

# Changes in the Content and $\alpha$ -Glucosidase Inhibition Efficiency of Active Substance in Mulberry Leaves during Natural Fermentation

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**Abstract:** Since mulberry leaves are rich in alpha-glucosidase inhibiting ingredients, they have been widely used in the treatment of diabetes since ancient times. Microbial fermentation is an effective way to increase the content and efficacy of active ingredients in medicinal plants. In this paper, the beneficial endophytic bacteria in fresh mulberry leaves were used for solid state fermentation, and the effects of fermentation on flavonoid content and  $\alpha$ -glucosidase inhibition in fresh mulberry were investigated. The results showed that the polysaccharide content of fresh mulberry leaves decreased rapidly during natural fermentation. The content of flavonoids in mulberry leaves decreased first and then increased. After 10 days of fermentation, the flavonoid content in mulberry leaves was  $3.858 \pm 0.193$  mg/g dried ML, which was 2.14 times that of MLF in unfermented mulberry leaves. The alkaloid content increased significantly, reaching the highest value of  $12.407 \pm 0.993$  mg/g dried ML on day 8 of fermentation, which was about 2.5-folds higher than that of unfermented mulberry leaves. The inhibitory rate of the mulberry leaf extract (MLE<sub>8</sub>) fermented for 8 d was higher than that of the unfermented fresh mulberry extract (MLE<sub>U</sub>) when compared with that of  $\alpha$ -glucosidase. At the concentration of 0.05 mg/ml, the inhibitory rate of  $\alpha$ -glucosidase from MLF-F8 increased to  $82.7 \pm 4.15\%$  while that from MLF-UF was only  $66.8 \pm 3.34\%$ . At the concentration of 5  $\mu$ g/ml, the  $\alpha$ -glucosidase inhibitory rate of MLA-F8 was  $87.5 \pm 6.55\%$ , while MLA-UF was only  $71.4 \pm 5.57\%$ .

**Keywords:** Diabetes mellitus, Mulberry leaves,  $\alpha$ -glucosidase inhibitors, solid-state fermentation, active compounds

## 1. Introduction

Only next to tumor and cancer, diabetes has become a disease that seriously endangers human life and health. According to the 10th edition of Diabetes Atlas released by the International Diabetes Federation, the number of diabetes patients in the world was 537 million in 2021, accounting for 10.5% of the world population. The number will keep rising to 643 million by 2023, and even reaching up to 783 million by 2045. Along with the rapid increase of the patient's number, the treatment cost of diabetes also keeps rising. The report also found that the direct expenditures associated with diabetes treatment increased from \$232 billion in 2007 to \$966 billion in 2021, which is expected to reach \$1 trillion. Therefore, diabetes mellitus has become an urgent medical task to develop effective and low-cost diabetes drugs, slow down the occurrence of complications, and improve the quality of life of patients as much as possible [1-2].

In recent years, due to the unique theory of traditional Chinese medicine, the system of prescription drugs has obvious advantages in the prevention and treatment of many complex diseases. For medicine and nutrition, the plant has many aspects in preventing and treating diabetes because it helps in psychological control by reducing blood sugar levels and can prevent or delay complications of diabetes. Hypoglycemic preparations derived from natural plants typically have advantages such as lower side effects, synergistic effects of multiple components, and higher efficacy [3-4]. Therefore, more and more attention has been paid to finding safe and economical natural hypoglycemic ingredients from medicinal plants.

Mulberry leaves (ML) are a kind of medicinal and edible homologous plant widely spread in the world. Mulberry leaves have a long history of being used to treat diabetes. With the development of

modern technology and medicine, the efficacy and mechanism of mulberry leaf in lowering blood sugar have been deeply studied. For example, Xie et al. [5] found that the TCM symptom scores and insulin resistance of the patients with type 2 diabetes with lung heat-fluid injury mellitus would be effectively improved by treated with mulberry leaf tea combined with metformin. It was suggested that mulberry extract combined maltodextrin could obviously reduce the increase of total blood glucose in patients after ingesting the maltodextrin for 120 min. More importantly, the increase in total insulin was also significantly suppressed during the same time period. Thaipitakwong et al. [6] found that mulberry leaves, as a dietary supplement, could reduce the digestion and absorption of starch in subjects, thereby benefit for the postprandial glucose control.

Alpha-glucosidase inhibitors can delay or prevent the hydrolysis of carbohydrates by inhibiting the activity of carbohydrate hydrolases, such as  $\alpha$ -amylase and  $\alpha$ -glucosidase. Therefore, by monitoring the activity of  $\alpha$ -glucosidase, real-time characterization of the decomposition level of food polysaccharides in the human body can be achieved, thereby achieving the effect of regulating blood sugar [7]. Nowadays, more and more researches have been focus on finding new natural hypoglycemic drugs that are green, safe, efficient, and inexpensive and have few side effects.

The active ingredients such as flavonoids, polysaccharides, and alkaloids in mulberry leaves (ML) have been proven to be effective  $\alpha$ -glucosidase, with significant effects on reducing blood sugar and blood lipids [8, 9]. In recent years, there have been a lot of studies on the hypoglycemic effect of  $\alpha$ -glucosidase inhibitors in ML. Cui et al [10]. Reported that the polysaccharide components obtained by low concentration alcohol precipitation had better  $\alpha$ -glucosidase inhibitory (AGI) effect than other polysaccharide fractions. Ji et al. [11] used  $\alpha$ -glucosidase inhibition model to evaluate the ML extracts containing different components, which indicated that enzyme inhibition rate increased with the increase of the concentration of each component, and the ML alkaloid (MLA) had the strongest inhibitory activity. In addition, the combination of MLA and mulberry leaf flavonoids (MLF), as well as MLA and MLP all showed synergistic effect, but there was no obvious synergistic effect between MLP and MLF, which confirmed the interaction of mulberry leaf components in the regulation of blood sugar use.

Microbial fermentation processing of medicinal plants has a long history. Fermentation processing plays a significant role in the extraction, separation and improvement of the active ingredients in medicinal plants. On the one hand, microorganisms can biotransform or decompose natural active compounds and produce new ingredients with new effects. For example, Guo et al. [12] comprehensively evaluated the fermentation performance of different edible fungi on mulberry leaves, which showed that *Monascus anka* was most suitable for producing quercetin and kaempferol. On the other hand, some microorganisms can secrete a variety of enzymes to destroy the dense cell wall structure of plants, thereby increasing its specific surface area. It will benefit for improving the dissolution rate and reducing the separation and purification cost of its active ingredients [13]. Moreover, solid state fermentation processing can also show the dual effect of biological transformation and promoting the extraction of active ingredients [14, 15].

In this paper, the beneficial endophytic bacteria rich in fresh mulberry leaves were used to study the solid-state fermentation of mulberry leaves, the appropriate fermentation parameters were determined, and the effects of fermentation on the flavonoids content and  $\alpha$ -glucosidase inhibition performance of mulberry leaves were studied.

## 2. Materials and Methods

### 2.1. Materials

Mulberry leaves, collected from Shangluo (Shanxi, China), were water washed and drained the surface moisture, then stored at 4°C for later use. The flour and corn cobs came from a local supermarket. Rutin, 1-deoxynojirimycin (DNJ), 4-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) and  $\alpha$ -glucosidase were prepared by Yuanye Biotechnology Co., Ltd (Shanghai, China). Raye saline, 4-hydroxy piperidinol, D-glucose, acarbose, etc. were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China).

## **2.2. Fermentation of Fresh Mulberry Leaves**

### **2.2.1. Processing of fresh mulberry leaves**

The surface of fresh ML was disinfected with 75% alcohol and then rinsed with sterile water. All the operation was conducted in a super clean bench. After air-dried the surface moisture, cut the ML into small pieces. Then, stored at 4°C for use. The fresh ML was dried at 60°C till constant weight and the moisture content of ML was calculated according to the weight loss of ML.

### **2.2.2. Solid state fermentation of fresh mulberry leaves**

First, corn flour and bran (w/w, 1:1) were mixed with distilled water evenly with a solid-to-liquid ratio of 1:4, and then sterilized at 121°C for 15 min. Then, in the super clean bench weighed 20 g fresh ML, added 5 g above mentioned sterilized corn flour and bran mixture and stirred evenly. The mixture was placed in an incubator and cultivated at 28-30°C statically. Samples were taken at appropriate interval and analyzed for relevant parameters. Three parallel experiments were designed for all the solid-state fermentation experiments.

### **2.3. Assay of the mulberry leaves polysaccharide content**

Mixed 4.0 g ML with 30 mL of distilled water, and then extracted ultrasonically at 80°C for 20 min. Then the supernatant was collected by filtration. The solid residuals were extracted again by repeat the above steps. Combined the supernatant to get the mulberry leaves polysaccharide extract (MLP). Then mixed 4 mL of the above MLP with 2 mL of 6 mol/L HCl. Reacted in 100°C water bath for 30 min. Then cooled to room temperature and adjust pH value to 8.0. Dilute the volume to 20 mL with distilled water. Then DNS method was used to measure the MLP content. Measured the absorbance at 550 nm with 754PC UV-Vis Spectrophotometer (Jinghua Science and Technology Co., Ltd., Shanghai, China). Using 1 mg/mL glucose as the standard, followed the above steps to draw a standard curve with absorbance (A) as the vertical axis and glucose concentration (C) as the horizontal axis. Then calculated the content of MLP according to the standard curve.

### **2.4. Assay of the mulberry leaves flavonoids content**

Added 40 mL of 70% ethanol solution with 4.0 g of ML and extracted ultrasonically at 50 °C for 40 min. After filtration, the solid residuals were extracted again by repeat the above steps. The two extracted supernatants were combined and used to determine the content of mulberry leaves flavonoid (MLF). NaNO<sub>2</sub>-Al(NO<sub>3</sub>)<sub>3</sub>-NaOH chromogenic method was used for the determination of MLF [16]. The absorbance of the sample was measured at 510 nm. Taken 0.2 mg/mL of rutin solution as standard, follow the above steps to draw a standard curve with absorbance (A) as the vertical axis and rutin concentration (C) as the horizontal axis. Then calculate the content of MLF using the standard curve.

### **2.5. Assay of the mulberry leaves alkaloids content**

Accurately weighed 5 g of ML and added with 50 mL of 25% ethanol-0.05 mol/L HCl solution. Then ultrasonically extracted in a 30 °C water bath for 20 min and collected the supernatant by filtration. The solid residuals were extracted again by repeat the above steps. Combined the supernatant and evaporated it to dryness through vacuum rotation. Dissolved the dried product with 0.05 mol/L HCl and fixed volume to 4 mL for subsequent determination of MLA. The detailed measure steps were described as follows [17]. Mixed the sample with Reye's salt solution and incubate in an ice bath for 2 hours. Centrifuged to collect sediment, and then add ice-bathed ethyl acetate. Mixed well and centrifuged again. The precipitate was dissolved in 70% acetone, and the absorbance of the solution was measured at 525 nm. Taken 4-hydroxypiperidinol as standard, draw the standard curve with the absorbance (A) as the vertical axis and 4-hydroxypiperidinol concentration (C) as the horizontal axis. Calculated the MLA content based on the standard curve.

### **2.6. $\alpha$ -glucosidase inhibitory activity assay**

Alpha-glucosidase inhibitory (AGI) rate of different inhibitors were assayed as described as Kwon et al in with a slight modification. The experiment was completed on a 96-well plate (MB-96B, Suzhou Chenghuai Technology Co., Ltd., China), and four groups were set up in the experiment. The sample group (S) was used 0.05 mg/mL MLF, 5  $\mu$ g/mL MLA, or 1 mg/mL MLP as inhibitor. The group without

adding  $\alpha$ -glucosidase was used as the sample blank (SB). The blank group (B) was only PNPG substrate. The group without adding inhibitor was used as control (C). The absorbance at 405 nm was measured.

The AGI rate was calculated according to the following formula (1).

$$\text{Inhibitory rate of } \alpha\text{-glucosidase (\%)} = \left(1 - \frac{A_S - A_{SB}}{A_C - A_B}\right) \times 100\% \quad (1)$$

Where,  $A_S$ ,  $A_{SB}$ ,  $A_B$  and  $A_C$  are the absorbance of the sample group, sample blank group, blank group and control group, respectively.

### 3. Results and Discussion

#### 3.1. Standard curve for the assay of main active components in ML

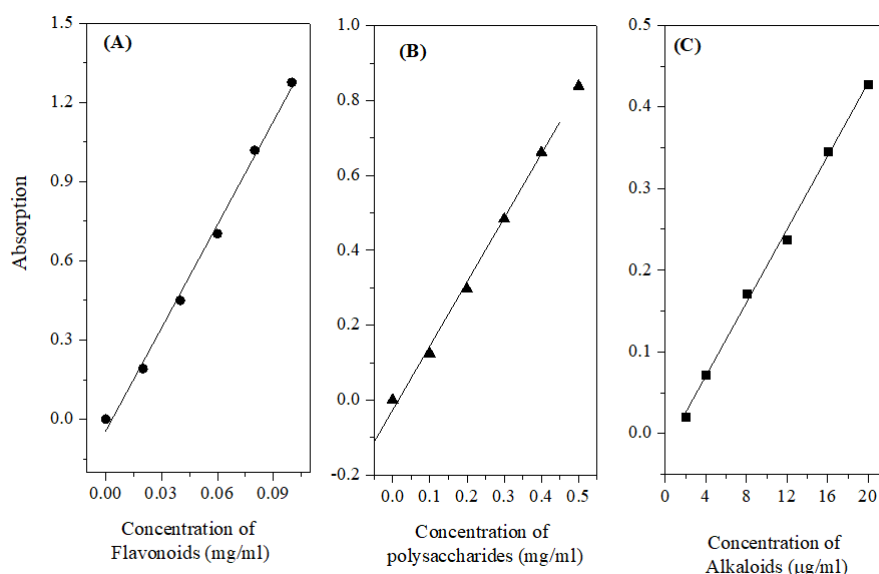


Figure 1: The standard curves for the spectroscopic assay of MLF (A), MLP (B), and MLA (C)

In this paper, the DNS method,  $\text{NaNO}_2\text{-Al}(\text{NO}_3)_3\text{-NaOH}$  chromogenic method and Reye's salt method were used for the content determination of MLP, MLF and MLA, respectively. The standard curves for MLP, MLF and MLA were  $Y=1.713 X -0.0273$  ( $R^2=0.996$ ),  $Y=13.011X-0.044$  ( $R^2=0.9945$ ) and  $Y=0.0225X-0.0196$  ( $R^2=0.997$ ), respectively. Since the correlation coefficients  $R^2$  were all higher than 0.99, the established methods could be used for the content determination of various active ingredients in ML accurately. Therefore, the above methods were used to investigate the variation of active compounds of fresh mulberry leaf during the solid state fermentation.

#### 3.2. Content variation of active substances in ML during the solid state fermentation

There are some microorganisms in fresh ML, also known as plant endophytes. A large number of studies have shown that the metabolites of some endophytes have the same or similar active compounds and function as the host plant. Therefore, plant endophytes are widely used in the biological modification and enrichment of active substances in Chinese herbal medicines [18, 19].

In this paper, the plant endophytes in fresh ML was used for the solid state fermentation of ML. The results indicated that the contents of MLP, MLF and MLA were all changed with the fermentation time (Fig.1). Generally, the polysaccharides content in ML was ranged from 78.65 mg/g dried ML to 233.47 mg/g dried ML [20]. The content of polysaccharides contained in the tested ML was  $112.09 \pm 8.6$  mg/g dried ML. The results showed that along with the fermentation the total polysaccharides content in the fermented ML (FFML) decreased rapidly from the 2nd day of fermentation and dropped to  $11.04 \pm 1.55$  mg/g dried ML at the 8d of fermentation (Fig.2A). It meant that the polysaccharides might be consumed by microbes to support their growth and metabolism.

Mulberry leaf flavonoids (MLF) have glucose-lowering, insulin resistance-improving, and anti-inflammatory effects [21, 22]. Therefore, it is one of the main active substances of mulberry leaves to exert hypoglycemic effect. The content of flavonoids in the used fresh ML was  $2.57 \pm 0.22$  mg/g dried ML. As the fermentation progressed, the content of flavonoids extracted from FFML showed a trend of decreasing first and then increasing. However, the MLF content obtained in the ML fermented for 2 d was reduced to  $1.80 \pm 0.11$  mg/g dried ML. Afterwards, the content of MLF began to increase gradually, and reached to  $3.86 \pm 0.19$  mg/g dried ML. It was 2.14 times of the MLF content in unfermented fresh ML (Fig. 2B).

Alkaloids are one of the most important hypoglycemic active substances in ML, especially its unique 1-deoxynojirimycin (DNJ) is a potent  $\alpha$ -glucosidase inhibitor. The total content of MLA in the used fresh ML in this paper was determined as  $3.57 \pm 0.29$  mg/g dried ML. After natural fermentation, the total alkaloids in the FFML showed a significant increase. The total MLA content measured in the FML that fermented for 8d was  $12.41 \pm 0.99$  mg/g dried ML, which was about 2.5-folds of that in unfermented ML (Fig.2C).

During microbial fermentation, a large number of enzymes are secreted, especially carbohydrate hydrolytic enzymes, such as cellulase, esterase, xylanase, and  $\beta$ -glucosidase, etc. [23]. When the carbon source in the system is insufficient, these enzymes would be secreted by microorganisms and will first hydrolyzed the easily biodegradable macromolecular carbohydrates in MLP. Therefore, some small molecular sugars would be released to maintain the further growth and metabolism of microorganisms, resulting in the significant decrease of MLP content as the fermentation progressed. During the fermentation process of plant raw materials, microorganisms can secrete various hydrolytic enzymes, such as glucosidase, amylase, cellulase, etc. [24]. They effectively decompose the cell wall structure, not only promoting the release of active substances in plant materials, but also enhancing the biological activity of plant materials. Therefore, much more free and bound flavonoids would be released in the ML extracts [25-26].

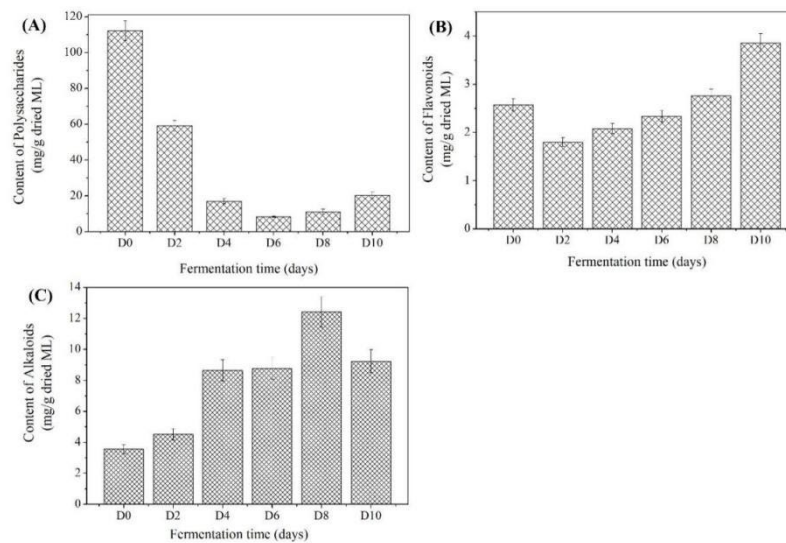


Figure 2: The variation of active substances in mulberry leaves at different fermentation time. A, mulberry leaf polysaccharides content (MLP); B, mulberry leaf flavonoids content (MLF); C, mulberry leaf alkaloids content (MLA)

### 3.3. Effects of fermentation on AGI rate of ML extracts

The AGI rate of MLP (1 mg/ml), MLF (0.05 mg/ml) and MLA (5  $\mu$ g/ml) that extracted from unfermented fresh ML (ML-UF) and 8 d fermented ML (ML-F8) were compared (Fig. 3). The results indicated that compared to ML-UF, fermentation could enhance the AGI activity of ML, since the  $\alpha$ -glucosidase inhibitory rate of MLF extracted from ML-UF and ML-F8 was  $66.8 \pm 3.34\%$  and  $82.7 \pm 4.15\%$ , respectively. This results was consistent with the reports of Guo et al., who also found that after the solid-state fermentation of *Monascus anka* the AGI activities of MLs was much higher than unfermented ML. For MLP, the AGI rate was increased from  $61.6 \pm 4.12\%$  of MLP-UF to  $78.4 \pm 5.77\%$  of MLP-F8. The inhibition rate of MLA extracted from ML-UF and ML-F8 also showed a

significant increase from  $71.4 \pm 5.57\%$  of MLA-UF to  $87.5 \pm 6.55\%$  of MLA-F8. The increase of AGI rate by active substances in FML was mainly attributed to the enzymes produced by fermentation, which could decompose the cell wall of plants and release active components substantially, and even produce new active substances. However, it was also reported that too long time fermentation might lead to further degradation of active ingredients. Therefore, appropriate fermentation period is of great significance for enriching active compounds of MLs and consequently improving  $\alpha$ -glucosidase inhibitory rate.

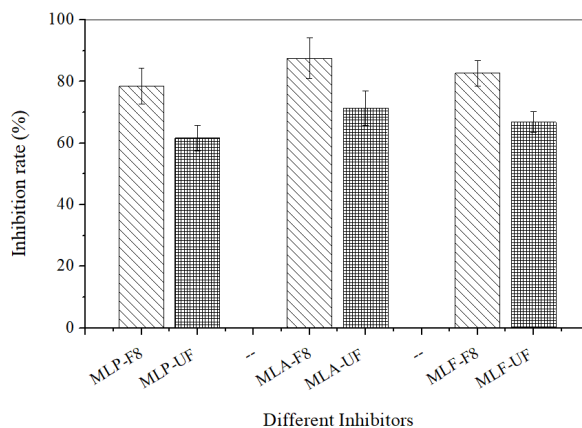


Figure 3: The  $\alpha$ -glucosidase inhibition rate of different mulberry leaves extracts. MLP-F8, MLA-F8 and MLF-F8 are polysaccharide, alkaloids and flavonoids extracted from mulberry leaves (ML) fermented for 8 d, respectively; MLP-UF, MLA-UF and MLF-UF are polysaccharide, alkaloids and flavonoids extracted from unfermented ML, respectively. The concentration for MLPs, MLAs and MLFs are 1 mg/ml, 5  $\mu$ g/ml, 0.5 mg/ml, respectively.

#### 4. Conclusion

Mulberry leaves are rich in resource and have great development prospects in the field of treating diabetes. In this paper, the contents of alkaloids, flavonoids and polysaccharides in the fresh mulberry leaves were determined by using Reye's salt method,  $\text{NaNO}_2\text{-Al}(\text{NO}_3)_3\text{-NaOH}$  chromogenic method and DNS method, respectively. Then, the natural solid state fermentation of fresh mulberry leaves using its endophytes was investigated. The content and  $\alpha$ -glucosidase inhibition rate of flavonoid and alkaloid obtained from fermented mulberry leaves both showed significant enhance compared to that from unfermented mulberry leaves. The research of this paper will provide reference is provided for the related research.

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