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Original paper

Optimization of Small Peptide Feed from Milk Thistle Residue by Synergistic Fermentation of Multiple Strains and Proteases

KANGTAO CAI[1], HUAYOU CHEN[1,2], XINYU HENG[1], LINGYU KANG[1], JUNMING WU[3], CHENXI LU[1], XIAOYU LIANG[1]

[1] College of Life Science, Jiangsu University, Zhenjiang, Jiangsu, 212013, China
[2] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, 100000, China
[3] Jiangsu Zhongxing Pharmaceutical Co., Ltd. Zhenjiang, Jiangsu, 212000, China

Abstract In order to improve the utilization rate of the milk thistle residue, this study used the synergistic fermentation of multiple strains and proteases to increase the small peptide content of the fermented feed produced by the milk thistle residue. Taking the small peptide content of the milk thistle residue fermented feed as an indicator, the optimal fermentation process was obtained by single-factor optimization experiments and the response surface methodology. The optimal fermentation process was as follows: fermentation time of 7 days, inoculum size of 15%, inoculation ratio of aerobic strains: anaerobic strains = 1: 2, solid-state fermentation water content of 66%, fermentation temperature of 36°C, and amount of protease was 0.25% acid protease+0.25% bromelain. Under the above process, the small peptide content of the fermented feed from milk thistle residue was greatly improved to 57.86%. These results inferred that the added proteases were beneficial to the growth of fermentative microorganisms, the secretion of protease and the increase of the small peptide content.

Keywords Milk thistle residue, protease activity, response surface methodology, small peptides, synergistic fermentation of multiple strains and proteases

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✉ *Corresponding author: KANGTAO CAI, College of Life Science, Jiangsu University, Zhenjiang, Jiangsu, 212013, China.
E-mail: hyc@ujs.edu.cn

Introduction

Milk thistle*(Silybum marianum (L.) Gaertn.*) residue after silymarin extracting is rich in protein, sugars and other substances (Hadolin et al., 2001; Li et al., 2013). Milk thistle residue has only a small peptide content of about 6% and is directly used for animal feeding, with poor palatability, low nutrient absorption rate(Li et al., 2011), which is often discarded as waste resulting in environmental pollution.

A peptide is defined as an organic molecule composed of two or more amino acid residues linked by peptide bonds (Wu, 2013). After proteins are hydrolyzed into small peptides, they can be absorbed by the gastrointestinal tract and enter the blood circulation in the form of dipeptides, tripeptides, etc.(Miriam et al., 2017). Absorption in the form of small peptides saves digestive energy and absorptive energy, and makes feed more nutritious(Mellor, 2000). In addition, small peptides also have biological functions such as anti-bacterial, anti-oxidation, anti-hypertensive, and improving the immune regulation ability of the body(López-Barrios et al., 2014; Ryder et al., 2016; Su et al., 2019).

In conventional feeds, due to the low small peptide content, the absorption rate of feed is very low in the digestive tract, which is easy to cause dyspepsia, diarrhea and other symptoms(Hou et al., 2017). The increase of the small peptide content in the fermented feed is conducive to improving the nutrient absorption, promoting the growth of feeding animals, enhancing the immunity of feeding animals, and reducing the use of antibiotics.

At present, methods for hydrolyzing proteins into small peptides include chemical hydrolysis, microbial fermentation (Smid and Lacroix, 2013), protease hydrolysis(Wang et al., 2014) and so on. The chemical hydrolysis is low in cost, but it will change the products of protein hydrolysis. For example, acid hydrolysis of the protein will lead to complete destruction of tryptophan and partial loss of methionine (Pasupuleki and Braun, 2010). Proteases secreted by microbial fermentation can hydrolyze proteins into small peptides (Sharma et al., 2017). But the ability of microorganisms to produce protease is limited, which greatly reduces the production efficiency (Dieterich et al., 2014). Protease hydrolysis does not cause any loss of amino acids (Miriam et al., 2017), but its production costs is very high, and the enzymatic hydrolysate of some proteins will form bitter substances(Kim et al., 2003). Therefore, in large-scale production and processing, a fermentation process with simple production process, high economic effectiveness, obvious increase of the small peptide content, safety and high efficiency is needed.

In the experiment, with the synergistic fermentation of multiple strains and proteases, the optimal fermentation process was obtained by single-factor optimization experiments and the response surface methodology, and the small peptide content was greatly improved. The added proteases were conducive to the growth of microorganisms and the secretion of protease, and promoted the fermentation.

Materials and Methods

Materials and reagents

The milk thistle was purchased from Zhenjiang Zhongxing Pharmaceutical Co., Ltd.. *Candida tropicalis* (CGMCC 2.1013), *Lactobacillus acidophilus* (CGMCC 1.1854) and *Lactobacillus casei*(CGMCC 1.8727) were purchased from the China General Microbiological Culture Collection Center, CGMCC; *Bacillus natto* and *Lactobacillus rhamnosus* were screened and preserved by our laboratory. All activities of added protease were 60000u.g^{-1}.

Solid-state fermentation process

Firstly, 150g milk thistle residue was mixed with 50g bean dregs. Secondly, aerobic strains (*Bacillus natto and Candida tropicalis*) and water were added. The mixture was stirred evenly and fermented under the aerobic condition at 30℃ for 12h. Then, anaerobic strains (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*), proteases and water were added. The mixture was stirred evenly and was put into a fermentation bag containing a one-way vent. Finally, The fermentation bag was sealed and the fermentation was conducted under the anaerobic condition at 30℃ in a constant temperature incubator.

Single-factor optimization experiments

Other factors remained unchanged, and different fermentation time(0, 1, 3, 5, 7, 9, 11, 13, 15 days), inoculum size(4%, 8%, 12%, 16%, 20%, 24%), inoculation ratio (aerobic strains: anaerobic strains= 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4), solid-state fermentation water content (30%, 40%, 50%, 60%, 70%, 80%) and temperature (20, 24, 28, 32, 36, 40℃) were selected for experiments. Three parallels were made for each factor and the results were averaged.

Selection of the types and amount of proteases

Based on the above experiments, 1% papain, 1% acid protease, 1% bromelain, 0.5% acid protease+0.5% bromelain, 0.5% acid protease+0.5% papain and 0.5% papain+0.5% bromelain were selected to be added respectively. The different amounts of proteases selected were 0%+0%, 0.025%+0.025%, 0.05%+0.05%, 0.25%+0.25%, 0.45%+0.45%, 0.65%+0.65%, 0.85%+0.85%, 1.05%+1.05%, 1.25%+1.25%.

Response surface methodology

The inoculum size, fermentation temperature and solid-state fermentation water content were selected as the factors to investigate, and the small peptide content was taken as the index. Box-Behnken Design (BBD) of Design Expert 8.0.6 software was used to optimize the experiment design. Experimental factors and levels are shown in Table 1.

Table1. Optimization of fermentation process factors and levels by response surface methodology

Level	A inoculum size %	B fermentation temperature ℃	C solid-state fermentation water content %
1	12	32	40
0	16	36	60
-1	20	40	80

Determination of the small peptide content

The determination of small peptide content (including small peptide, free amino acid, etc.) referred to the Chinese national standard GB/T 22492-2008.

Molecular weight distribution of feed

The methods of extracting protein from fermented feed referred to Tang (2007). SDS-PAGE referred to Li et al.(2013). 1g dried sample was mixed with 30 mL 0.03 mol L^{-1} Tris-HCl solution, soaked for 1 hour, centrifuged at 16520g for 10min. The supernatant was identified by SDS-PAGE.

Determination of number of fermentative microorganisms, protease activity, and pH

According to the optimized fermentation process, feeds with and without protease were prepared. Number of fermentative microorganisms was determined according to ISO 4833:2003. Acid, neutral, alkaline proteases and pH were determined according to the method reported by Elfalleh et al. (2017).

Results and discussion

Effect of the fermentation time

As shown in Figure 1(a), the small peptide content increased gradually with the increase of fermentation time. From the first day to the seventh day, fermentative microorganisms multiplied and secreted a large amount of proteases, which led to a rapid increase in the small peptide content. After the seventh day, nutrients decreased and the growth of fermentative microorganisms slowed down, resulting in a slow increase in the small peptide content. Considering that the fermentation time in actual production cannot be increased indefinitely, the optimal fermentation time was 7 days.

Effect of the inoculum size

As shown in Figure 1(b), when the inoculum size was 16%, the small peptide content was the highest. When the inoculum size was low, the initial proteases secreted by microorganisms was less and the protein degradation was slow(Hou et al., 2017). When the inoculum size was high, nutrients were rapidly consumed by microorganisms in the early stage of fermentation, which made the metabolism of microorganisms be inhibited without secreting a large amount of proteases(Shu et al., 2015). Therefore, the optimal inoculum size was 16%.

Effect of the inoculation ratio

As shown in Figure 1(c), when the inoculation ratio was aerobic strains: anaerobic strains=1:2, the small peptide content was the highest. Anaerobic strains had a great influence on the protein degradation into small peptides. However, when the amount of anaerobic strains was too high, lactic acid bacteria secreted a lot of organic acid, which inhibited the metabolism of fermentative microorganisms and affected protein degradation(Yang et al., 2018). Therefore, the optimal inoculation ratio was aerobic strains: anaerobic strains=1:2.

Effect of the solid-state fermentation water content

As shown in Figure 1(d), with the increase of water content, the small peptide content increased gradually. An increase in water content would promote the dissolution of nutrients in water, which accelerated the speed of nutrient transfer and facilitated the metabolism of microbial proteases, so the small peptide content increased rapidly (Sharma et al., 2017). When the water content was over 60%, excessive water caused a decrease in the porosity of the substrate, thereby decreasing the gas exchange and affecting the growth of microorganisms and degradation of proteins (Mahadik et al., 2002). In addition, excessive water content will promote the growth of harmful bacteria and affect the quality of fermented feed. Therefore, the optimal solid-state fermentation water content was 60%.

Effect of the fermentation temperature

In multi-strain mixed solid-state fermentation, the optimal growth temperature of microorganisms may not be the optimal temperature for protein degradation. As shown in Figure 1(e), the small peptide content increased first and then decreased, reaching the maximum at 36 °C. At lower temperatures, the fluidity of cell membranes decreased, nutrient delivery slowed down, Microbial growth was inhibited and microbial protease secretion decreased (Shu et al., 2015). At higher temperatures, fermentative microorganisms grew rapidly and proteases activity increased (Mendoza et al., 2009), but biologically active substances in microbial cells changed, such as rapid inactivation of proteases, resulting in slow growth, premature aging and even death of microorganisms. Therefore, the optimal fermentation temperature was 36°C.

Effect of the types of proteases

The protease cleavage sites of different proteases were different, and the activity of each protease was greatly affected by temperature, pH and other factors(Castro et al., 2015; Yuan et al., 2017). The added proteases can synergize with microorganisms to degrade protein more thoroughly, enrich the types of small peptides, and significantly improve the degradation efficiency. As shown in Figure 1(f), 0.5% acid protease+0.5% bromelain increased the small peptide content most. It showed that 0.5% acid protease+0.5% bromelain were more suitable for this solid-state fermentation. Therefore, acid protease+bromelain were selected to be added.

Effect of the amount of acid protease+bromelain added

As shown in Figure 1(g), the small peptide content increased continuously with the increase of the amount of acid protease+bromelain added. But when the amount continued to increase, the small peptide content increased slowly. When the amount of acid protease+bromelain was 0.45%+0.45%~1.25%+1.25%, the increase of small peptide content was only about 10% higher than that of 0.25% acid protease+0.25% bromelain. It indicated that adding too much protease did not have a particularly good degradation effect, which was easy to cause waste. Considering the cost control, the optimal amount of protease added was 0.25% acid protease+0.25% bromelain.

Response surface analysis

The inoculum size, fermentation temperature and solid-state fermentation water content were selected as independent variables, and the small peptide content was used as the response value. The experiment of three factors and three levels was designed. The experimental schemes and results are shown in Table 2.

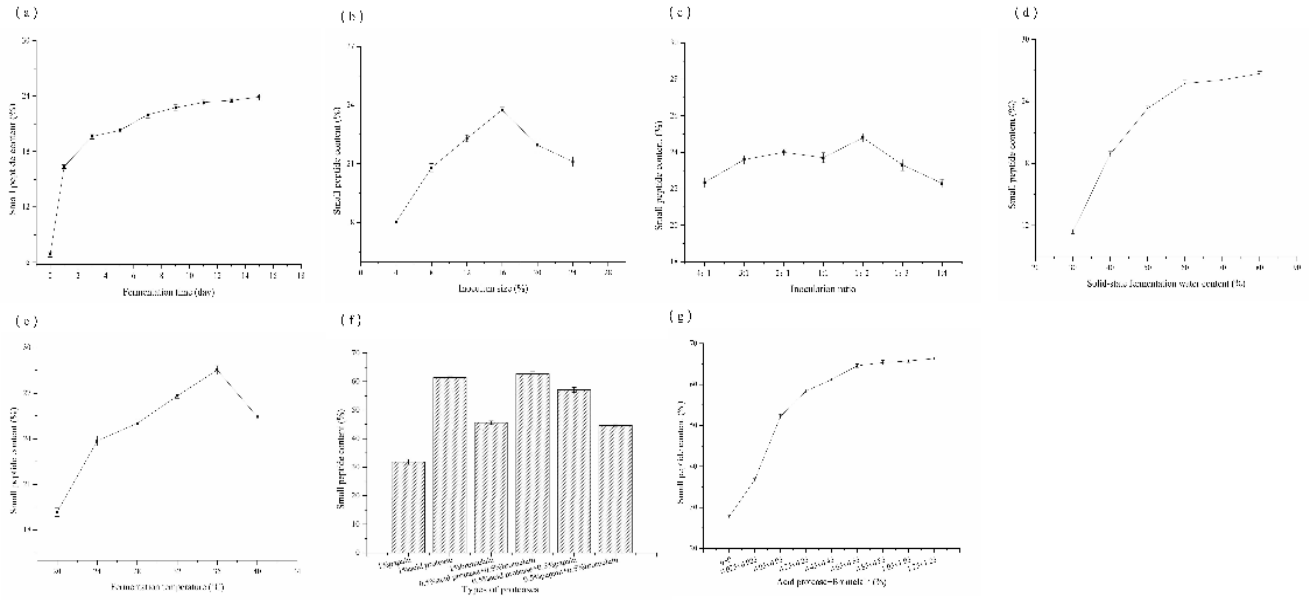

Figure 1. (a) Effect of the fermentation time on the small peptide content. (b) Effect of the inoculum size on the small peptide content. (c) Effect of the inoculation ratio on the small peptide content. (d) Effect of the solid-state fermentation water content on the small peptide content. (e) Effect of the fermentation temperature on the small peptide content. (f) Effect of the types of proteases on the small peptide content. (g) Effect of the amount of acid protease+bromelain on the small peptide content.

Table 2. Experimental design and results for response surface analysis

Number	A	B	C	Small peptide content(%)
1	0	1	1	48.91
2	0	1	-1	45.23
3	-1	1	0	53.87
4	1	0	-1	43.22
5	-1	0	1	53.31
6	-1	0	-1	43.89
7	0	-1	-1	38.47
8	0	0	0	56.89
9	1	-1	0	48.47
10	0	0	0	56.83
11	1	1	0	54.89
12	0	0	0	57.83
13	0	-1	1	52.46
14	1	0	1	51.63
15	-1	-1	0	52.8
16	0	0	0	56.52
17	0	0	0	57.83

The quadratic multiple regression equation of A (inoculum size), B (fermentation temperature), C (solid-state fermentation water content) was:

Small peptide content =57.18-0.71*A+1.34*B+4.44*C+ 1.34*A*B-0.25*A*C-2.58*B*C-1.46*A^2-3.21*$B^2$7.70*C^2

The variance analysis is shown in Table 3. The regression model for small peptide content was extremely significant ($p<0.0001$), indicating that there were significant differences in the small peptide content under different conditions. The lack of fit was not significant (0.2355>0.05), indicating that the equation can describe the relationship between different conditions and the small peptide content well. The coefficient of variation (1.45< 5 %) indicated that the model was reproducible (Wanasundara et al., 1996). The R^2 and Adj R^2 of the model were 0.9927 and 0.9834, respectively, indicatig that the fitting degree was good and the error was small. Adeq precision(33.672>4) indicated that the equation model had a good predictive effect (Kwatia et al., 2017).

In the model established in this experiment, the effects of factors C, B^2 and C^2 on the small peptide content were extremely significant ($p<0.01$); the effects of factors A, B, AB, BC and A^2 on the small peptide content were significant ($p < 0.05$).

Three dimensional (3D) response surface and contour plots are shown in Figure 2. The 3D response surface plot reflected the significance of each factor. The steeper the 3D response surface plot was, the greater the influence of this factor on the response values was(Wang et al., 2014). The contour plot showed the significance of the interaction between the two variables. The more the contour lines tend to ellipse, the stronger the interaction between the two factors. As shown in Figure 2, contour lines were elliptical and dense, indicating that the interaction between inoculum size and fermentation temperature, fermentation temperature and solid-state fermentation water content had significant effects on the small peptide content. The 3D response surface plot also proved this argument. The results of the response surface analysis were consistent with the results of the analysis of variance.

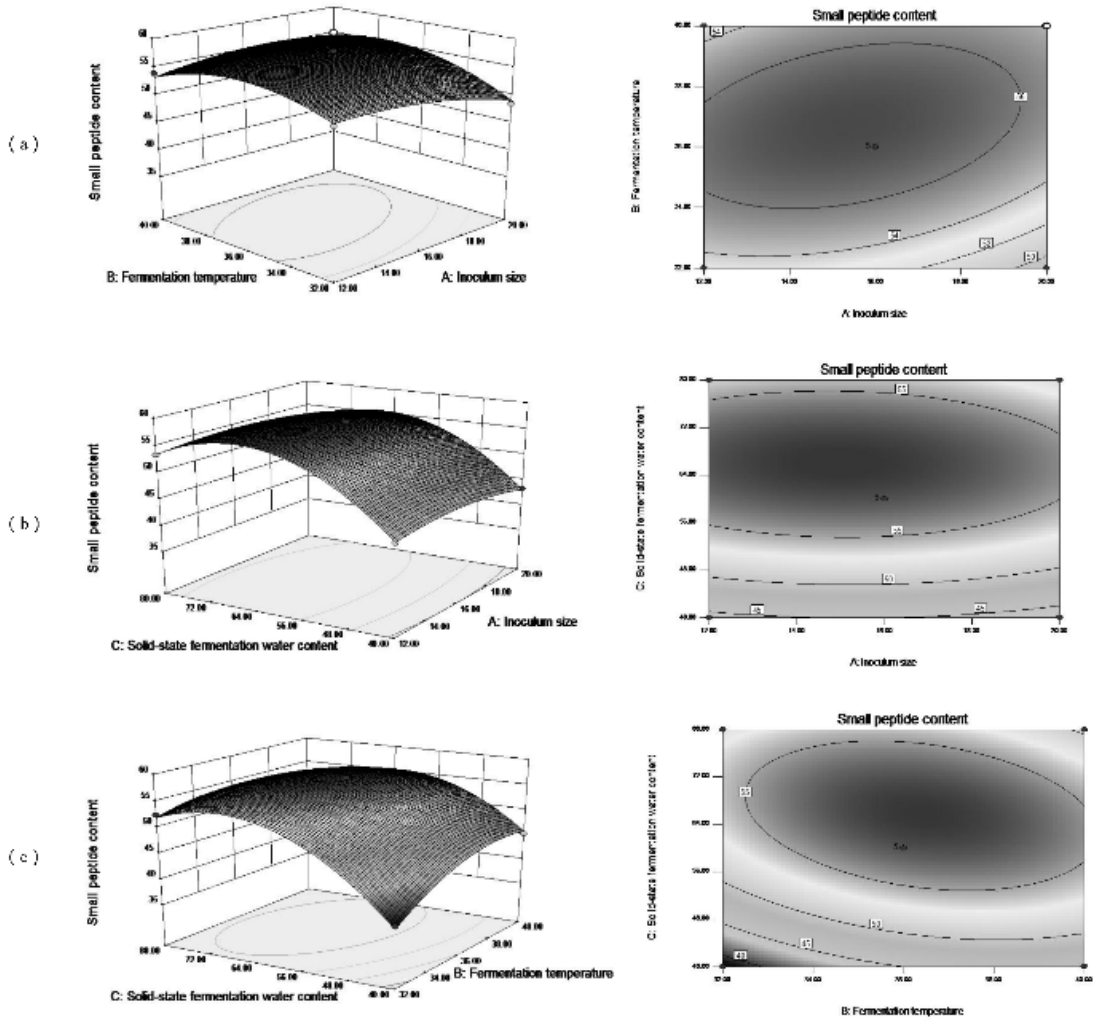

Figure 2. (a) 3D response surface and contour plots showing the effects of incoulum size and fermentation temperature when the solid-state fermentation water content was kept at an optimized level of 60%. (b) 3D response surface and contour plots showing the effects of incoulum size and solid-state fermentation water content when fermentation temperature was kept at an optimized level of 36°C. (c) 3D response surface and contour plots showing the effects of fermentation temperature and solid-state fermentation water content when the inoculum size was kept at an optimized level of 16%.

Table 3. Analysis of variance of the regression model

Source	Sum of squares	Df	Mean square	F-value	P-value Prob>F
Model	533.02	9	59.22	106.43	<0.0001
A	4.00	1	4.00	7.20	0.0314
B	14.31	1	14.31	25.72	0.0014
C	157.53	1	157.53	283.08	<0.0001
AB	7.16	1	7.16	12.86	0.0089
AC	0.26	1	0.26	0.46	0.5202
BC	26.57	1	26.57	47.75	0.0002
A^2	9.02	1	9.02	16.21	0.0050
B^2	43.35	1	43.35	77.90	<0.0001
C^2	249.89	1	249.89	449.04	<0.0001
Residual	3.90	7	0.56		
Lack of fit	2.41	3	0.80	2.16	0.2355
Pure error	1.49	4	0.37		
Cor total	536.91	16			

C.V.%1.45; R^2=0.9927; Adj R^2=0.9834; Pred R^2=0.9239; Adeq precision=33.672

Verification experiment

The optimal fermentation process predicted by the response surface experimental equation was as follows: inoculum size 15.01%, fermentation temperature 36.17 ℃, solid-state fermentation water content 65.69%. Under the process, the theoretical small peptide content was 57.928%. In order to facilitate the verification, the parameters were corrected as follows: inoculum size 15%, fermentation temperature 36℃, solid-state fermentation water content 66%. The experiment was repeated 3 times, and the average of small peptide contents was 57.86%, which was close to the theoretical value.

Molecular weight distribution of feed

SDS-PAGE pattern is shown in Figure 3. Compared with the unfermented raw materials, the protein bands in the fermented feed basically disappeared. The protein in the milk thistle residue fermented feed was basically hydrolyzed into small peptides, and the experimental results were consistent with those of Li et al.(2013).

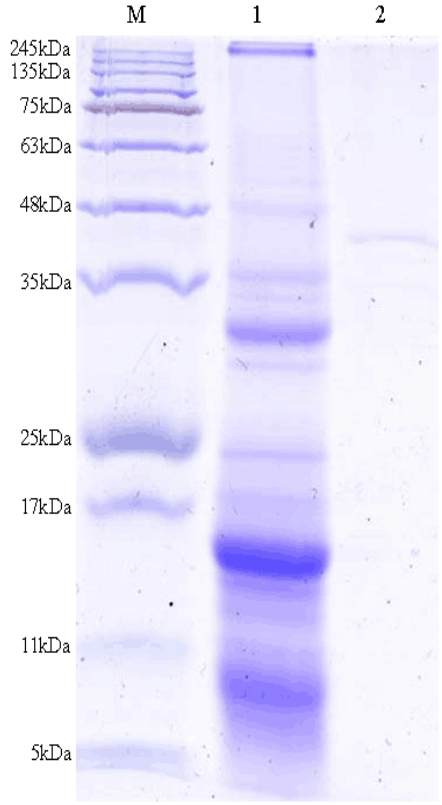

Figure 3. Molecular weight distribution of feed. M: protein molecular weight markers (5~245kDa); 1: raw materials before fermentation; 2: fermented feed.

Changes of the number of fermentative microorganisms

As shown in Figure 4, the changes of fermentative microorganisms were first increased and then decreased slightly. The number of microorganisms in the feed supplemented with protease reached the maximum on the fifth day, while the number of microorganisms in the feed without protease reached the maximum on the seventh day. The maximum number of microorganisms in the protease-added feed was greater than that without protease. At the beginning of fermentation, the added protease degraded the large molecular proteins in the feed into small peptides and amino acids, which was more conducive to the absorption and utilization, and the fermentative microorganisms multiplied quickly. With the progress of fermentation, nutrients were continuously consumed, microorganisms such as lactic acid bacteria secreted a large amount of organic acids, the pH of the feed decreased, and the growth of fermentative microorganisms was inhibited.

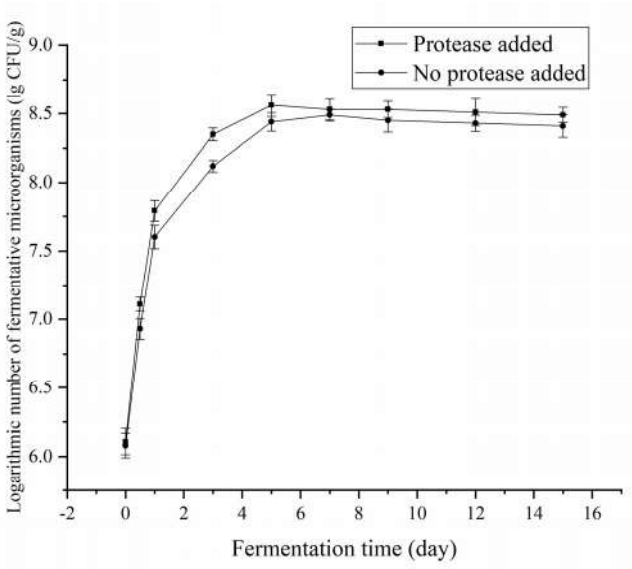

Figure 4. The changes of the number of fermentative microorganisms in fermented feed.

Changes of protease activity

The level of protease activity in fermented feed played a vital role in protein degradation. As shown in the Figure4(a)(b)(c), the addition of acid protease and bromelain made the activity of acid and neutral protease much higher than that of the non-added protease, the activity of the alkaline and acid protease reach the highest value in advance, and the highest value of alkaline protease activity is higher than that without addition. At the initial stage of fermentation, the added protease promoted that fermentative microorganisms, especially *Bacillus natto*, rapidly proliferated and secreted a large number of proteases, so the activities of alkaline proteases and acid proteases were constantly increasing. Protease activity increased slowly in the feed without protease. In the early stage of fermentation, the added protease was partially inactivated, which made the neutral protease activity decrease, but as microorganisms secreted the neutral protease, the protease activity started to increase. In the middle of fermentation, oxygen was gradually depleted, and the growth of Bacillus natto was inhibited. Yeast and lactic acid bacteria became the dominant microorganisms, and they continuously secreted organic acids, which reduced the pH and activity of alkaline and neutral proteases (Elfalleh et al., 2017). The optimal pH of acid protease was lower, so the acid protease activity was

less affected. In the later stage of fermentation, nutrients in the feed were consumed in large quantities, a large amount of organic acids were produced, and the pH of the feed was low. The growth of fermentative microorganisms and the secretion of proteases were inhibited, and the activity of all three proteases was reduced.

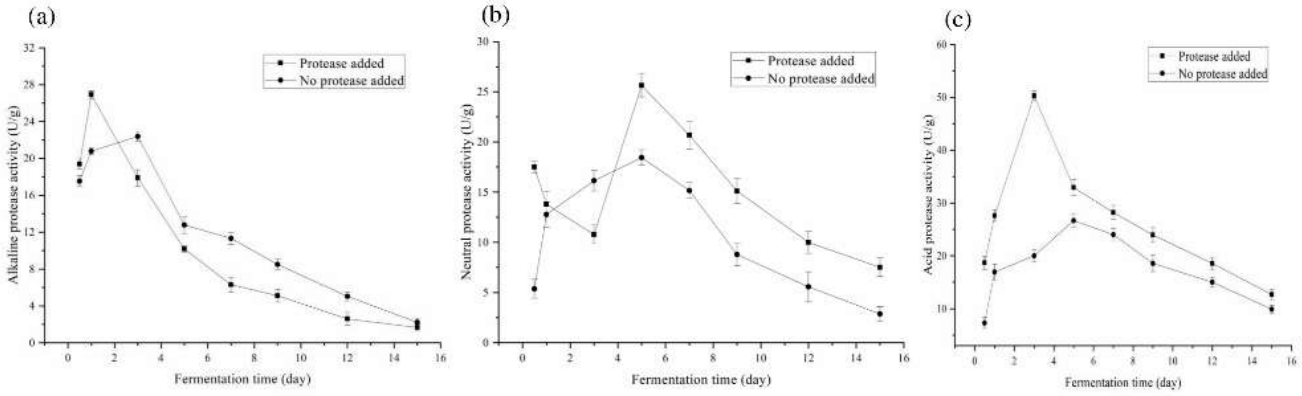

Figure 5. (a)The changes of acid protease activity in fermented feed. (b)The changes of neutral protease activity in fermented feed. (c) The changes of alkaline protease activity in fermented feed.

Changes of pH

As shown in Figure 6, the pH of the feed decreased continuously, and the pH of the feed with protease decreased faster than that without protease. The added protease enriched the nutrients in the feed, which promoted the proliferation of microorganisms such as lactic acid bacteria and accelerated the secretion of organic acids such as lactic acid and acetic acid.

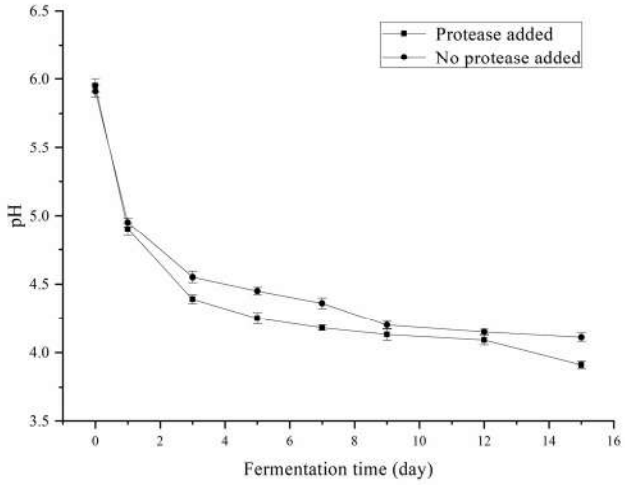

Figure 6. The changes of pH in fermented feed.

Conclusion

The optimal fermentation process obtained in this experiment was fermentation time of 7 days, inoculum size of 15%, inoculation ratio of aerobic strains: anaerobic strains=1: 2, solid-state fermentation water content of 66%, fermentation temperature of 36℃, amount of proteases added was 0.25% acid protease+0.25% bromelain. Under the fermentation process, the small peptide content of the milk thistle residue fermented feed increased significantly to 57.86%. The added protease greatly promoted the growth of fermentative microorganisms, the secretion of protease and the increase of small peptide content. This study will provide a good reference for the efficient utilization of the biological feed of the milk thistle residue.

Acknowledgments

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