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The impact of *Aspergillus Niger* and *Neurospora sitophila* co-fermentation on the soluble dietary fiber of soybean residue

Zituo He[1, 3], Zhiying Xu[1], Yiming Sun[1, 2, 4]

[1]Guangxi University of Chinese Medicine, Nanning, Guangxi, 530200, China
[2]Corresponding author

[3]1471500580@qq.com
[4]sunyiming_1226@hotmail.com

Abstract. Soybean residue, as a by-product of soybean, contains over 90% insoluble dietary fiber (IDF) and is underutilized. This study employed *Aspergillus Niger* and *Neurospora sitophila* (1:1) as mixed fermentation strains to ferment soybean residue's insoluble dietary fiber. Through a single-factor study investigating fermentation time, temperature, and inoculum size, the optimal fermentation conditions for the mixed culture to increase the extraction rate of soluble dietary fiber from soybean residue were explored. After 8 days of fermentation at 22°C with an inoculum size of 5%, the extraction rate of soluble dietary fiber reached 38.30%. The fermented soybean residue exhibited a threefold increase in swelling capacity, a 2.5-fold increase in water retention, a 1.56-fold increase in oil retention, and a 1.2-fold increase in oil absorption compared to the unfermented residue. This method provides a research basis for enhancing the extraction rate, obtaining nutritionally rich, and cost-effective soluble dietary fiber from soybean residue.

Keywords: Soybean residue, Soluble dietary fiber, *Aspergillus Niger*, *Neurospora sitophila*

1. Introduction

As a major producer and consumer of soy products, China produces approximately 20 million tons of soybean residue annually [1]. Fresh soybean residue, with its high moisture and protein content, is susceptible to putrefaction, making it challenging for storage and transportation. Moreover, soybean residue contains over 90% [2-3] insoluble dietary fiber (IDF), composed of cellulose, hemicellulose, and lignin, leading to poor texture and rough quality [4-5]. IDF, unable to be directly utilized, often ends up as waste, diminishing soybean utilization and causing environmental pollution [6]. Research indicates that for dietary fiber to be of high quality, soluble dietary fiber (SDF) should comprise over 30%-50% of the total fiber content [7], with SDF exhibiting higher content and more significant effects on human health compared to IDF [8].

In recent years, numerous studies and product developments have been dedicated to utilizing various methods to process soybean dregs dietary fiber, aiming to modify it and enhance its solubility [9-10]. Common methods for modifying dietary fiber include acid treatment, alkali treatment, enzymatic treatment, fermentation, ultrasound-assisted enzymatic treatment [11-12], irradiation technology [13], drying method [14], or steam explosion method [15-16]. Acid and alkali treatments are cost-effective and easy to operate, but they involve significant chemical residues and require a longer processing time.

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Enzymatic methods, such as the co-fermentation method with Aspergillus and lactic acid bacteria [17], or the complex microbial fermentation method [18], yield good results but are costly, have strict conditions, and are difficult to scale up for large-scale production. Fermentation involves the microbial degradation of phytic acid, large molecular polysaccharides, proteins, and other components in raw materials to modify dietary fiber. This process converts insoluble dietary fiber (IDF) into more soluble dietary fiber (SDF), producing high-quality SDF that is easy to control and adjust. It is a promising method for obtaining SDF [19]. The use of fermentation for the modification of dietary fiber results in soybean dregs products with superior color, texture, aroma, and dispersibility compared to chemical methods. Additionally, it maintains high water-holding capacity and extraction efficiency [20]. In fermentation, commonly employed methods include single or multiple strains in co-fermentation, which successfully modify dietary fiber, enhancing the nutritional value of soybean dregs products and obtaining other nutrients, such as functional oligosaccharides and peptides [17-19].

Aspergillus niger is often used as a common strain to improve the characteristics of dietary fiber. Its primary mechanism involves the production of various enzymes such as cellulases, hemicellulases, ligninases, and pectinases, which hydrolyze dietary fiber, thereby increasing the proportion of soluble content in dietary fiber. Additionally, *Aspergillus niger* exhibits characteristics such as rapid growth, short fermentation time, production of multiple enzyme types, high safety, and absence of toxic toxins. Consequently, it is widely employed as a crucial enzyme preparation in various manufacturing processes [21-23].

Neurospora sitophila, also known as chain mold, thrives in low-growth environments and has been found on the bark of burnt trees. Research indicates its superiority in fermenting leguminous materials [24]. *Neurospora sitophila* contains abundant substances beneficial to the human body, such as proteins, vitamin B_{12}, and is commonly used in the fermentation industry [25].

This study utilized fresh soybean residue and a mixture of *Aspergillus niger* and *Neurospora sitophila* (in a 1:1 ratio) as co-fermentation strains to extract soybean residue SDF using a fermentation method. The research measured the extraction rate of SDF in total dietary fiber and investigated the impact of fermentation time, inoculation volume, and temperature through orthogonal experiments. The aim was to obtain nutritionally rich and cost-effective soybean residue SDF. This approach not only reduces environmental pollution from soybean residue but also yields high-quality soluble dietary fiber, aligning with the theme of waste utilization and environmental protection, showcasing promising prospects. It provides a new developmental direction for soybean residue resources.

2. Materials and Methods

2.1. Materials and Equipment

2.1.1. Materials and Reagents. Soybean residue - purchased from Nanning Xianhu University City Market; Anhydrous ethanol - from Wuhan Canos Technology Co., Ltd.; Aspergillus niger - from Beijing NACHRAL Bio-technology Co., Ltd.; Neurospora sitophila (ICC40204) - from Beijing NACHRAL Bio-technology Co., Ltd.; Potato Dextrose Agar (PDA) culture medium - from Guangdong Huankai Microbial Technology Co., Ltd.; Food-grade anhydrous citric acid - from Weifang Yingxuan Industrial Co., Ltd.; Sabouraud agar - from Guangdong Huankai Microbial Technology Co., Ltd.; Anhydrous sodium carbonate - from Guangdong Guanghua Technology Co., Ltd.; pH test paper - from Hangzhou San Science and Technology Co., Ltd.

2.1.2. Instruments and Equipment. LRH-250A biochemical incubator - from Shaoguan Taihong Medical Equipment Co., Ltd.; DHG-9053A electric constant temperature blast drying oven - from Shanghai Boxin Instrument Equipment Factory; ZWY-240 constant temperature culture oscillator - from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.; BM1800 binocular digital electronic microscope - from Nanjing Jiangnan Yongxin Optical Co., Ltd.; 02270113 hemocytometer - from

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Shanghai Qiujing Biochemical Reagents Instrument Co., Ltd.; TDZ5-WS medical centrifuge - from Hunan Xiangyi Laboratory Instrument Development Co., Ltd.

2.2. Methods

2.2.1. Experimental Procedure. Fresh soybean residue → sterilization → cooling → inoculation → fermentation → sterilization → distilled water rinsing → pH adjustment → filtration → filtrate → rotary evaporation → 95% ethanol precipitation → centrifugation → residue → drying → SDF

2.2.2. Activation of Strains and Preparation of Spore Suspensions

(1) Activation of *Aspergillus niger* and preparation of spore suspension

Following the method described by Li Weiwei [26] et al., an appropriate amount of potato dextrose agar (PDA) powder was weighed, dissolved in sterile water in a beaker, heated until fully dissolved, and then sterilized at 121°C for 15 minutes. The PDA solution was poured into Petri dishes to create PDA agar plates. The *Aspergillus niger* strain was thawed at room temperature, inoculated onto the PDA agar plates, activated, and expanded under conditions of 28°C for 4 days. After rinsing with 0.9% saline solution and gentle scraping with an inoculating loop to remove excess *Aspergillus niger* from the surface of the plates, the suspension was thoroughly washed and filtered through four layers of sterile gauze into a conical flask.

(2) Activation of *Neurospora sitophila* and preparation of spore suspension

Referring to the method in the paper by Wang Qianman [25], an appropriate amount of Sabouraud agar powder was weighed, dissolved in 500 ml beaker, heated until fully dissolved, and then sterilized at 121°C for 15 minutes. The Sabouraud agar solution was poured into Petri dishes to create Sabouraud agar plates. The *Neurospora sitophila* strain was thawed at room temperature, inoculated onto the Sabouraud agar plates, activated, and expanded under conditions of 28°C for 4 days. After rinsing with 0.9% saline solution and gentle scraping with an inoculating loop to remove excess *Neurospora sitophila* from the surface of the plates, it was transferred to a sterilized conical bottle containing glass beads. The bottle was placed on a shaker and shaken for 2 hours.

(3) Cell Counting by Hemocytometer and Microscopic Observation for Determination of Inoculation Volume

Following the method described by Wang Qianman [25], 1 mL of spore suspension was diluted 10 times with physiological saline solution. A hemocytometer was cleaned with alcohol and air-dried. A drop of diluted spore suspension was placed on the edge of the coverslip, allowing it to fill the counting chamber. The formula for cell counting is as follows:

$$Cell\ count\ = \frac{Number\ of\ cells\ in\ 100\ cells}{100} \times 400 \times 104 \times dilution\ factor \quad (1)$$

2.2.3. Preparation of Fermented Soybean Residue. A conical flask containing 100 grams of fresh soybean residue with appropriate moisture content was sealed with six layers of gauze, sterilized at 121°C for 15 minutes, cooled to room temperature, and then inoculated with a 4% mixed spore suspension (Aspergillus niger spore suspension: Neurospora sitophila spore suspension = 1:1). After thorough mixing, it was incubated in a constant temperature incubator at 28°C for 8 days to obtain the co-fermented soybean residue by Aspergillus niger + Neurospora sitophila, with untreated soybean residue used as a control.

2.2.4. Extraction Rate of Soybean Residue SDF

2.2.4.1. Extraction of Soybean Residue SDF. Following the method by Zhao Taixia [27], fresh soybean residue was sterilized at 121°C for 20 minutes. After cooling, it was separately inoculated with Aspergillus niger and Neurospora sitophila spore suspensions. After an 8-day fermentation, the mixture was sterilized, rinsed with distilled water 1-2 times, adjusted to neutral pH with a 2% Na2CO3 solution,

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vacuum-filtered, the filtrate was rotary-evaporated, and then precipitated with 4 times 95% ethanol. The precipitate obtained after centrifugation at 3500 rpm for 10 minutes was dried in an oven at 70°C to obtain SDF.

2.2.4.2. Extraction of Soybean Residue IDF. The residue obtained after vacuum filtration in the above experiment was dried at 70°C to obtain IDF.

2.2.4.3. Calculation of Soybean Residue SDF Extraction Rate. Soluble dietary fiber in soybean residue (%)=

$$\frac{\text{Mass of soluble dietary fiber}}{\text{Mass of soluble dietary fiber} + \text{mass of insoluble dietary fiber}} \times 100\% \quad (2)$$

2.2.5. Single-Factor Experiment and Orthogonal Design of Soybean Residue SDF Extraction Rate. This experiment investigated the impact of three factors—fermentation temperature (A), fermentation time (B), and inoculation volume (C)—in the co-fermentation of Aspergillus niger and Neurospora sitophila on the extraction rate of SDF from soybean residue. Single-factor experiments were conducted to determine the levels for orthogonal experiments, aiming to identify the optimal conditions for extracting SDF from co-fermented soybean residue by Aspergillus niger and Neurospora sitophila.

2.2.6. Determination of Dietary Fiber Characteristics

2.2.6.1. Determination of Swelling Capacity. Following the method by Li Anping [28], 0.10 grams (m) of soluble dietary fiber was placed in a 50 mL centrifuge tube, and its initial volume V1 (mL) was recorded. Then, 5 mL of distilled water was added, shaken, and placed in a constant temperature incubator at 25°C for 24 hours. The final volume V2 (mL) of soluble dietary fiber after swelling was measured, and the swelling capacity was calculated.

$$Swelling\ Capacity(mL/g) = \frac{V_2 - V_1}{m} \quad (3)$$

2.2.6.2. Determination of Water-Holding Capacity. Following the method by Li Anping [28], 0.10 grams (m_1) of soluble dietary fiber was placed in a 50 mL centrifuge tube, 25 mL of distilled water was added, and it was soaked at 25°C for 1 hour. After centrifugation at 2500 rpm for 10 minutes, the supernatant was decanted, the residual water on the centrifuge tube walls was dried, and the tube containing soluble dietary fiber was weighed as m2 (g). The water-holding capacity of soluble dietary fiber was then calculated.

$$Water - holding\ Capacity(g/g) = \frac{m_2 - m_1}{m_1} \quad (4)$$

2.2.6.3. Fermented Soybean Residue Adsorption Characteristics

1) Adsorption Capacity for Unsaturated Fat

This experiment, following the method by Sangnark A [29], placed 1 gram of soybean sample (m) in a pre-weighed centrifuge tube (m_1) and added 8 grams of peanut oil, mixed evenly, and allowed to stand for 30 minutes. After centrifugation at 4500 rpm for 25 minutes and decanting the upper layer of free fat, the tube containing soluble dietary fiber was weighed (m_2). The oil-holding capacity of soluble dietary fiber was calculated.

$$Oil - holding\ Capacity(g/g) = \frac{m_2 - m - m_1}{m} \quad (5)$$

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2) Adsorption Capacity for Saturated Fat

Following the method by Sangnark A [29], a 50 mL centrifuge tube, labeled as W1, was used. Exactly 1 gram of soybean residue sample, labeled as W2, was accurately weighed and placed into the tube. Then, 8 grams of lard were added, and the tube was left to stand in a water bath at 37°C for 1 hour. Subsequently, it was centrifuged at 4000 rpm for 20 minutes. After decanting the upper layer of oil and wiping dry the residual free lard, the weight was recorded as W3. The oil absorption capacity of soluble dietary fiber was calculated as:

$$Oil\ Absorption(g/g) = \frac{W_3 - W_1 - W_2}{W_2} \quad (6)$$

2.3. Data Processing

Experimental data were processed and optimized using Box-Behnken analysis in Design Expert 11 software.

3. Results and Analysis

3.1. Impact of Single-Factor Experiment Results on Soybean Residue SDF Extraction Rate

3.1.1. Influence of Fermentation Time on Soybean Residue SDF Extraction Rate

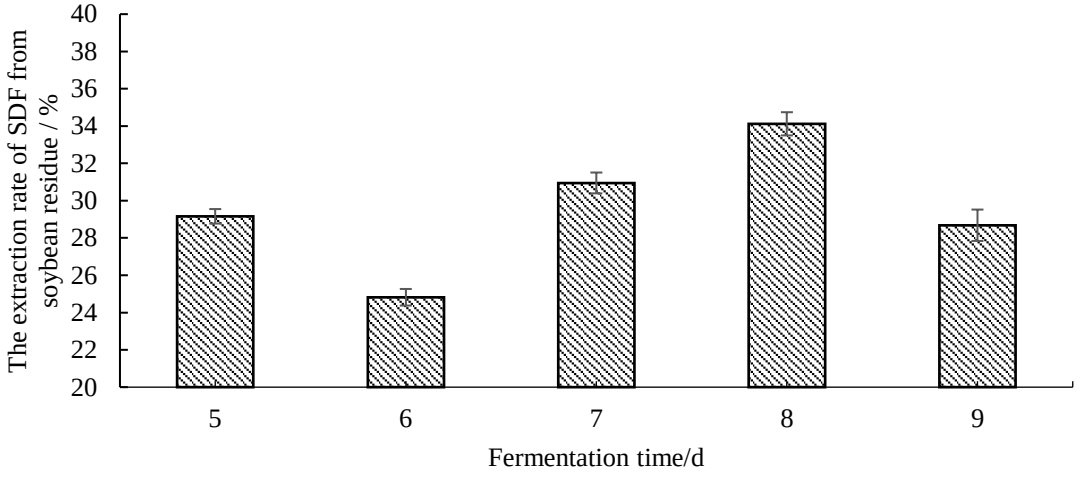

Figure 1. Effect of fermentation time on the extraction yield of SDF from soybean dregs

Under conditions where fermentation temperature, inoculation volume, and other factors remained constant, the extraction rate of SDF from soybean residue exhibited a trend of initially decreasing and then increasing. Studies by Wang Qianman [25] and Gao Daxiang [30] mentioned that during the initial stage of soybean residue fermentation, the growth rate of the strains was slow, resulting in less production of cellulase and amylase. Towards the later stages, due to a gradual decrease in nutrients, the growth conditions of the strains weakened, leading to slow or no growth. Consequently, both excessively short and prolonged fermentation times can affect the extraction rate of soluble dietary fiber from soybean residue.

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3.1.2. Influence of Fermentation Temperature on Soybean Residue SDF Extraction Rate

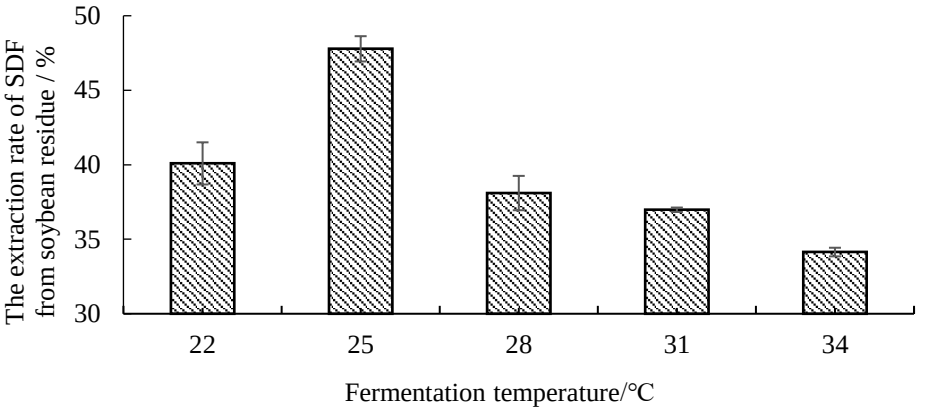

Figure 2. Influence of fermentation temperature on the extraction yield of SDF from soybean dregs

Under constant conditions of fermentation time, inoculation volume, and other factors, the extraction rate of SDF from soybean residue gradually decreased with an increase in temperature. Although the suitable range for microbial growth is between 25°C and 34°C, the highest extraction rate of soybean SDF was observed at 25°C during the experiment. It is speculated that the optimum temperature for cellulase production through the co-fermentation of these two strains might be between 22°C and 28°C. In the research by He Xiaozhe [31], it was noted that excessively high temperatures cause rapid aging of microorganisms, leading to a decreased capacity to produce cellulase and amylase. Conversely, low temperatures slow down microbial growth and enzyme production.

3.1.3. Influence of Inoculation Amount of Strains on Soybean Residue SDF Extraction Rate

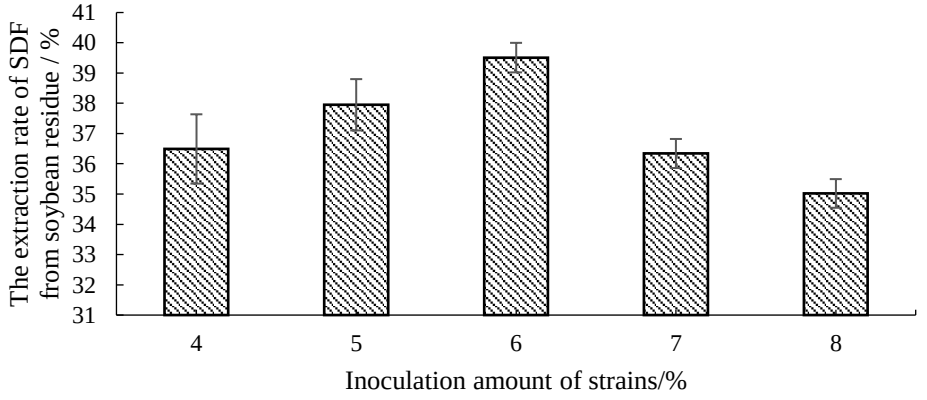

Figure 3. Influence of inoculation amount of strains on the extraction rate of SDF from soybean dregs

Under conditions where fermentation time, fermentation temperature, and other factors remained constant, the extraction rate of SDF from soybean residue gradually increased with an increase in the inoculation amount of strains. At an inoculation volume of 6%, the extraction rate of SDF from soybean residue reached its peak, after which the rate declined with further increases in inoculation volume. Tian Yahong [32] highlighted that insufficient inoculation volume leads to a lower production of metabolic byproducts, resulting in inadequate degradation of nutrients in soybean residue, requiring a longer adaptation period. Conversely, excessive inoculation volume may lead to intra-strain competition, restricting the strains from obtaining sufficient nutrition, thus limiting their fermentation. The research

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by He Xiaozhe [31] also suggested that different proportions of strains can affect the fermentation rate of soybean residue.

3.2. Orthogonal Experimental Results and Analysis of Soybean Residue SDF Extraction Rate

Through single-factor experiments, the optimum ranges for three factors—fermentation temperature (A), fermentation time (B), and inoculation volume of strains (C)—were determined, with each factor having three levels. According to the principles of orthogonal experimental design, an L9 (34) orthogonal experimental table was used to study the interaction of these three factors on the extraction rate of SDF from soybean residue, determining the optimal fermentation extraction conditions. The orthogonal experimental design and results are presented in Table 1.

From the range analysis in Table 2, it is evident that the primary and secondary order of factors' influence is B > A > C. The optimal combination is identified as B1A2C2, representing a fermentation time of 8 days, a fermentation temperature of 22°C, and an inoculation volume of strains at 5%.

Table 1. Orthogonal Test Results and Analysis of SDF Preparation Process Optimization

Number	A	B	C	D(Null)	SDF/DF(%)
1	1	1	1	1	31.51
2	1	2	2	2	32.07
3	1	3	3	3	28.57
4	2	1	2	3	38.30
5	2	2	3	1	29.87
6	2	3	1	2	32.18
7	3	1	3	2	34.60
8	3	2	1	3	28.82
9	3	3	2	1	24.63
k_1	30.72	34.80	30.84	28.67	
k_2	33.45	30.25	31.67	32.95	
k_3	29.35	28.46	31.01	31.90	
R	4.10	6.34	0.83	4.28	
Optimization conditions	A_2	B_1	C_2		
Degree of influence	$B_1>A_2>C_2$				

Table 2. Analysis of Variance of Orthogonal Test Results

Source of deviation	deviation	Df	Mean Square Deviation	F-value	Sig.
A	52.31	2	26.15	2.31	
B	128.40	2	64.20	5.67	*
C	2.30	2	1.15	0.10	
Error	124.52	11	11.32		

Note: * Represents significance level, where more * indicates a higher degree of significance.

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3.3. Results of Dietary Fiber Characteristics Determination

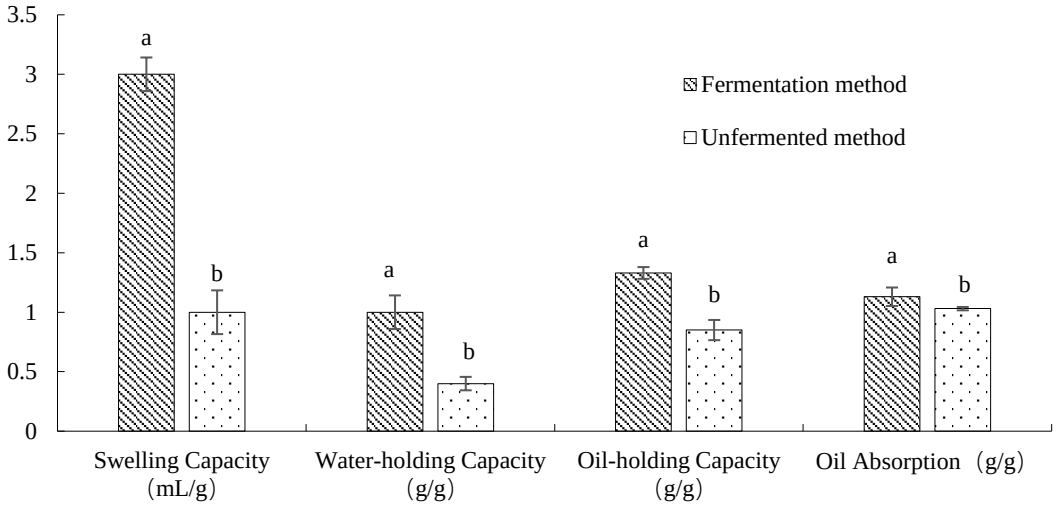

Figure 4. Comparison of dietary fiber characteristics between fermented and unfermented methods

Dietary fiber's structure contains numerous hydrophilic groups, thus exhibiting high water-holding capacity [32-33]. Different dietary fibers possess varying water-holding capacities. Well-characterized SDF absorbs a considerable amount of water in the intestines, increasing fecal volume and speed, thereby reducing the duration of toxin presence in the body and lowering disease occurrence. Swelling capacity and water-holding capacity are characteristics used to measure the quality of soybean residue dietary fiber's physical properties, and the greater the swelling and water-holding capacities, the better the physiological activity of soybean residue dietary fiber [27]. In this study, the extracted SDF from fermented soybean residue showed higher rates compared to unfermented, indicating enhanced water absorption after fermentation, as also evidenced by Tuo Zongcai [34] in their paper.

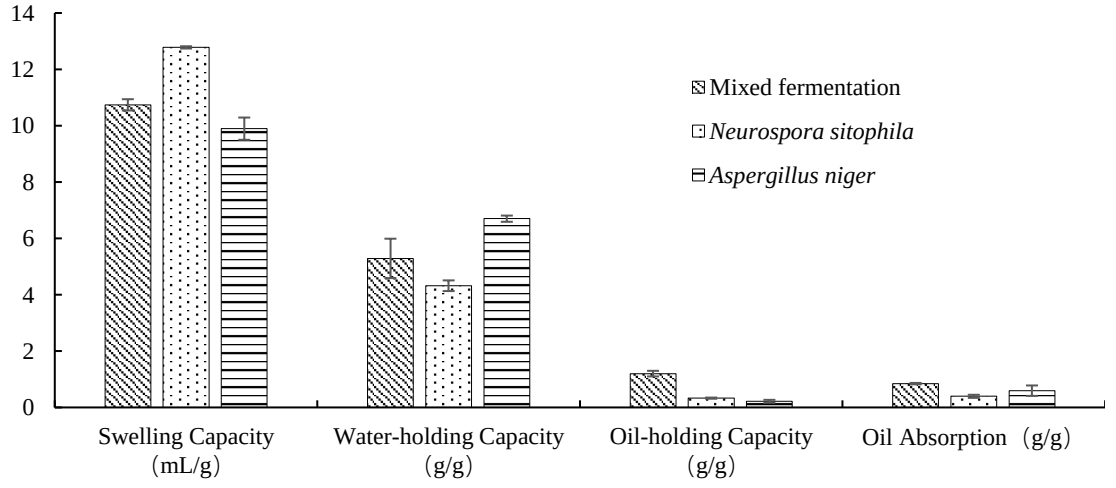

Figure 5. Comparison of dietary fiber characteristics between mixed fermentation and single strain fermentation

Comparing the physical properties of mixed fermentation with single-strain fermentation, it was observed that both swelling capacity and water-holding capacity of dietary fiber from mixed fermentation of soybean residue were significantly improved. Dietary fiber can adsorb organic

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molecules within the body such as cholesterol and bile, influencing the substances in cholesterol, thereby accelerating the excretion of fats [35-36].

4. Conclusion

This study employed soybean dregs as raw material, utilizing *Aspergillus niger* and Rhizopus oligosporus in fermentation to consume carbon and nitrogen sources within soybean dregs. It effectively converted macromolecules such as proteins and starch into smaller molecules, further enhancing the extraction rate of SDF by degrading cellulase and hemicellulase. The optimal fermentation conditions were determined through single-factor experiments and orthogonal experiments. Comparative analysis was conducted on the expansion, water-holding capacity, unsaturated fat adsorption, and saturated fat adsorption of soybean dregs SDF before and after fermentation. Key research findings include:

For every 100g of fresh soybean dregs, the extraction rate of SDF varied with fermentation time. The lowest SDF extraction rate occurred on the 6th day, while the highest, reaching 34.12%, was achieved on the 8th day. This indicates that the fermentation time significantly influences the extraction rate of soybean dregs SDF. Short fermentation periods result in inadequate utilization of carbon and nitrogen sources, leading to minimal conversion of IDF into SDF. An appropriately extended fermentation duration effectively exhausts these sources, enhancing the SDF extraction rate.

For every 100g of fresh soybean dregs, the highest extraction rate of SDF, reaching 47.78%, was observed at 25°C, declining gradually as the temperature increased. This decline resulted from the temperature's impact on the growth metabolism of *Aspergillus niger* and Rhizopus oligosporus, affecting the optimal temperature for cellulase and hemicellulase enzyme activity, thus influencing the fermentation effect on soybean dregs and subsequently reducing the SDF extraction rate.

For every 100g of fresh soybean dregs, the highest extraction rate of SDF reached 39.51% at an inoculation amount of 6%. Prior to reaching 6%, the extraction rate increased with the inoculation amount, indicating incomplete fermentation due to low coverage and slow fermentation. After surpassing 6%, the increased inoculation led to rapid early-stage growth, producing aging cells that inhibited the conversion of IDF to SDF, thereby reducing the SDF extraction rate.

The optimal fermentation conditions for microbial fermentation of soybean dregs to produce SDF were determined as follows: 8 days fermentation time, 22°C fermentation temperature, and 5% inoculation amount, resulting in an SDF extraction rate of 38.30%. Compared to unfermented soybean dregs, fermented soybean dregs significantly improved the extraction rate of soybean dregs SDF.

The fermented soybean dregs demonstrated increased swelling capacity, water-holding ability, and adsorption characteristics compared to the unfermented. This indicates significant modifications in soybean dregs after microbial fermentation, enhancing its binding capacity to water, which plays a vital role in preventing diseases like atherosclerosis and hyperlipidemia. Improving the extraction rate of soybean dregs SDF can provide substantial guidance for future research.

In this experiment, the starch, protein, and fat in soybean dregs were not removed. Studies by Tuo Zongcai [34] found only 2.5% fat content in soybean dregs, while Wang Bo [37] found that starch and fat in soybean dregs were below 1%. These studies indicated a low proportion of starch and fat in soybean dregs, exerting minimal influence on the fermentation process, thus not requiring removal. Although soybean dregs contain higher protein and ash content, these nutrients can be consumed by fermentation or washed away during the process, hence eliminating the need for removal.

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