted responses for calcium conversion are shown in Table 3.

Researchers attempted to fit the response variable to a quadratic model in order to correlate the response variable to the independent variables. The behavior of the system was explained by the following quadratic polynomial equation:

$$Y = 30.24-0.18X_1-0.26X_2+1.19X_3-1.06X_{12}-2.09X_{22}-0.069X_{32}-0.37X_1X_2-0.61X_1X_3-1.04X_2X_3$$

The optimum levels of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour and surface plots. The statistical significance of the model was determined by F test and the analysis of variance for the fitted

Table 4: Variance of RSD experiments

Source	Sum of squares	df	Mean square	F value	Prob>F	Significance
Model	70.052360	9	7.783596	14.613040	0.0001	**
X_1	0.316769	1	0.316769	0.594706	0.4584	
X_2	0.696960	1	0.696960	1.308483	0.2793	
X_3	14.126510	1	14.126510	26.521320	0.0004	**
X_{12}	3.100695	1	3.100695	5.821290	0.0365	*
X_{22}	12.054270	1	12.054270	22.630860	0.0008	**
X_{32}	0.013131	1	0.013131	0.024652	0.8784	**
X_1X_2	1.093647	1	1.093647	2.053228	0.1824	
X_1X_3	2.996474	1	2.996474	5.625625	0.0392	*
X_2X_3	8.596524	1	8.596524	16.139240	0.0024	**
Residule	5.326474	10	0.532647	-	-	-
Lack of fit	3.883448	5	0.776690	2.691183	0.1506	-
Pure error	1.443026	5	0.288605	-	-	-
Cor total	75.378830	19	-	-	-	-

quadratic polynomial model is shown in Table 4. The value of P_{model}>F was <0.0001 indicating that the model was significant at the probability level of a = 0.01. However, the lack of fit was observed to be insignificant (Plack of fit>F = 0.1506) implying that the obtained model was adequate to represent the experimental data.

A high value of the correlation coefficient (R = 0.9293) indicating that 92.93% of the variability in the response could be explained by the secondorder polynomial prediction equation given below indicated a good agreement between the experimental and predicted values of cell viability thus suggesting a high significance for the model. The significance of each coefficient of the model was determined by F test, this meant that X_3, X_{22}, X_{32}, X_2X_3 were highly significant ($p<0.001$) and the X_{12}, X_1X_3 were significant ($0.01<p<0.05$). X_1, X_2, X_1X_2 were insignificant($p>0.05$) that every factors may be the highly significant.

The factors on respond value was $X_3>X_2>X_1$ indicating glucose/isomaltooligosaccharide may be the most important protective agent influencing the resistance of *L. rhamonosus* to calcium conversion, argnine/ methionine as follow vitamin PP/B_2 was the lowest.

The isoresponse contour and surface plots of RSM as a function of two factors at a time holding all other factors at fixed level (zero for instance) are helpful for understanding both the main and the interaction effects of these two factors (Liu *et al*., 2003).

The response values for the variables can be predicted from these plots. Figure 5-7 represent the isoresponse contour and surface plots for conversation of calcium (%) of *L. rhamnosus*.

The effect of varying concentration of amino acid and vitamin on the conversation of calcium of *L. rhamnosus* while other two viariables were fixed at central concentration is shown in Table 4. So, the interaction between the amion acid and vitamin was insignificant.

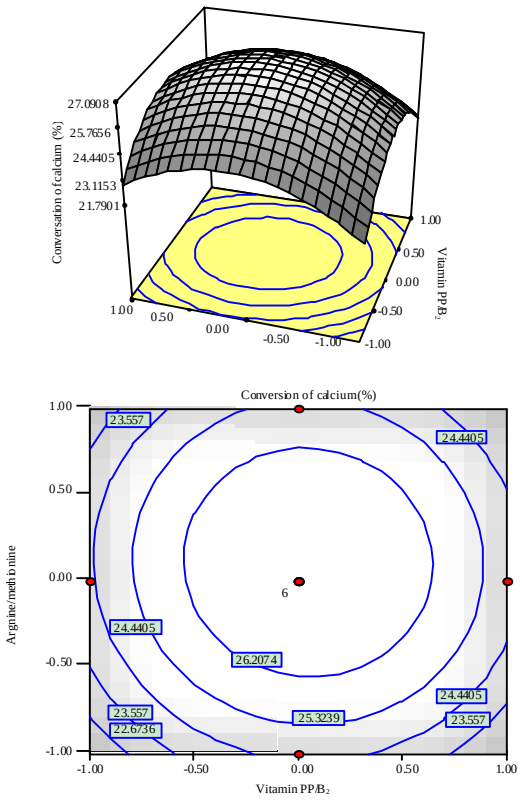

Fig. 5: Effects of conversion rate of calcium on interaction of vitamin and amino acid

Holding amino acid factors at zero level, researchers can know interaction effects on witamin and prebiotica for the conversation of calcium polynomial equation:

$$Y = 33.896-2.62X_1-4.42X_2-1.06X_{12}-0.37X_1X_2-2.09X_{22}$$

The effect of varying concentration of prebiotics and vitamin on the conversation of calcium of *L. rhamnosus* is shown in Table 4.

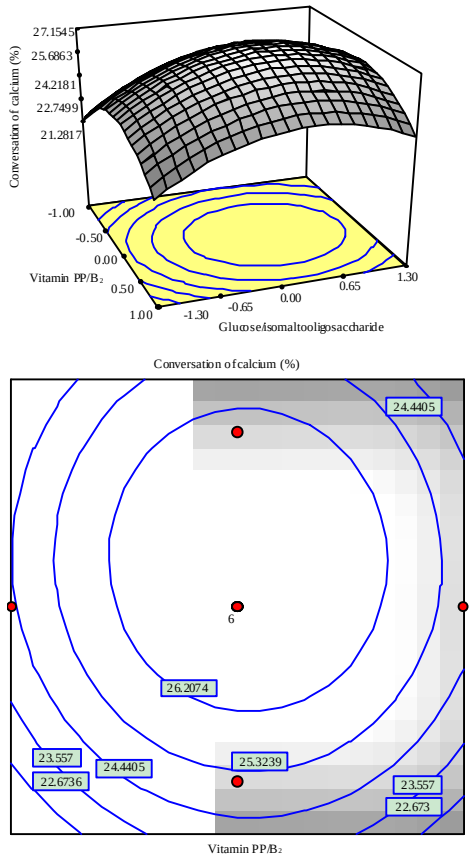

Fig. 6: Effects of conversion rate of calcium on the interaction of vitamins and prebiotics

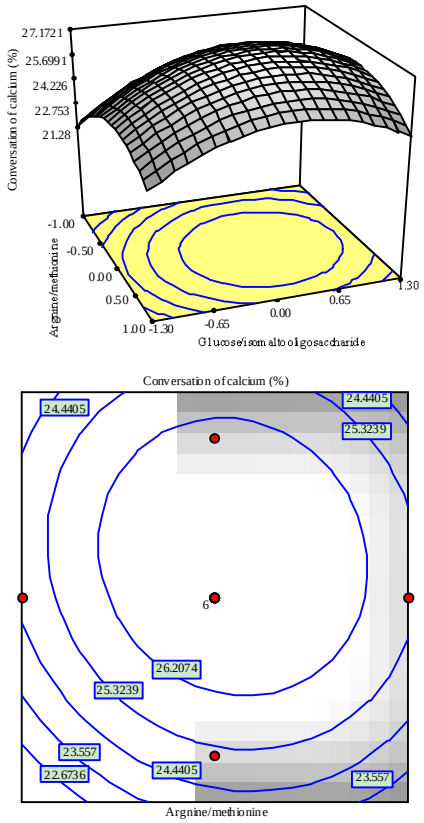

Fig. 7: Effects of conversion rate of calcium on the interaction of amino acids and glycogen

So, the interaction between the prebiotics and vitamin was significant. Holding vitimin factors at zero level, researchers can know interaction effects on vitamin and prebiotica for the conversation of calcium polynomial equation:

$$Y = 21.36-1.4X_1-0.89X_3-1.06X_{12}-0.069X_{32}-0.61X_1X_3$$

The effect of varying concentration of prebiotics and amino acid on the conversation of calcium of *L. rhamnosus* is shown in Table 4. So, the interaction between the prebiotics and amino acid was significant. Holding vitimin factors at zero level, researchers can know interaction effects on amino acid and prebiotica for the conversation of calcium polynomial equation:

$$Y = 20.16-1.37X_2-0.64X_3-2.09X_{22}-0.069X_{32}-1.04X_2X_3$$

From equations derived by differentiating equations, the optimum values for the independent variables investigated were nicotinic acid/vitamin B_2 = 2.82:1, arginine/met = 1.82:1, glucose/isomaltooligosaccharide = 3.80:1. The best conversion rate of calcium was 28.32%. To confirm the results, conversation of calcium in this optimum protective medium and a viability of 96.05% (theory conversation of calcium was 27.55%). The good correlation between these results verified the goodness of fit of the model.

CONCLUSION

Analyzing the response surface contour and surface plots, prebiotics were the most effective for calcium conversion, amino acids second and vitamin minimum the last. The optimal combination of the prebiotics and growth factors were determined as nicotinic acid: vitamin B_2 = 2.82:1, arginine: met = 1.82:1, glucose: isomaltooligosaccharide = 3.80:1. The best conversion rate of calcium was 28.32%.

ACKNOWLEDGEMENTS

This research was financially supported by the Support Project of Science and Technology of Guizhou

province of the People's Republic of China (GZNY NO.: [2007] 3021) and Guizhou technical system of modern beef industry (GZCYTX-0301-03).

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