



# Article Optimization of Solid-State Fermentation Conditions of Quercus liaotungensis by Bacillus subtilis

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**Abstract:** The current study aimed to investigate the solid-state fermentation process of *Quercus liaotungensis* (QL) by *Bacillus subtilis* (BS). The parameters included the inoculation amount, the soybean meal addition amount, the fermentation temperature and the ratio of material to water. The optimal process was determined based on the nutritional value, tannin content and DPPH clearance of QL after fermentation. The results showed that: (1) The parameters of the optimal process included inoculating 10<sup>6</sup> BS per gram of QL, then adding 10% soybean meal, the ratio of material to the water of 100:80, and temperature at 33 °C for 72 h. (2) In the optimum fermentation conditions, the crude fiber content, and the ether extract content of QL decreased by 66.94% and 66.96%, respectively (p < 0.05). Moreover, the crude protein content and the ash content increased by 65.81% and 4.63%, respectively, after fermentation (p < 0.05). Additionally, the tannin content decreased by 62.77% (p < 0.05), and the DPPH scavenging rate decreased by 45.45% (p < 0.05) after fermentation, respectively. In summary, the QL significantly improved the nutritional value after the solid-state fermentation with BS.

**Keywords:** *Bacillus subtilis;* solid fermentation; *Quercus liaotungensis;* nutritional value; DPPH clear rate; tannin

# 1. Introduction

The animal husbandry industry converts plant-derived nitrogen into animal protein in the form of meat, egg, or milk for human society [1]. However, the rapid development of animal husbandry consumes large amounts of crops, mainly cereals and soybeans, threatening human food security [2]. The discovery and development of new feed ingredients could be a benefit for saving food consumption in animal husbandry [3].

*Quercus liaotungensis* is a deciduous tree of the *Fagaceae Quercus genus*, widely distributed in Northern China. The seed of *Quercus liaotungensis* is a starch-rich acorn that has the potential to be used in animal feed [3]. Unfortunately, *Quercus liaotungensis* is hindered by its own anti-nutritional factors, such as tannins and non-starch polysaccharides [4]. The high content of tannin would likely reduce the palatability and nutritional value of the feed.

Solid-state fermentation of probiotics is one of the important methods to reduce anti-nutritional factors and improve feed quality [5,6]. Probiotics degrade macromolecular substances in feed ingredients, such as sugars and proteins, to generate small molecules that are more easily absorbed by the animal gut, helping to improve the nutrient digestibility of the feed [6–8]. In addition, inactivated by-products of beneficial microorganisms, defined as epibiotics, also help to condition the gut health of animals [9]. Moreover, certain strains have the ability to degrade certain anti-nutritional factors [10]. Especially, *Bacillus subtilis* (BK16), a beneficial and efficient bacteria degrading tannin, was selected for targeted application in the solid-state fermentation of *Quercus liaotungensis* in the current study.



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**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Therefore, the current study aimed to investigate the solid-state fermentation process of *Quercus liaotungensis* (QL) by the *Bacillus subtilis* (BK16). The parameters included the inoculation amount, the soybean meal addition amount, the fermentation temperature, and the ratio of material to water.

## 2. Materials and Methods

# 2.1. Strains and Culture Medium

The *Bacillus subtilis* (BK16) was provided by Hunan BKS Biotech Co., Ltd. (Changsha, China). Moreover, the *Quercus liaotungensis seed* (QLS) was provided by Hongyi Biotechnology Co., Ltd. (JinZhong, China), which was crushed and sieved with 60 meshes for later use. The QLS contains  $5.47\% \pm 0.09\%$  crude protein (CP),  $4.93\% \pm 0.11\%$  crude fiber (CF) and  $12.65\% \pm 0.10\%$  tannin from the results of chemical analysis.

Nutrient broth medium (NB): Glucose 3.5 g, peptone 0.83 g, yeast powder 0.5 g, beef extract 5 g, dipotassium hydrogen phosphate 0.35 g, calcium carbonate 0.25 g, distilled water volume to 1000 mL, natural pH, 121 °C sterilization for 20 min. After cooling, the freeze-dried powder of *Bacillus subtilis* resuscitation was added and cultured in a 37 °C incubator for 24 h.

Solid fermentation medium: accurately weigh 30 g *Quercus liaotungensis* powder into a 250 mL triangle bottle, add soybean meal in proportion, add different proportions of water, stir evenly, and sterilize at 121 °C for 20 min at natural pH.

#### 2.2. Orthogonal Test to Optimize Fermentation Conditions

Under the condition of natural pH, an L9 ( $3^4$ ) orthogonal test design was adopted for inoculum size, fermentation temperature, material–water ratio and soybean meal addition (Table 1). 10<sup>6</sup> CFU/mL of activated strains were added to the solid fermentation medium with the ratio of material to water of 1:1.5, 1:1, 1:0.8, the additional amount of soybean meal of 1%, 5%, 10% and the fermentation temperature of 28 °C, 33 °C and 38 °C respectively. 10<sup>7</sup> CFU/mL *Bacillus subtilis* was also added to the solid fermentation medium with the ratio of material to water of 1:1.5, 1:1, 1:0.8, the additional amounts of soybean meal of 1%, 5%, and 10% and the fermentation temperatures of 28 °C, 33 °C, 38 °C, respectively, and 10<sup>8</sup> CFU/mL. Similarly, four factors and three levels were orthogonal. After the fermentation, the samples were dried at 65 °C and crushed through a 40-mesh sieve. Then the fermentation products were sampled for chemical analysis, including CP, ether extract, ash, energy, CF, DPPH free radical scavenging rate and tannin content.

	Factors				
Levels	Substrate/Moisture (A)	Inoculation (B) cfu/mL	Soybean (C)	Fermentation Temperature (D)	
1	1:1.5	10 <sup>6</sup>	1%	28 °C	
2	1:1	$10^{7}$	5%	33 °C	
3	1:0.8	10 <sup>8</sup>	10%	38 °C	

**Table 1.** Orthogonal experiment design for  $L_9$  (3<sup>4</sup>).

According to the orthogonal design, the optimal conditions of solid-state fermentation of *Quercus liaotungensis* by *Bacillus subtilis* were determined, and the fermentation time was designed by single factor experiment, and the fermentation time was set to 1 d, 2 d and 3 d, respectively. According to the optimal conditions after the last batch of fermentation, three different times were set respectively, and then the optimal fermentation conditions were selected according to them.

#### 2.3. Determination of Conventional Nutrients, DPPH Clear Rate and Tannin Content

The fermentation product was dried at 65  $^{\circ}$ C, crushed and passed through a 40-mesh sieve, and the crude protein (CP) was determined by the Kjeldahl method. Dry matter

(DM) was determined by the oven-drying method. Coarse fiber (CF) was measured by the filter bag manual method. Crude fat (EE) was determined by Soxhlet extraction. Crude ash (Ash) was measured by the muffle furnace charcoal burning method.

The liquid to be tested, F-D chromogenic agent and sodium carbonate solution (1 mol/L) were mixed into a volumetric flask at a ratio of 1 mL:2.5 mL:10 mL, and then fixed to 50 mL and bathed at 50 °C for 5min. After cooling, the absorbance of the solution at 760 nm was measured, and the experiment was repeated 3 times with a 0 mL standard liquid system as the control. Calculate the tannin concentration in the solution according to the standard curve and regression equation, and then calculate the tannin extraction amount by the following formula [11].

tannin extraction amount (%) = 
$$\frac{C \times D \times V}{m} \times 100\%$$

In the formula: C is tannin mass concentration (mg/L); D is the dilution multiple; V is the volume of the liquid to be measured (L); m is the sample mass (g).

The DPPH clearance rate was determined by spectrophotometry:

Determine the absorbent value (A0) of 0.1 mL distilled water and 3.9 mL DPPH • mixture and 0.1 mL distilled water and 3.9 mL 95% ethanol mixture (A2). A sample solution of 0.1 mL with different mass concentrations was added to the 3.9 mL DPPH solution as the experimental group, and 0.1 mL Vc solution with different mass concentrations was added as the control group. After shaking, the above solutions were incubated at room temperature for 30 min and the absorption bending values (A1) were measured at 517 nm. Three replicates were set up for each sample [12]. The calculation formula is:

DPPH clearance rate (%) = 
$$(\frac{A0 - A1}{A0}) \times 100\%$$

#### 2.4. Data Statistics and Analysis

The data were preprocessed by Excel 2019 and then processed and analyzed by SPSS 20.0. Single-factor screening test data were analyzed by single-factor ANOVA, and multiple comparisons were made by Duncan's method. The orthogonal test data were analyzed by range analysis. The results of the experiment all used the mean and standard deviation, and the significant level was p < 0.05.

# 3. Results and Analysis

From the R-value of the range analysis (Table 2), it can be seen that the order of the influence of various factors on protein is C > D > B > A; that is, the addition of soybean meal has the greatest influence on tannin degradation, followed by temperature, and furthermore, the inoculum size has the smallest influence on protein content, which is the ratio of material to water. According to the K value of crude protein content, the best combination is A2B3C3D1. That is, the ratio of material to water is 1:1, the inoculum size is  $10^8$  CFU/mL, the addition of soybean meal is 10%, and the fermentation temperature is 28 °C.

#### 3.1. Orthogonal Test Results

3.1.1. Effect of Bacillus subtilis Inoculation Amount on Nutritional Value of Quercus liaotungensis

It can be seen from Table 3 that different inoculum sizes have different effects on the tannin content of *Quercus liaotungensis* during solid-state fermentation. When inoculum size is  $10^6$  CFU/mL, the tannin content in *Quercus liaotungensis* is the lowest, but the difference is not significant (p > 0.05). When the inoculum size was  $10^6$  CFU/mL and  $10^7$  CFU/mL, the CP content was significantly higher than  $10^8$  CFU/mL (p < 0.05), but there was no significant difference between the inoculum size of  $10^6$  CFU/mL and  $10^7$  CFU/mL (p > 0.05). After comprehensive consideration,  $10^6$  CFU/mL inoculum was selected as the best inoculum.

E		Factors			<b>T</b> 0/	
Experiment Number	Α	В	С	D	- Iannin %	CP %
1	1	1	1	1	11.75	6.09
2	1	2	2	2	10.80	6.94
3	1	3	3	3	7.43	8.51
4	2	1	2	3	8.49	7.01
5	2	2	3	1	8.06	9.02
6	2	3	1	2	8.22	5.82
7	3	1	3	2	5.79	6.49
8	3	2	1	3	11.06	5.72
9	3	3	2	1	9.14	7.78
Tannin %						
K1	10.00	8.68	10.34	9.65		
K <sub>2</sub>	8.26	9.97	9.48	8.27		
K <sub>3</sub>	8.66	8.26	7.09	9.00		
R	1.74	1.71	3.25	1.38		
Optimum combination		$A_1B_2$	$C_1D_1$			
CP %						
$K_1$	7.18	6.53	5.88	7.63		
K <sub>2</sub>	7.28	7.23	7.24	6.42		
K3	6.66	7.37	8.01	7.08		
R	0.62	0.84	2.13	1.21		
Optimum combination		$A_2B_3$	$C_3D_1$			

Table 2. Range analysis table of orthogonal experiment result (DM basis) %.

 $K_1$ ,  $K_2$  and  $K_3$  represent the average values of the results at levels 1, 2 and 3, respectively. R  $K_1$ ,  $K_2$ , and  $K_3$  represent the extreme difference under various factors.  $K_1$ ,  $K_2$  and  $K_3$  stand for the mean value of the results under levers 1,2 and 3, respectively. R stand for the range of  $K_1$ ,  $K_2$  and  $K_3$  under each factor.

Table 3. Effect of inoculation on the nutritional value of Quercus liaotungensis (DM basis) %.

Therese	Inoculation of Inoculum			
Items	10 <sup>6</sup>	<b>10</b> <sup>7</sup>	10 <sup>8</sup>	
Tannin % CP %	$\begin{array}{c} 5.73 \pm 0.39 \\ 8.78 \pm 0.15 \ ^{a} \end{array}$	$\begin{array}{c} 6.07 \pm 0.29 \\ 8.69 \pm 0.09 \ ^{\rm a} \end{array}$	$\begin{array}{c} 6.02 \pm 1.28 \\ 8.23 \pm 0.21 \ ^{\rm b} \end{array}$	

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

#### 3.1.2. Effect of Soybean Meal Addition on Nutritional Value of *Quercus liaotungensis*

It can be seen from Table 4 that with the increase of soybean meal, the tannin content in *Quercus liaotungensis* showed a significant downward trend. When the addition of soybean meal was 10%, the tannin content decreased to the lowest level of 7.29%, which was significantly lower than other groups (p < 0.05). The CP content increased with the addition of soybean meal. When the addition of soybean meal was 10%, the highest CP content was 9.43%, which was significantly lower than that of other groups (p < 0.05), which indicated that the fermentation effect of *Quercus liaotungensis* was the best when adding 10% soybean meal.

Table 4. Effect of soybean on the nutritional value of Quercus liaotungensis (DM basis) %.

T.	Soybean Meal Addition Soybean			
Items	1%	5%	10%	
Tannin % CP %	$9.67 \pm 0.25$ a $6.44 \pm 0.49$ c	$8.30 \pm 0.22^{\ b}$ $7.71 \pm 0.31^{\ b}$	$7.29 \pm 0.13$ <sup>c</sup> $9.43 \pm 0.13$ <sup>a</sup>	

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

#### 3.1.3. Effect of Fermentation Temperature on Nutritional Value of *Quercus liaotungensis*

From Table 5, it can be seen that the content of tannin decreases at first and then increases. When the temperature is 33 °C, the content of tannin is the lowest, 6.02%, with no significant difference (p > 0.05). When the fermentation temperature was 28 °C, the highest CP content was 9.39%, which was significantly lower than other groups (p < 0.05). It may be that too low a temperature is not conducive to the growth of *Bacillus subtilis*; If the temperature is too high, the faster the water loss of the solid-state fermentation medium will be, and the more unfavorable the fermentation of *Bacillus subtilis* will be. Considering comprehensively, the fermentation temperature can be selected between 28 °C and 33 °C.

Table 5. Effect of fermentation temperature on nutritional value of Quercus liaotungensis (DM basis) %.

Items	Fermentation Temperature		
	28 °C	33 °C	38 °C
Tannin % CP %	$\begin{array}{c} 7.47 \pm 0.27 \\ 9.39 \pm 0.33 \ ^{\rm a} \end{array}$	$6.02 \pm 1.28 \\ 8.23 \pm 0.21 \ ^{ m b}$	$6.70 \pm 0.13 \\ 8.42 \pm 0.14$ <sup>b</sup>

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

#### 3.1.4. Effect of Feed-Water Ratio on Nutritional Value of *Quercus liaotungensis*

Water is one of the important factors affecting microbial fermentation. Too low will affect the absorption of nutrients by microorganisms and reduce the metabolic rate of bacteria. Too high will affect the permeability of the culture medium, which is not conducive to microbial growth. As can be seen from Table 6, when the ratio of material to water is 1:0.8, the tannin content in *Quercus liaotungensis* is 7.16%, which is significantly lower than in other groups (p < 0.05), but there is no significant difference between the ratio of material to water being 1:1 and 1:0.8 (p > 0.05); When the ratio of material to water is 1:0.8, the highest CP is 9.35%, but the difference is not significant (p > 0.05). Considering comprehensively, the best ratio of material to water is 1:0.8.

Table 6. Effect of substrate/moisture on the nutritional value of Quercus liaotungensis (DM basis) %.

The second	Substrate/Moisture			
Items	1:1.5	1:1	1:0.8	
Tannin % CP %	$7.83 \pm 0.26 \text{ a} \\ 9.24 \pm 0.36$	$7.35 \pm 0.21^{\text{ b}} \\ 8.96 \pm 0.06$	$7.16 \pm 0.10^{\text{ b}} \\ 9.35 \pm 0.25$	

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

#### 3.2. Single Factor Test Results

According to the results of the orthogonal test, the nutritional value of *Quercus liaotungensis* fermented by the solid state is the highest when the inoculum size is  $10^6$  CFU/mL, the fermentation temperature is  $33 \,^\circ$ C, the ratio of material to water is 1:0.8, and the addition of soybean meal is 10%. As can be seen from Table 7, with the increase in fermentation time, the tannin content gradually decreased. When the fermentation time was 3 days, the tannin content decreased to 4.71%, which was significantly lower than in other groups (p < 0.05). The content of CP increased with the increase of fermentation time, and the highest content of CP was 9.08% when the fermentation time was 3 days, which was significantly higher than other groups (p < 0.05), so the nutritional value of *Quercus liaotungensis* was the highest when the fermentation time was 3 days.

The second	Fermentation Time/d			
Items	1 d	2 d	3 d	
Tannin %	$6.58\pm0.13$ $^{\rm a}$	$6.00\pm0.17^{\text{ b}}$	$4.71\pm0.10~^{\rm c}$	
CP %	$8.28\pm0.12^{\text{ b}}$	$8.65\pm0.13^{\text{ b}}$	$9.08\pm0.10~^{\rm a}$	

Table 7. Effect of fermentation time on the nutritional value of *Quercus liaotungensis* (DM basis) %.

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

#### 3.3. Fermentation Results under Optimal Conditions

It can be seen from Table 8 that after solid-state fermentation of *Quercus liaotungensis* under the optimal conditions, compared with the control group, after BS fermentation, the Tannin content of *Quercus liaotungensis* decreased from 12.65% to 4.71% (p < 0.05), the DPPH clear rate decreased from 73.33% to 40% (p < 0.05) and the CP content increased from 5.47% to 9. EE content decreased from 4.51% to 1.49% (p < 0.05), Aah content increased from 2.81% to 2.94% (p < 0.05), CF content decreased from 4.93% to 1.63% (p < 0.05), and GE content increased from 4.08 kcal/g to 4.31 kcal/g.

Table 8. Change of nutritional value of Quercus liaotungensis before and after fermentation (DM basis) %.

Items	Control Group	Fermentation Group	Improvement Rate
Tannin %	$12.65\pm0.10$ $^{\rm a}$	$4.71\pm0.10^{\text{ b}}$	-62.77
DPPH clear rate %	$73.33\pm0.02~^{a}$	$40.00\pm0.03$ <sup>b</sup>	-45.45
CP %	$5.47\pm0.09$ <sup>b</sup>	$9.07\pm0.10$ $^{\mathrm{a}}$	65.81
EE %	$4.51\pm0.39$ <sup>a</sup>	$1.49\pm0.27~^{ m b}$	-66.96
Ash %	$2.81\pm0.06~^{\rm b}$	$2.94\pm0.03$ a	4.63
CF %	$4.93\pm0.11$ a	$1.63\pm0.07$ b	-66.94
GE (kcal/g)	$4.08\pm0.01~^{\rm b}$	$4.31\pm0.01$ a	5.64

In the same row, values with different small letter superscripts mean significant difference (p < 0.05), while with the same or no letter superscripts mean no significant difference (p > 0.05).

# 4. Discussion

Determining the appropriate amount of bacterial inoculation and fermentation duration is of great significance for the fermentation process, which is related to several key parameters, such as reaction rate, oxygen consumption, fermentation product concentration, and bacterial contamination of the fermentation process [13]. In the current study, it was found that the optimal inoculation amount of *Quercus liaotungensis* fermented by BS was 10<sup>6</sup> CFU/mL, which was similar to previous research results (fermentation time is 4 d) [14,15]. However, the optimal fermentation time is longer than the optimal fermentation time obtained in this experiment, probably because the nutritional value of *Quercus liaotungensis* is higher than that of tea residue or sweet potato residue, and the growth of microorganisms ends early.

Temperature affects the growth of microorganisms and the activity of enzymes and is, therefore, one of the most important parameters affecting fermentation results [16]. In the current study, *Bacillus subtilis* was used to ferment *Quercus liaotungensis* in order to degrade the tannin content in *Quercus liaotungensis*. The results showed that the tannin content was the lowest when the fermentation temperature was 33 °C, which indicated that the output of tannase was the highest at this temperature. Guan Jun found that the temperature of 28~32 °C when *Bacillus subtilis* fermented compound feed was beneficial to the growth of *Bacillus subtilis* [17], which was consistent with the results of this experiment, indicating that *Bacillus subtilis* grew fastest, produced enzymes and degraded tannin at this temperature. The previous study showed that the crude protein content of sweet potato residue fermented by *Bacillus subtilis* is the highest at 28 °C [18], which is consistent with the results of this experiment.

In the process of microbial fermentation, the fermentation time is a big factor that affects the enzyme production of bacteria. The anti-nutritional factors and fibers in the fermentation substrate cannot be sufficiently degraded to achieve the desired effect when the fermentation time is too short, whereas prolonged fermentation could lead to a weakening phase of the strain, excessive consumption of nutrients or secondary metabolism. With the increase in the temperature of the culture medium, the protease production rate will slow down, and the probability of substrate contamination will increase. Therefore, choosing the appropriate fermentation time can reduce the cost [8,19]. The best fermentation time of *Bacillus subtilis* is generally about 72 h [8,20,21], and the best fermentation time of this experiment is 3 d, which is consistent with the research results of this experiment.

Given the low crude protein content of *Quercus liaotungensis*, providing an additional nitrogen source is beneficial to promote the growth of bacteria during fermentation. Adding appropriate carbon sources and nitrogen sources can accelerate the growth of *Bacillus subtilis*, reduce the diarrhea period of the strain, improve the fermentation efficiency, and increase the yield of *Bacillus subtilis*. In this study, the optimum addition of soybean meal was 10%. At this time, the content of crude protein was 9.07%, and the content of tannin was the lowest. When 20% soybean meal was added to fermented cassava residue, the crude protein content reached the highest of 18.6% [22], which indicated that the crude protein content was closely related to the addition of soybean meal.

Water plays an important role in the metabolic process of microorganisms, and the production of enzymes is greatly affected by the water activity of substrates. The lower water content will limit the growth and diffusion of microorganisms, the heat transfer will also be affected, and the diffusion rate of solute and water molecules will decrease. Increasing the initial water content of materials can improve the utilization and absorption of nutrients while reducing the inhibition of metabolites on fermentation strains [23]. The water content in the fermentation process should be based on the water content of fermentation substrate materials and the water content required in the microbial metabolism process. The best ratio of material to water in this experiment is 1:0.8, which is consistent with Xu Yunhe's research [21] but different from Fu Min's research [24]. The possible reason is that the mixed bacteria need less water, while the fermentation of *Bacillus subtilis* needs more water, which may be related to the different water content required by the growth and metabolism of the bacteria.

### 5. Conclusions

The results of the single factor and the orthogonal test showed that the optimum conditions for solid-state fermentation of *Quercus liaotungensis* by *Bacillus subtilis* were as follows: inoculum size of 10<sup>6</sup> CFU/mL, fermentation temperature of 28~33 °C, fermentation time of 3 days, a solid-water ratio of 1:0.8, and soybean meal addition of 10%.

Under the optimum fermentation conditions, tannin, DPPH clear rate, CF, and EE of *Quercus liaotungensis* decreased significantly, while CP and GE contents increased significantly, and the tannin content of anti-nutritional factor decreased from 12.65% to 4.71%.

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