

# Effect of *Eurotium cristatum* fermentation on the $\alpha$ -glucosidase inhibitory activity of mulberry leaves alkaloids

Yangcheng Gao<sup>1,a\*</sup>, Rong Chen<sup>2,b</sup>, Yinqi Zhao<sup>3,c</sup>

<sup>1</sup>Michigan State University, ML, USA

<sup>2</sup>Providence country day school, RI, USA

<sup>3</sup>International department of Beijing No.35 High School, Beijing, China

<sup>a</sup>3078918589@qq.com, <sup>b</sup>3215606702@qq.com, <sup>c</sup>3413662781@qq.com

\*Corresponding author: 3078918589@qq.com

These authors contributed equally to this work

**Abstract:** *Eurotium cristatum* CY-1 was used for the solid-stated fermentation of mulberry leaves (ML) with the purpose of enhancing the alkaloids content and  $\alpha$ -glucosidase inhibitory activity of ML through microbial fermentation. The results indicated that *Eurotium cristatum* CY-1 grown well when cultivated with MLs as substrate. The maximum cell dry weight of 393.32 mg was reached on the 10 d of fermentation. The alkaloids content of ML was increased with the fermentation time, and the maximum value of 8.711 mg/g was obtained at 8 d of fermentation, which was increased by 171.96% compared to that extracted from unfermented ML. The  $\alpha$ -glucosidase inhibitory efficiency was also improved along with the fermentation time. The IC<sub>50</sub> values of alkaloid extracted from 8d-fermented mulberry leaves was 3.94  $\mu$ g/mL, but 6.82  $\mu$ g/mL of the alkaloid extracted from the unfermented ML. Therefore, the fermentation of *Eurotium cristatum* could benefit for enhancing the alkaloid content and  $\alpha$ -glucosidase inhibitory efficiency of ML. It would provide a valuable strategy for obtain safe and low-cost  $\alpha$ -glucosidase inhibitors from natural herbal medicines.

**Keywords:** Mulberry leaf, *Eurotium cristatum*, Alkaloids,  $\alpha$ -glucosidase inhibitory, Diabetes

## 1. Introduction

Recently, as one of the most prevalent worldwide chronic diseases, diabetes becomes the third threatening illness after cancer and cardiovascular and cerebrovascular diseases [1, 2]. There have been 463 million diabetics all around the globe, according to the International diabetes federation (IDF)'s diabetes Atlas of 2019. It is predicted that the number of the global diabetics, furthermore, is going to be around 700 million by 2045 [3]. Generally, the diabetes can be classified into 4 types based on pathogenesis: type I diabetes, type II diabetes, specific types of diabetes and gestational diabetes, among of which type 2 diabetes accounts for more than 90% [4].

To delay the hyperglycemia after dining,  $\alpha$ -glucosidase inhibitors can slow down the hydrolysis rate of carbohydrate in the gut by declining glycosidase activity, are superior to another blood glucose-lowering pharmaceuticals in regulating postprandial blood glucose. And it can also effectively delay the progression of pre-diabetic patients to type II diabetes [5]. In this case,  $\alpha$ -glucosidase inhibitors are currently more mature clinical pharmaceuticals for the treatment of diabetes. The most popularly clinic used  $\alpha$ -glucosidase inhibitor, including acarbose, voglibose, and miglitol, although it has been reported that some of them have serious side effects, especially for the gastrointestinal tract, and can cause physical dependence [6]. Therefore, developing, multi-targeted, safe and low-cost  $\alpha$ -glucosidase inhibitors from natural herbal medicines obtained increasing concerns [7].

Mulberry leaf, containing abundant secondary metabolites like flavonoids, alkaloids and polyphenols, is an traditional herbs with a multitude of pharmacological efficiency like antioxidant, hypolipidemic, hypoglycemic, antitumor, and immune effects, etc., owing to the consist of flavonoids, alkaloids, polysaccharides, amino acids and other chemical components. Therefore, in recent years, the exploitation of mulberry leaves as special medicine for treating chronic diseases and freeing people from sub-healthy state has an exciting market prospect. As an important natural active ingredients, many researches have proved that the alkaloids in mulberry leaves, especially 1-Deoxynojirimycin, are potent inhibitors of  $\alpha$ -

glucosidase with good water solubility, high biological activity and no cytotoxicity [8,9].

However, due to the low concentration of alkaloids in plants, the cost of alkaloids extraction and purification is relatively high which inhibit the development of natural ingredient. So it is significant to enrich the active ingredients for reducing their preparation cost. Microbial fermentation of herbal medicines has a long history. There is much attention focus on the modernization research of herbal pharmaceuticals. Alongside this, the utilization of modern biotechnology to monitor the microbial fermentation process of herbal medicines and the changes of its parameters has greatly improved the quality of modern fermented herbal medicines.

In this article, the effect of *Eurotium cristatum* CY-1 fermentation on the  $\alpha$ -glucosidase inhibitory activity of mulberry leaves alkaloids extract was investigated with the aim of achieving enrichment of alkaloid active substances in mulberry leaves through microbial fermentation and improving their anti-glycemic efficacy.

## 2. Materials and Methods

### 2.1. Materials and Reagents

Mulberry leaves were obtained from the market of Shangluo (Shanxi, China). Wheat bran was purchased from Feitian Agricultural Development Co., Ltd. (China, Henan). Alpha-glucosidase (from yeast), 4-hydroxypiperidinol standard, pNPG (4-nitrophenyl- $\alpha$ -D-glucopyranoside), glucosamine standard solution, were purchased from Yuanye Biotechnology Co., Ltd. (China, Shanghai). Acarbose was purchased from Aladdin Bio-Chem Technology Co., Ltd (China, Shanghai). All of the other used reagents or chemicals in this study were of analytical grade.

### 2.2. Solid-state fermentation of *E. cristatum* CY-1 with mulberry leaves as medium

*E. cristatum* CY-1 used for mulberry leaves fermentation was previously isolated from Fu-brick dark tea by our laboratory. The mulberry leaves medium was prepared as the following procedures. Weigh 9 g of fresh mulberry leaves and 1 g dry wheat bran, then distilled water was added to adjust the solid-to-liquid ratio of the medium to 1:3. After been stirred evenly under natural pH, the mixture was sterilized at 121 °C for 20 min and used as the fermentation medium of *E. cristatum* CY-1 after been cooled.

The inoculum of *E. cristatum* CY-1 was obtained by cultivated *E. cristatum* CY-1 in potato dextrose broth medium at 28-30 °C with 180-200 rpm for 3-5 days. Inoculated *E. cristatum* CY-1 inoculum into the mulberry leaves medium to adjust the final liquid-solid ratio to 1:4, and then the mixture was statically cultivated at 30 °C. Samples were taken at one day interval and stored in a refrigerator at 4 °C for use. Each experiment was conducted by three parallels.

### 2.3. Determination of Biomass of *E. cristatum* CY-1

Dried the fermented residues at 60°C to constant weight. Then, the glucosamine content in the fermented residues was used to monitor the biomass of *E. cristatum* CY-1 during the solid state fermentation [10]. Weighed 1 g of the dried residues (accurate to 0.001 g) soaked in 10 ml strong HCl solution (36.9%) for 24 h. The mixed solution was added into 40 ml of distilled water and hydrolyzed at 121 °C for 2h. Collected the supernatant by centrifuged at 8000 rpm for 10 min (H2050R-1, Xiangyi Centrifuge Instrument Co., LTD, Shanghai, China). Then, fixed the volume of the supernatant to 50ml. Taken 10 ml of the supernatant and neutralized with NaOH, then fixed to 25 ml. Mixed 1ml of the above-mentioned glucosamine extract with 1ml of Ehrlich's reagent and incubated at 90 °C for 1 h. Then, 6 ml of ethanol was added into the solution and reacted at 65 °C for 10 min. The absorbance at 520 nm of the solution was analyzed using a UV-Visible spectrophotometer (754PC, Jinghua Technology Co., LTD, Shanghai, China). The cell dry weight of *E. cristatum* CY-1 in the fermented residues could be calculated according to the standard curve.

### 2.4. Extraction and determination of total alkaloids from mulberry leaves

The extraction and determination of total alkaloids in fresh mulberry leaves and fermented residues were conducted as described by Cao et al [8] with slight modification. The extraction of total alkaloids was carried out as following: Accurately weighed 5 g of fresh mulberry leaves or wet fermented residues, added with 50 mL of 25% ethanol-0.05M hydrochloric acid solution. Stirred evenly, the mixture was

ultrasonically extracted at 30 °C for 20 min. The supernatant was collected by centrifuged at 8000 rpm for 10 min.

The above-mentioned alkaloids extracts were concentrated and evaporated to dry. The dried alkaloids extracts was dissolved with 0.05 mol/L HCl and fixed to 4 ml. Taken 2 ml of this solution and centrifuged at 8000 rpm for 10 min. The supernatant was added with 3 mL of 0.02 g/mL Reye's salt solution, and reacted in ice bath for 2 h. Then, the precipitate was collected by centrifuging at 8000 rpm for 10 min, washed with cooled ethyl acetate, and dissolved in 10 ml of 70% acetone. Using 4-hydroxypiperidinol as standard, the content of alkaloids in the solution was determined by measuring the absorbance at 520 nm with an ultraviolet-visible spectrophotometer (754PC, Jinghua Technology Co., Ltd, Shanghai, China). According to the standard curve, the content of total alkaloids in fresh mulberry leaves and fermented residues can be calculated.

### 2.5. $\alpha$ -glucosidase inhibition rate analysis

The inhibition rate of alkaloids extracts on  $\alpha$ -glucosidase was conducted on a 96-well plate [11, 12]. Four groups, namely substrate blank group, control group, sample blank group, and sample group, were set in this experiment. The volume and order of the each reagent was added according Table 1. After been reacted at 37°C for 30 min, 100  $\mu$ L 1 mol/L Na<sub>2</sub>CO<sub>3</sub> was added to terminate the reaction. The absorbance was measured at 405 nm using a microplate reader (MB-96B, Chenghuai technology Co., Ltd, Suzhou, China). The concentration of the inhibitor required for inhibiting 50% of the enzyme activity under the assay conditions was defined as the IC<sub>50</sub>.

Table 1 The adding volume and order of reagents in different group of  $\alpha$ -glucosidase inhibition rate assay

Reagent	Substrate blank group (B)	Control group (C)	Sample blank group (SB)	Sample group (S)
PBS buffer ( $\mu$ l)	80	70	60	50
Alkaloid extracts ( $\mu$ l)	0	0	20	20
$\alpha$ -glucosidase ( $\mu$ l)	0	10	0	10
Stirred evenly, reacted at 37°C for 15 min				
pNPG ( $\mu$ l)	20	20	20	20
Stirred evenly, reacted at 37°C for 30 min				
Na <sub>2</sub> CO <sub>3</sub> ( $\mu$ l)	100	100	100	100

The inhibitory rate on  $\alpha$ -glucosidase activity of alkaloid extracts was calculated according to formula (1).

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

Where, AB is the absorbance of substrate (pNPG); AC is the absorbance of pNPG reacted with  $\alpha$ -glucosidase; ASB is the absorbance of sample and pNPG; AS is the absorbance of pNPG reacted with  $\alpha$ -glucosidase under the existence of inhibitor.

### 2.6. Data analysis

The data was analyzed with Origin 8.0 software package. All the experiments were conducted in triplicate and presented as the mean  $\pm$ SD. ANOVA at probability level (P) $\leq$ 0.05 was used to determine the statistically significant differences between the mean samples.

## 3. Results and Discussion

### 3.1. Fermentation process of *E. cristatum* CY-1 grown on mulberry leaves medium

*E. cristatum* is a non-toxic and safe probiotic fungus that have been reported played a vital role in the formation or conversion of biological and flavor compounds of many plants during the solid state fermentation, such as Fu-brick dark tea [13], Hippophae rhamnoides leaves [14], red bean [15]. In this paper, *E. cristatum* CY-1 was used for the mulberry leaves fermentation. The cell dry weight of the *E. cristatum* CY-1 and the weight loss rate of the mulberry leaves medium were both increased gradually

with the extension of the fermentation time, and stabilized after 8 d of fermentation, with less fluctuation (Fig.1). The cell dry weight of *E. cristatum* CY-1 reached a maximum of 393.32 mg on the 10 d, an increase of 292.66 mg compared to 100.66 mg on the 2 d of fermentation. The weight loss rate of mulberry leaves medium increased steadily to a maximum of 45.24% on the 12 d, an increase of 39.88% compared to 5.36% on the 2 d. All these results suggested that the fresh mulberry leaves can be used as nutrition and energy for the grown of *E. cristatum* CY-1.

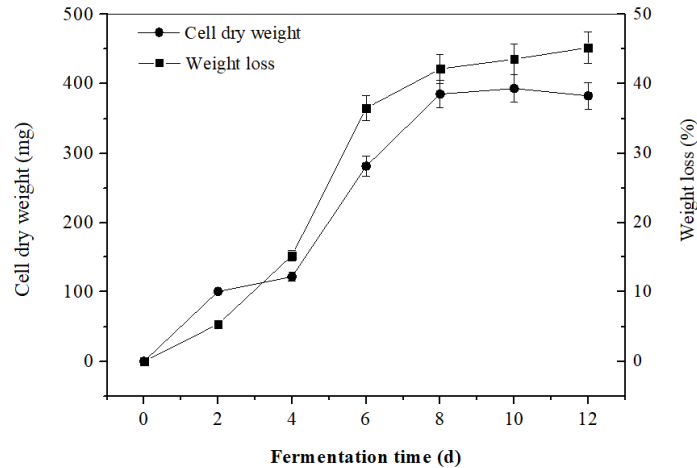


Fig.1 The cell dry weight of *E. cristatum* CY-1 and weight loss of mulberry leaves medium during the fermentation process

### 3.2. Variation of alkaloid content in mulberry leaves during the fermentation

It was reported that *E. cristatum* can secrete several enzymes including  $\alpha$ -amylase,  $\alpha$ -glucosidase, cellulase, protease, pectinase, tanninase, etc., benefit for increasing the content and improving the function of activate ingredients [16, 17]. Zou et al. reported that *E. cristatum* during SSF of *Ginkgo biloba* leaves increased flavonoids content [16]. Xiao et al found that black soybean processed by SSF with *E. cristatum* would greatly increase the contents of protein, essential amino acids, and some minerals (e.g., calcium, phosphorus, and magnesium) [18]. Du et al fermented *P.lobata* with *E. cristatum* which gave the results that the total flavonoids of *P.lobata* increased significantly compared with unfermented *P.lobata* [19].

When fermented mulberry leaf with *E. cristatum* CY-1, the content of alkaloids extracted from the fermented mulberry leaves gradually increased with the extension of fermentation time, reaching the maximum value at day 8 and then rapidly decreased. It reached  $8.711 \pm 0.405$  mg/g on the 8 d, which was increase by 171.96% compared to 3.203 mg/g of unfermented mulberry leaves. Therefore, *E. cristatum* CY-1 has good potentiality on enhance the content of alkaloids in mulberry leaves.

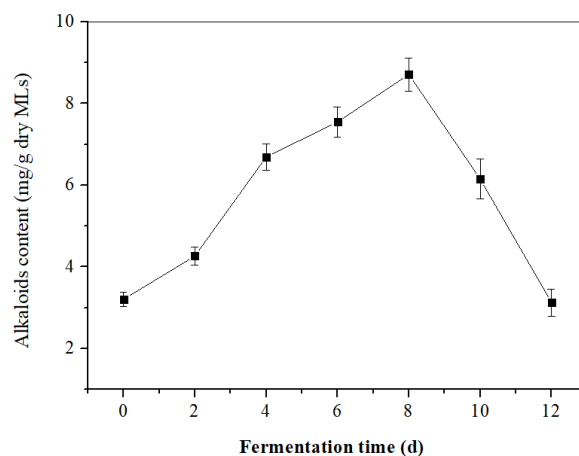


Fig.2 The variation of total content of alkaloids in mulberry leaves during the fermentation of *E. cristatum* CY-1

### 3.3. Effect of fermentation on inhibitory rate of alkaloids from mulberry leaf

In this paper, the  $\alpha$ -glucosidase inhibition of alkaloid extracts from unfermented mulberry leaves and the 8d-fermented mulberry leaves was investigated. The results indicated that with the increase of concentration, the  $\alpha$ -glucosidase inhibitory rate of alkaloids extracted from unfermented mulberry leaves and 8d-fermented mulberry leaves were both increased (Fig.3). However, alkaloids from 8d-fermented mulberry leaves were significantly more efficient than that from unfermented mulberry leaves. At the same concentration of 0.020 mg/ml, the inhibition rate of alkaloids from unfermented mulberry leaves was only 25.48% which was 65.16% of alkaloids from 8d-fermented mulberry leaves. Therefore, the fermentation of *E.cristatum* CY-1 would improve the  $\alpha$ -glucosidase inhibitory rate of mulberry leaves alkaloid. The IC<sub>50</sub> values of alkaloid extracted from 8d-fermented mulberry leaves was 3.94  $\mu$ g/mL, while it was 6.82  $\mu$ g/mL to unfermented one. Actually, many researches have reported that *E. cristatum* has great potential to improve the nutritional value and biological activity of substrates. Xiao et al [18] reported that *E. cristatum* can enhance the antioxidant and  $\alpha$ -glucosidase inhibitory activities for the accumulation of phenolic compounds during the fermentation of black soybean. Du et al [19] found that the antioxidant activity of fermented *P.lobata* was superior to the unfermented *P.lobata*. Li et al [20] suggested that *E.cristatum* fermented okara can reduce postprandial blood glucose levels through the in-vitro  $\alpha$ -glucosidase activity assays and in-vivo mice studies.

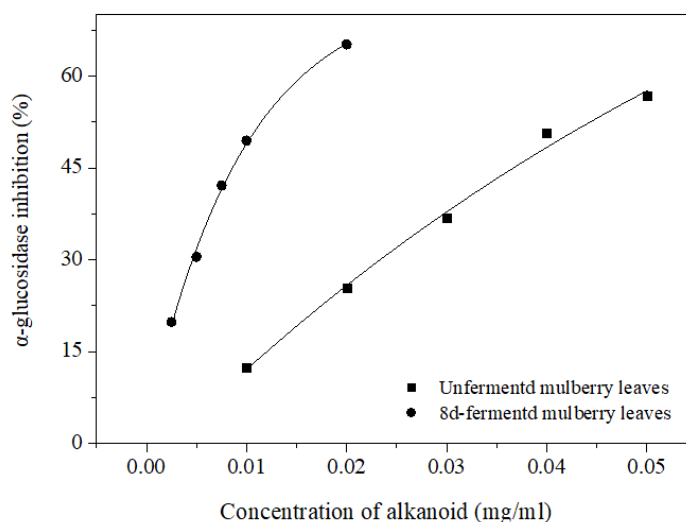


Fig.3 The difference of  $\alpha$ -glucosidase inhibition of mulberry leaves alkaloids extracted from different mulberry leaves (■ unfermented mulberry leaves; ● 8d-fermented mulberry leaves)

## 4. Conclusions

The present investigation demonstrated that the solid-stated fermentation with *Eurotium cristatum* was an effective approach to enhance the yield of Alkaloids from fresh mulberry leaves. The higher contents of alkaloids  $11.84 \pm 0.58$  mg/g were obtained after 8 d of fermentation, which was increased by 294.76% compared to 3.49 mg/g of unfermented mulberry leaves. Meanwhile, the  $\alpha$ -glucosidase inhibitory efficiency was improved along with the fermentation time. The IC<sub>50</sub> values of alkaloid extracted from 8d-fermented mulberry leaves was 3.94  $\mu$ g/mL, while it was 6.82  $\mu$ g/mL to unfermented mulberry leaves. It could be concluded that the fermentation of *Eurotium cristatum* not only benefit for the enrichment of mulberry leaves alkaloid and but also for enhancing the  $\alpha$ -glucosidase inhibitory efficiency of mulberry leaves alkaloid. The study would provide a valuable strategy for high-value alkaloid production from plant leave resources.

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