




# *Penicillium* fermentation combined with enzyme treatment to enhance the release of phenolic acids from wheat bran

Guangyao LI<sup>1</sup>, Xinxiang CHAI<sup>1</sup>, Zhiyi ZHONG<sup>1</sup>, Yan FENG<sup>1</sup>, Haiyan SUN<sup>2\*</sup>, Baoshi WANG<sup>1\*</sup> 

## Abstract

Phenols from wheat bran play an important role in improving the physiological function of human body because of their potential antioxidant capacity. However, most of the phenols in wheat bran exist in the form of insoluble binding, which limits their application in the field of food and medicine. In this paper, the modification of wheat bran by *Penicillium* solid-state fermentation (SSF) was studied. It was found that the content of free phenolic acids in wheat bran was significantly correlated with the activity of key hydrolytic enzymes in the fermentation process. On the fifth day of *Penicillium* fermentation, the content of free phenolic acids was the highest, which was 750.98 µg/g, which was 1.78 times higher than that of the control. The release of free phenolic acids was highest when wheat bran was treated with compound enzyme at 300 U/mL. Furthermore, the structure of wheat bran was significantly changed by fermentation with enzyme addition. On the 5th day of fermentation, the content of free phenolic acids was as high as 1500 µg/g, which was twice as much as that of fermented bran and 3.56 times that of unfermented bran.

**Keywords:** wheat bran; SSF; phenolic acid; enzyme treatment.

**Practical Application:** Fermentation with enzyme addition enhanced the release of free phenolic acids from wheat bran, which laid the foundation for the high-value utilization of wheat bran.

## 1 Introduction

Wheat bran is a by-product of extracting wheat flour and germ in the process of flour processing (Khan et al., 2021). Due to the high content of crude fiber and poor taste, it is generally used as feed or wine raw material with low added value, or as agricultural waste is directly discarded and harms the environment. How to realize the high value utilization of wheat bran is an important problem to be solved urgently (Călinoiu et al., 2019). In recent years, with the in-depth study of the composition and structure of wheat bran, it has been found that wheat bran is rich in phenols which are beneficial to human health (Lee et al., 2020; Xu et al., 2018). Pathological studies show that these phenols have important functional properties and pharmacological effects, and have potential application value in the prevention of many degenerative diseases, such as obesity, cancer, diabetes, cardio-cerebrovascular diseases and so on (Safrida et al., 2022; Zhao et al., 2017). Phenols in wheat bran mainly exist in two forms: soluble free phenol and insoluble-bound phenol. Generally, soluble polyphenols do not react with other molecules physically or chemically, and can be extracted by traditional polar water/organic solvents (Călinoiu & Vodnar, 2018). However, insoluble-bound phenols are usually combined with plant cell wall polysaccharides in the form of ester bonds or covalent bonds, so it is not easy to extract them by traditional methods, resulting in the difficulty of effective utilization of these highly active substances (Wang et al., 2022b). In recent years, more and more studies have shown that the content and antioxidant activity of insoluble-bound phenols in wheat bran are

higher than those of soluble polyphenols (Mazahir et al., 2022). Through the analysis of high performance liquid chromatography, most phenols were bound to wheat bran in insoluble form, and their antioxidant activity and content were higher than those in free form (Zhang et al., 2018b). Abd Razak et al. used *Monascus* and *Rhizopus oligospora* to ferment rice bran, which significantly improved the antioxidant activity, which may be related to the increase in the content of phenolic substances (Abd Razak et al., 2015). Therefore, the efficient release of bound phenols from wheat bran is an effective strategy to improve its bioavailability and health benefits in human body.

A large number of studies have shown that wheat bran modification is an effective means to solve the above problems, but the traditional modification methods (strong acid and strong alkali hydrolysis, microwave-assisted extraction and steam explosion, etc.) are helpful to the release of insoluble-bound phenols (Chen et al., 2018; Kong et al., 2020), but these methods are high-energy and inefficient, destroy the structure of phenols and bring huge burden to the environment. In recent years, solid state fermentation (SSF) technology has made important progress in promoting the release of active substances from wheat bran (Zhang et al., 2018a). SSF involves the growth and product formation of microorganisms on solid particles without or near free water. Because of its cost-effectiveness and environmental advantages, SSF has been identified as a powerful tool for the release of phenols from wheat bran (Baoshi et al., 2020). Specially,

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<sup>1</sup>School of Life Science and Technology, Henan Collaborative Innovation Center in Modern Biological Breeding, Henan Institute of Science and Technology, Xinxiang, China

<sup>2</sup>Hainan Key Laboratory of Tropical Microbe Resources, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China

\*Corresponding author: wangbaoshifsd@126.com; sunhaiyan@itbb.org.cn

filamentous fungi are considered to be the most suitable strains for SSF because of their good binding ability with agricultural waste matrix and good environmental tolerance (Schmidt et al., 2014). In the process of SSF of filamentous fungi, the hyphae are often closely wound in the bran medium and secrete a variety of hydrolytic enzymes (glucoamylase, cellulase and esterase, etc.), which can constantly destroy the chemical bond and change the fiber structure (Gupta et al., 2016; Liu & Qu, 2019), thus promoting the transformation and release of insoluble phenols. The antioxidant activity and content of oat phenols were improved by constructing the co-culture and fermentation system of *Monascus* and *Bacillus subtilis*, which may be closely related to the hydrolase secreted by microorganisms during the SSF process (Chen et al., 2020). In general, the single strain fermentation system, the insoluble phenols are often released incompletely because of the low enzyme activity. In order to further increase the release of phenols from wheat bran, the cell wall of wheat bran needs to be degraded by enzyme to realize the synergistic fermentation with additional enzyme. Liu et al. used lactic acid bacteria fermentation and complex enzyme hydrolysis to significantly increase the total phenol and antioxidant activities of  $\alpha$ -amylase cooking pretreatment rice bran aqueous solution (Liu et al., 2017b). Therefore, fermentation with enzyme addition was helpful to improve the deficiency of enzyme secretion in a single-strain fermentation system, and is an important way to achieve efficient release of phenols from wheat bran (Wang et al., 2022a).

This study focused on the relationship between the hydrolase series produced by microorganisms, the modification of bran and the release of soluble polyphenols during the fermentation of wheat bran by *Penicillium*. At the same time, the strategy of SSF coupled with compound enzyme treatment was proposed to release soluble phenolic acids from wheat bran, which may provide a theoretical basis for the high value utilization of phenolic substances from bran.

## 2 Materials and methods

### 2.1 Strain and medium

*Penicillium* A1 was stored in the laboratory, grown in PDA medium and cultured at 28 °C for 8-10 days to obtain mature spores. The spores in eggplant bottles were washed with sterile water, shaken and dispersed, and a certain concentration of spores' suspension was prepared.

### 2.2 Wheat bran fermentation process

The fermentation medium was mixed evenly with bran and water at 1:1 (w/w), and sterilized at 121 °C for 30 min. The *Penicillium* A1 spores were inoculated at an inoculum volume of  $1 \times 10^6$  /g wheat bran and fermented at 28 °C for 7 days.

### 2.3 Enzymatic treatment process

A certain amount of unfermented/fermented bran was accurately weighed and placed in a 50 mL centrifuge tube, and 1 mL of different concentrations of exogenous hydrolase was added, respectively. After the treatment, the mixture was fully

stirred and placed in a dark environment at 30 °C for 6 h. Then the yield of free phenolic acid in hydrolysate was detected.

### 2.4 *Penicillium* fermentation process with enzyme addition

On the basis of the above experiments, to further strengthen the release of free phenolic acids in bran, a compound enzyme preparation was added at 300 U/g during the *Penicillium* A1 fermentation.

### 2.5 Analysis method

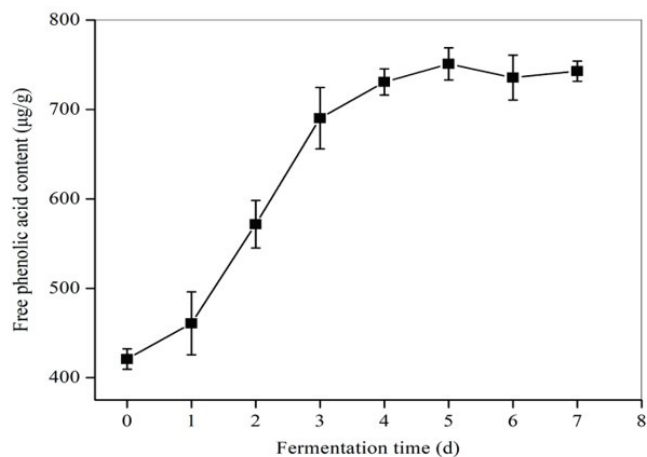
The  $\alpha$ -Amylase activity was determined by the method of Bei et al. (2018) and the cellulase activity was determined by the method of DNS. A certain amount of bran sample was added to a 50 mL centrifuge tube with 60% (v/v) ethanol solution, sonicated at 50 °C with ultrasonic power 60 W, and then centrifuged (7000 rpm for 5 min), which was then assayed. The content of free phenolic acid was detected using Folin-Ciocalteu's reagent (Bei et al., 2018).

## 3 Results and discussion

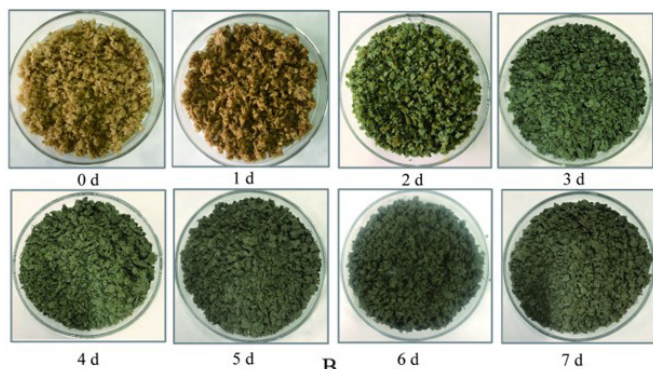
### 3.1 Free phenolic acid content and its morphological changes during bran fermentation

The trend of free phenolic acid content of bran before and after fermentation, and the results are shown in Figure 1A. After fermentation by *Penicillium*, the free phenolic acid content of bran showed an increasing trend with the increase of fermentation days, reaching the highest level of 750.98  $\mu\text{g/g}$  at the 5th day of fermentation, which was 330.13  $\mu\text{g/g}$  higher than that of unfermented bran. This may be closely related to the fact that the microorganisms produced hydrolytic enzymes during the fermentation process broke the chemical bond between the bound phenols and the plant cell wall, thus degrading the bran structure and releasing more free phenolic acids. The free phenolic acid content decreased slightly after 5 d of fermentation. This phenomenon is attributed to the fact that: firstly, the microorganisms may produce a number of oxidative enzymes to oxidize the phenolics during the fermentation process. Secondly, with the prolongation of fermentation time, the substrate of fermentation gradually decreased, and microorganisms may use some phenolic substances in bran as carbon source for their own metabolism, thus resulting in the reduction of soluble polyphenols content (Wang et al., 2016).

While studying the release pattern of free phenolic acids from the bran, the changes of bran morphology during 7 d of fermentation were systematically observed, and the results are shown in Figure 1B. With the extension of fermentation time, the color of fermented bran gradually deepened from green to greenish gray. After 3 d of fermentation, the bran became loose, decreased in weight, and decreased in moisture content. This phenomenon may be attributed to the facts that at the initial stage of fermentation, the higher water content in the bran substrate condensed with each other to form larger particles, while *Penicillium* absorbed water for its own metabolism during the growth process, resulting in a decrease in water content in the substrate, resulting in dry and loose wheat bran in the



A



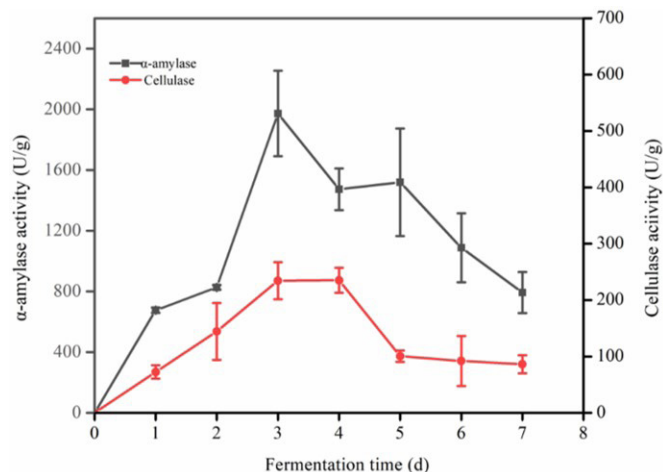
B

**Figure 1.** The content of free phenolic acid (A) and the change of bran morphology (B) during bran fermentation.

later stage of fermentation. Additionally, in the fermentation process, the mycelium of *Penicillium* could penetrate the bran substrate, while secreting a variety of hydrolytic enzymes can quickly and efficiently break up the rigid structure of the bran tissue, resulting in a looser bran.

### 3.2 Changes in key hydrolytic enzyme systems during bran fermentation

To elucidate the relationship between the hydrolytic enzymes produced by microorganisms and the release of free phenolic acid content in bran during the fermentation of *Penicillium*, we measured the activity of key hydrolytic enzymes produced during the fermentation process with the results shown in Figure 2. Interestingly, the two enzyme activities showed different laws. In comparison, the  $\alpha$ -amylase enzyme activity was significantly higher than that of the cellulase, probably due to the facts that the microorganisms preferentially attacked the dextrin layer of bran during fermentation, which had a higher starch content and a lower cellulose content. Overall, it was found that the activity of  $\alpha$ -amylase and cellulase both showed a trend of increasing first and then decreasing. The activity of  $\alpha$ -amylase reached the maximum of 1972.40 U/g at the 3rd day of fermentation. The activity of  $\alpha$ -amylase increased sharply from 0 to 3 d of fermentation, probably because the fermentation conditions were more suitable and the microorganisms had strong metabolism



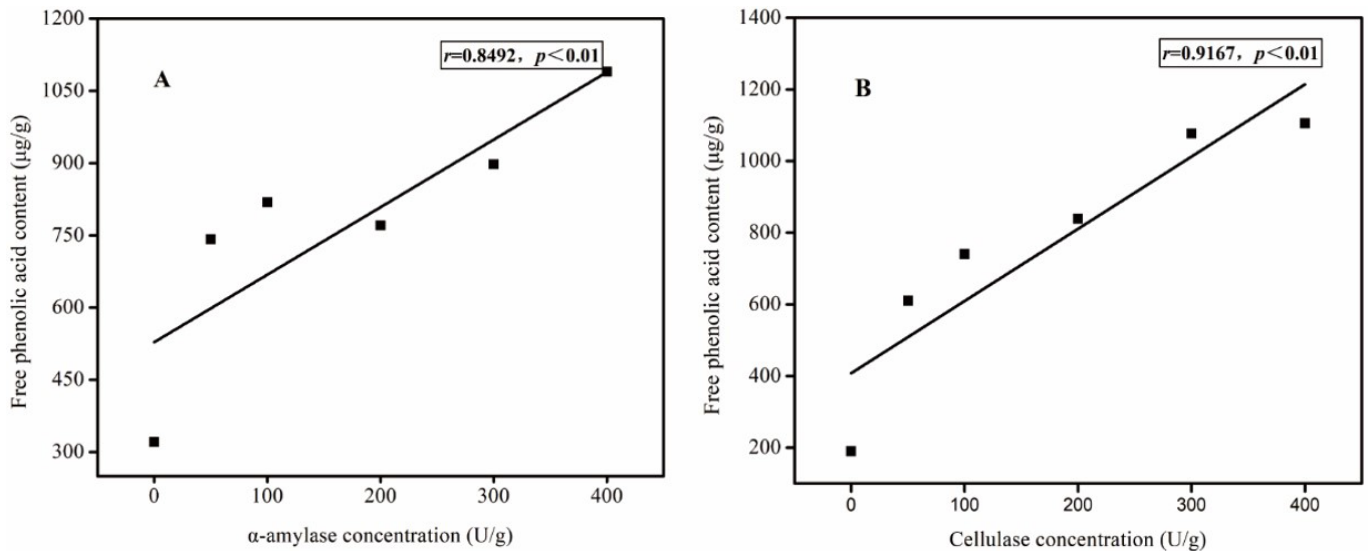
**Figure 2.** Changes in the activities of key hydrolases ( $\alpha$ -amylase and cellulase) during *Penicillium* fermentation.

and enzyme production ability. When the fermentation time exceeded 3 d, the activity started to decrease as the fermentation proceeded.

This may be due to the fact that most of the starch particles in wheat bran are distributed in the aleurone layer, and the aleurone layer is first degraded by microorganisms in the fermentation. With the progress of fermentation, after the aleurone layer is degraded, there is no inducing factor to induce the synthesis of  $\alpha$ -amylase (Yin et al., 2018). The highest cellulase activity of 235.19 U/g was reached at the 4th day of fermentation, which is consistent with the results of Yan et al. research on the change of cellulase activity of sugarcane (Yan et al., 2020). In the first 3 d of fermentation, the activity of cellulase showed a linear increase trend, and after 4 d of fermentation, the activity began to decline slowly, probably because the substrate cellulose was gradually consumed with the prolongation of fermentation time.

### 3.3 Relationship between exogenous hydrolase addition and bran free phenolic acid release

To further demonstrate the correlation between the free phenolic acid release content of bran and the activity of hydrolytic enzymes produced by microorganisms, we added different concentrations of exogenous  $\alpha$ -amylase and cellulase to treat bran, and the results are shown in Figure 3. The correlation R-values between exogenous  $\alpha$ -amylase and cellulase and free phenolic acid release from bran were 0.8492 and 0.9167 ( $p < 0.01$ ), respectively, as shown in Figure 3A and Figure 3B. This indicated that the release of free phenolic acids was significantly correlated with  $\alpha$ -amylase, cellulase in the process of wheat bran fermentation by *Penicillium*. This phenomenon is consistent with the study by Wang et al. that during the co-fermentation of guava leaves by *Monascus* and *Bacillus* BS2, the release of soluble polyphenols was significantly correlated with the activities of  $\alpha$ -amylase and cellulase (Wang et al., 2018). Similarly, Chen et al. co-cultured *Monascus* and *Bacillus subtilis* for fermentation with oats as raw material and found that the hydrolase activities of cellulase and  $\alpha$ -amylase were significantly correlated with the total phenolic



**Figure 3.** Relationship between the release of free phenolic acid from bran and the concentration of key hydrolytic enzymes A:  $\alpha$ -amylase B: Cellulase.

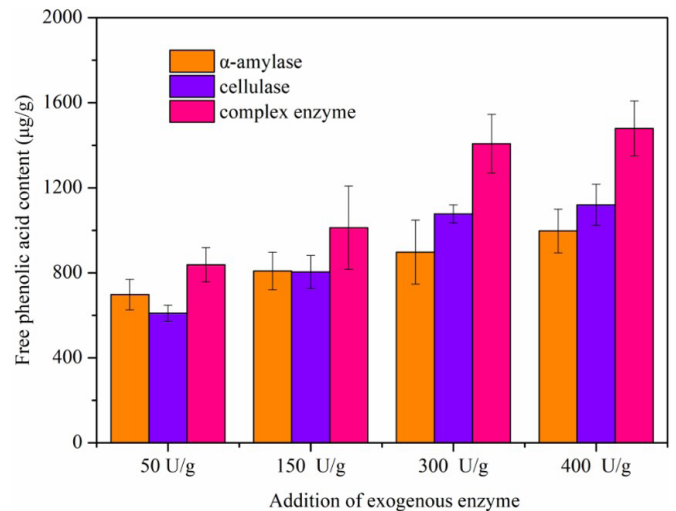
content (Chen et al., 2020). In conclusion, these results indicate that the release of free phenolic acids is closely related to the key hydrolase systems secreted during fermentation.

### 3.4 Effect of exogenous complex enzyme treatment on the release of free phenolic acid from bran

The above experiments have proved that the hydrolase produced by *Penicillium* in the process of bran fermentation can significantly affect the presence of phenolic compounds in grains, and exogenous enzyme is closely related to the release of free phenolic acids from bran. To further enhance the release of free phenolic acids from bran, different concentrations of the complex enzymes were examined to treat bran, and the results are shown in Figure 4. With the increase of exogenous enzyme concentration, the release of free phenolic acid from bran gradually increased, and when the increase continued to 300 U/mL, the free phenolic acid content increased at a slow rate. Combining the cost of exogenous enzyme addition, the release of free phenolic acid tended to the highest value of 1407.35  $\mu\text{g/g}$  when bran was treated with exogenous enzyme concentration of 300 U/mL, which was 1.57 times higher than that  $\alpha$ -amylase and 1.31 times higher than that cellulase. Many studies have shown that the release of phenolics in cereals is not only related to  $\alpha$ -amylase and cellulase, but also to the content of some esterases (Yin et al., 2018). Similarly, treatment of wheat bran with arabinofuranosidase and xylanase resulted in increased free phenolic acid content and enhanced antioxidant capacity (Xue et al., 2020). In conclusion, the combined enzyme treatment of bran could further promote the release of free phenolic acid, and the effect was better than that of single enzyme treatment.

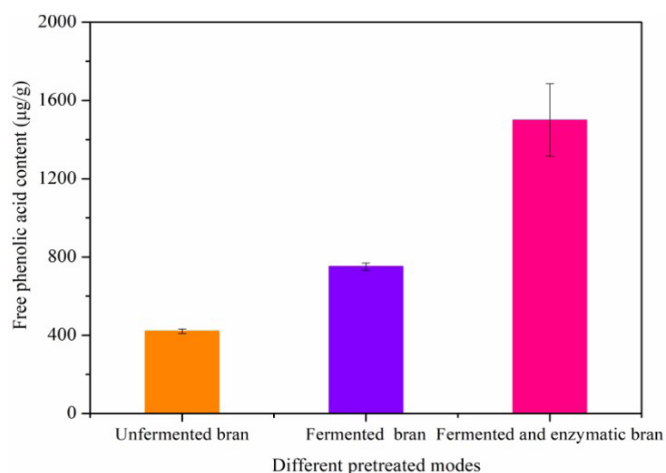
### 3.5 Effect of synergistic fermentation with complex enzyme addition on the release of free phenolic acid from bran

*Penicillium* fermentation was able to promote the release of free phenolic acids in bran, but in the fermentation system, the



**Figure 4.** Effects of different concentrations of exogenous enzymes on the release of free phenolic acids.

free phenolic acids could not be fully released, probably due to the relatively low enzymatic activity of the key hydrolases produced. In order to further enhance the release of free phenolic acids in bran, compound enzyme was added during the fermentation process. The release of phenolics was highest in the synergistic fermented bran with compound enzyme addition, which was twice as much as that of the fermented bran and 3.56 times as much as that of the unfermented bran as shown in Figure 5. Although *Penicillium* fermentation could increase the content of free phenolic acids in bran, the free phenolic acids were not fully released due to the complexity of plant cell wall structure and the limitation of microbial enzyme production capacity. In contrast, the release of free phenolic acids from bran was further enhanced by the treatment of synergistic fermentation with compound enzyme addition. Similarly, Liu et al. added



**Figure 5.** Release of free phenolic acids from bran under different treatments.

glucosidase and acidic cellulase to the fermentation system after fermentation treatment of rice bran, and the release of free phenolic acid was further enhanced (Liu et al., 2017a). Therefore, the synergistic fermentation with enzymes addition is an important way to achieve efficient release of bran phenolics.

Promoting the release of active phenolics from cereal by-products by means of SSF is a hot topic of interest for current researchers. We found that the release of free phenolics is caused by the disruption of bran structure by relevant hydrolytic enzymes secreted by microorganisms. Among them,  $\alpha$ -amylase can hydrolyze starch to oligosaccharides by excising its 1,4-glucosidic bonds; cellulase is a multi-component enzyme consisting of endocytic cellulase,  $\beta$ -glucosidase, and cellobiose hydrolase, which can endo- or exo-cut the ends of cellobiose chains attached between plant cell walls to bioconvert cellulose to soluble sugars (Nawrot et al., 2010). In summary, possible enzymatic mechanism of free phenolic acids release during bran fermentation by *Penicillium* coupled with enzyme addition is as follows. Firstly, the hydrolases such as  $\alpha$ -amylase and cellulase produced during *Penicillium* fermentation can break the chemical bonds linking insoluble bound phenols to lignin, cellulose, hemicellulose and other polysaccharide structures, thus converting insoluble phenolic substances into soluble free states and thus increasing release of free phenolic acids. Secondly, the cellulase produced during the fermentation of *Penicillium* can directly destroy the fiber structure of the plant cell wall, thus releasing the intracellular phenolic substances. Finally, *Penicillium* fermentation process secreted hydrolytic enzyme system, so that the bran to form a porous loose structure can make organic solvents easier to enter, thus releasing free phenolic acids.

Collectively, *Penicillium* fermentation treatment of wheat bran promoted the release of free phenolic acid content, and showed an increasing trend with the increase of fermentation days, and reached the highest at the 5th day of fermentation. It was also found that the release of free phenolic acids was closely related to the key hydrolytic enzymes secreted during *Penicillium* fermentation. Furthermore, the free phenolic acid content of wheat bran treated by *Penicillium* fermentation with

enzyme addition was the highest, which was 3.56 times higher than that of unfermented bran. Thus, fermentation with enzyme addition technology is an important way to realize the high value of bran, which is expected to provide a certain reference for the comprehensive utilization of agricultural processing by-products.

#### 4 Conclusion

SSF by *Penicillium* promoted the release of free phenolic acids from wheat bran, and the content of free phenolic acids was the highest (750.98  $\mu\text{g/g}$ ) at 5 days of fermentation. It was found that the content of free phenolic acids was closely related to the activity of key hydrolase systems. Furthermore, the content of free phenolic acids was 3.56 times higher than that of unfermented bran by the strategy of fermentation with enzyme addition. This study is expected to provide high value-added utilization of wheat bran and related functional food development.

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