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ENZYMES PRODUCED BY *PAENIBACILLUS AMYLOLYTICUS* TO IMPROVE THE SENSORY QUALITY OF THE UPPER LEAVES IN FLUE-CURED TOBACCO

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Abstract

Enzyme preparation was utilized to overcome the deficiencies of high nicotine content and low sugar/nicotine ratio in the upper flue-cured tobacco leaves. A strain of *Paenibacillus amylolyticus* with high amylase yield was screened out from the surface of tobacco leaves for the production of the enzyme preparation. To isolate and detect the intracellular substances of tobacco leaves, the method of gas chromatography-mass spectrometry (GC-MS) was carried out. Both cluster heatmap analysis and principal component analysis (PCA) demonstrated that active enzymes had an obvious effect on tobacco leaves, while the changes brought by inactivated enzymes on tobacco leaves were non-significant. Orthogonal partial least-squares discriminant analysis (OPLS-DA) revealed that active enzymes could lower the nicotine content of the upper tobacco leaves, and elevate the content of higher fatty acids, terpenoids, and their derivatives. As a result, the physiological strength and pungent odor brought by tobacco leaves were diminished, while the aroma volume and quality were enhanced. Moreover, the macromolecule-degrading enzymes in the enzyme preparation degraded the starch, pectin, and cellulose in flue-cured tobacco, contributing to a higher level of reducing sugars and thereby a higher sugar/nicotine ratio. Taken together, the present investigation presented a method to apply microbial enzyme preparations in the processing of flue-cured tobacco, thereby providing new insights into the damage reduction and quality improvement of tobacco leaves.

Key words: *Paenibacillus amylolyticus*; Enzyme preparation; Flue-cured tobacco; Sensory quality.

Introduction

Tobacco (*Nicotiana tabacum* L.), a distinctive cash crop cultivated in more than 120 countries and regions, holds significant importance (Ye *et al*., 2017). The majority of freshly harvested tobacco leaves are converted into flue-cured tobacco, later transformed into cigarettes, a specific addictive consumable (Wu *et al*., 2021b). The composition of tobacco leaves exhibits variations in quality across different sections (Chen *et al*., 2021). The upper leaves are abundant in nicotine with a low sugar/nicotine ratio (Lin & Zhang, 2016, Zhang *et al*., 2018), so cigarettes made from them are associated with poor sensory coordination and unpleasant sensory experiences such as obsession and strong irrigation (Thuerauf *et al*., 1999, Shen *et al*., 2019, Fan *et al*., 2021). That's why the upper leaves are barely adopted as the ingredient of high-quality cigarettes. Currently, how to enhance the quality of upper flue-cured tobacco leaves has emerged as a central concern in the tobacco sector.

Biological enzymes can catalyze the degradation of starch, pectin, cellulose, proteins, and other macromolecular substances in tobacco leaves (Dai *et al*., 2020, Tao *et al*., 2022). Besides, they can also help in elevating the sugar/nicotine ratio and facilitate the Maillard reaction (Hu *et al*., 2022). Utilizing these capabilities, biological enzymes possess a pivotal role in enhancing the caliber of tobacco leaves. In the procedures of tobacco re-drying, enzyme preparations boast greater advantages over microbial agents, because they are associated with stable and controllable catalytic reactions and can be inactivated instantaneously under high temperatures during the shred drying stage. However, the majority of enzyme preparations are produced by commercial enzymes (Xiao *et al*., 2014, Wu *et al*., 2021a, Zheng *et al*., 2022) that are inappropriate for industrial applications due to the high cost.

In contrast, enzymes generated during the metabolism of tobacco-derived microorganisms are able to accelerate the alcoholization of tobacco leaves and thereby are a cheap substitute for commercial enzymes. Among the tobacco-derived microorganisms, *Paenibacillus amylolyticus*, with strong amylase-producing capacities (Ikram *et al*., 2012), possesses the potential to lift the sugar/nicotine ratio in tobacco leaves. In the present study, a strain of *Paenibacillus amylolyticus* with high amylase yield was obtained from the surface of tobacco leaves. To clarify whether this bacterial strain was capable of improving the sugar/nicotine ratio in upper leaves, the extracellular enzymes were prepared into a natural enzyme preparation, which was applied during the re-drying process. The investigation focused on exploring the impact of the enzyme preparation on the upper leaves of tobacco, particularly analyzing its effect on the sensory characteristics and revealing the mechanisms underlying it. This study aims to provide insights into reducing damage and enhancing the overall quality of tobacco leaves.

Material and Methods

Preparation of tobacco samples, microbial media, and reagents: The flue-cured tobacco leaves were obtained from B03, Nanping, Fujian Province. The starch-screening medium contained 10 g/L soluble starch, 5 g/L NaCl, and 20 g/L agar. The tryptic soy broth (TSB) liquid medium (pH = 7.2) consisted of 15 g/L tryptone, 5 g/L soy peptone, and 5 g/L NaCl, and the TSB solid medium was supplemented with agar at a concentration of 20 g/L, following the composition of the liquid medium. In the fermentation

medium (pH = 7.0), there was 2 g/L soluble starch, 10 g/L peptone, 3 g/L beef extract, 0.2 g/L manganese sulfate, and 5 g/L dipotassium hydrogen phosphate.

Strain screening: In a 250 mL shake flask, 10 g of flue-cured tobacco leaves and 60 mL of phosphate-buffered saline (PBS) buffer (pH = 7.4) were combined. The flask was then placed on a shaker operating at 200 r/min and shaken for a duration of 40 minutes. This is followed by the infiltration through 2 layers of gauze to obtain the extract. Then the tobacco leaves were washed with a small volume of PBS buffer (40 mL in total) several times. After the extracts were pooled, bacterial sludge was concentrated using the 0.22 μm filter membrane and subsequently redissolved in 10 mL PBS buffer to obtain the bacterial suspension. The suspension was subjected to gradient dilution and coated in a TSB solid medium for separation. The strains of various morphologies were spotted into the amylase-screening medium and cultured for 36 h at 30°C, followed by plate staining with dilute iodine solution (containing 0.088 g/L I_2 and 40 g/L KI) for 10 min. After the dilute iodine solution was discarded, the diameters of hydrolytic circles and colonies in the various strains were measured via a millimeter-scale ruler. The HC value (hydrolytic circle diameter/colony diameter) was calculated to evaluate the enzyme-producing capacities of different strains (Cheng *et al*., 2018).

Strain identification: The DNA of the target strain was extracted via the bacterial DNA extraction kit for polymerase chain reaction (PCR) amplification, during which the primers of 27F (5'-AGAGTTTGATCMTGG CTCAG-3') and 1492R (5′-TACGGYTACCTTGTTAC GACTT-3′) were adopted and the specific program was introduced in a previous study (Mahima *et al*., 2020). The amplification products were sent to Sangon Biotech (Shanghai) Co., Ltd. for gene sequencing. The obtained DNA sequences were matched with the species included in the nucleotide database of the National Center for Biotechnology Information (NCBI) based on species similarity. Finally, a phylogenetic tree was illustrated. Specifically, the screened high-quality strain *Paenibacillus amylolyticus* LX-3 was uploaded its 16S rDNA gene sequence to NCBI and obtained the registration number OR781603.

Production of enzyme preparation: The *Paenibacillus amylolyticus* on the surface of the TSB solid medium was prepared into bacterial suspension with sterile normal saline (NS). The suspension was then transferred to the TSB liquid medium and cultured for 12 h (37°C, 200 r/min). The cultured seed liquid was incubated in a 200 L fermenter containing 100 L fermentation medium for 18 h (37°C, 150 r/min), during which the mixture was sampled every 3 h to measure bacterial concentration and amylase activity. The bacterial concentration was characterized by OD600 and amylase activity was determined via the iodine colorimetric method (Zhou *et al*., 2021a). The determination method was properly adjusted based on the enzyme activity definition: Amylase activity is quantified by measuring the quantity of amylase needed to break down 1mg of soluble starch by a 1mL sample of extracellular fermentation broth over a 5-minute period under specific conditions of temperature (37°C) and pH (5.8). Following the fermentation process, the fermentation broth was firstly filtered through the ceramic membrane of 50 nm for bacterial removal and then passed through the ultrafiltration membranes of 150 kDa and 10 kDa successively for concentration. The solid content after concentration was 1.5% (w/v). The concentrate was compounded with 3.0% (w/v) skim milk powder and the mixture were subsequently spray-dried into an enzyme preparation.

Measurement of chemical component content and enzyme activity in the enzyme preparation: The levels of proteins, polysaccharides, and reducing sugars in the enzyme preparation were determined via the Coomassie Brilliant Blue (Grintzalis *et al*., 2015), phenol-sulfuric acid (Xi *et al*., 2010), and 3,5-dinitrosalicylic acid (DNS) (Zhou *et al*., 2021a), respectively. In the present research, the determination method of amylase activity introduced in the literature (Zhou *et al*., 2021a) was adopted and appropriately adjusted based on the definition of amylase activity. The amylase activity of one unit was defined as the amount of amylase necessary for 1 g of enzyme preparation to degrade 1 mg of soluble starch within duration of 1 minute at a temperature of 37°C and pH level of 5.8. Pectinase activity was measured based on the method provided in previous research (Yahya *et al*., 2022), which was properly modified according to the definition of pectinase activity. The amount of pectinase necessary for the degradation of citrus pectin by 1 g of enzyme preparation to produce 1 μg of galacturonic acid in 1 min (50°C, pH = 9.4) is defined as one unit of pectinase activity. Cellulase measurement was performed following the literature (Wu *et al*., 2022), with necessary adjustments corresponding to the definition of cellulase activity. The activity of cellulase was determined by the amount of enzyme preparation (1 g) needed to generate 1 μg glucose by degrading carboxymethylcellulose sodium within a minute, at a temperature of 50°C and pH value of 5.0. Similarly, the determination method of protease activity described in the reference (Zhou *et al*., 2021b) was properly adjusted based on the definition of protease activity and utilized in the current experiment. A single unit of protease activity was determined as the amount of protease necessary for 1 g of enzyme sample to produce 1 μg L-tyrosine by decomposing casein within duration of 1 min, under the conditions of 40°C and pH 7.5.

Enzyme preparations for processing flue-cured tobacco: The active enzyme preparation, weighing 0.0500 g, was dissolved in 10 mL of secondary reverse osmosis water at a temperature of 40°C. This resulting solution was then evenly sprayed onto the surface of 50 g of flue-cured tobacco leaves using a nebulizer. Then the leaves were sealed in PE pouches. After 4 h of natural alcoholization at 30°C, 70 s hot air dehydration (shred drying) at 135°C, and natural cooling, the re-drying process finished and the leaves were included in the TAE group (experimental group was processed with active enzymes). The re-dried tobacco leaves of the TW (blank group was processed with water) and TIE (control group processed with inactive enzymes) groups were obtained in a similar approach. The

difference was that the liquid active enzyme preparation was replaced by 40°C secondary reverse osmosis water in the TW group, while in the TIE group; the liquid active enzyme preparation was inactivated by boiling water before spraying.

Sensory evaluation of tobacco leaves: The evaluation team consisted of 7 qualified professional evaluators. Based on the Chinese Tobacco Industry Standards (YC/T 138-1998, TC/T 138-2014), tobacco leaves in the TW, TIE, and TAE groups were assessed in terms of the following 9 indicators: sweetness, aroma quality, aroma volume, offensive odor, exquisite taste, taste concentration, physiological strength, pungent odor, and aftertaste.

Measurement of tobacco aroma precursors: 2.00 g of tobacco leaves, along with 10 mL of dichloromethane and 200 µL of phenylethyl acetate (internal standard, 1.03 mg/mL), were added to the extraction flask. Following a 30-minute ultrasound treatment at 50°C and 300 W, the mixture was filtered through a 0.22 µL filtration membrane. This process yielded the extraction solution, which was then analyzed using gas chromatography-mass spectrometry (GC-MS) to isolate and detect the target compounds. The GC-MS procedures outlined by the literature (Qi *et al*., 2022) were followed.

Detection of tobacco-related chemical components: The tobacco leaves in the TW, TIE, and TAW groups were ground to fine powder of at least 60 mesh with a grinder. The content of starch, pectin, cellulose, protein, and reducing sugar in tobacco leaves was determined according to the Chinese tobacco industry standards YC/T 216-2013, YC/T 346-2010, YC/T 347-2010, YC/T 249-2008, YC/T 159-2002, respectively.

Statistical analysis

In the experiment, all the detections were conducted in parallel three times. Mean and standard derivation (SD) values were illustrated in graphs. To assess the statistical variations among groups, a one-way analysis of variance (ANOVA) was conducted. A significance level of $p<0.05$ was adopted to determine whether the differences in observed value were substantial.

Results

Strain screening, identification, and enzyme preparation production: By comparing the strains with high HC values, it was discovered that LX-3 had the strongest amylase-producing capacity (Fig. 1A). The alignment of 16S rDNA sequences revealed that this bacterial strain was *Paenibacillus amylolyticus* (Fig. 1B). The growth and metabolism curves of the strain in the fermenter showed that the strain reached the stable phase of growth at 9 h of fermentation and entered the stable phase of amylase production at 15 h of fermentation (Fig. 1C). To ensure the stability of the enzyme preparations of different batches, the fermentation cycle could be extended slightly to 18 h. At this time, the amylase activity of the fermentation broth was 427.63 ± 9.17 U/mL. The fermentation broth was prepared as an enzyme preparation after sterilization, concentration, and spray-drying (Fig. 1D). The amylase activity of the enzyme preparation was 118.00 ± 0.44 U/mg.

Effects of enzyme preparation on the sensory quality of upper flue-cured tobacco leaves: Measuring the effects of the enzyme preparation highly relies on the sensory characteristics of tobacco leaves, which serves as a fundamental and pivotal factor. Sensory evaluation (Fig. 2) indicated that compared with the tobacco leaves in the TW group, those in the TIE group exhibited a minor improvement in the sensory quality, while the leaves in the TAE group showed a greater improvement in this aspect. The specific manifestations included alleviated offensive odor, reduced physiological strength and pungent odor, enhanced sweetness, improved aroma volume and quality, smooth smoke, and favorable flavor.

GC-MS analysis was conducted on the intracellular extracts of tobacco leaves in each group to delve into the reasons behind the effects of the enzyme preparation on tobacco sensory quality. In total, 51 substances were detected and were subjected to cluster heatmap analysis (Fig. 3A) and principal component analysis (PCA, Fig. 3B). It was found that the substances in tobacco leaves of TW and TIE groups were hard to distinguish, but those of the TAE group presented with visible differences. The finding was consistent with the result of sensory evaluation, that is, the quality and flavor of tobacco leaves underwent modifications due to the presence of active enzymes.

Following the significance analysis on the 51 detected substances, orthogonal partial least squares-discriminant analysis (OPLS-DA) and calculation of variable importance in projection (VIP) values were carried out on the substances with significantly different content in the tobacco leaves of the three groups. Finally, the substances with different features among the three groups having VIP values ≥ 1 were screened out (Fig. 3C). There were multiple substances with different features between the TAE and TIE groups as well as between the TAE and TW groups. Most of them were identical. However, the number of substances with different features between the TIE and TW groups was smaller. The difference implied that active enzymes in the enzyme preparation played a major part in affecting the quality of tobacco leaves. Regarding the substances with distinct characteristics between the TAE and TIE groups, it was observed that active enzymes led to a decrease in the levels of nicotine (M4) and scopoletin (M35). Conversely, there was an increase in the levels of cis-7-dodecen-1-yl acetate (M45), eicosane (M51), higher fatty acids including 7,10,13-hexadecatrienoic acid, methyl ester (M28), n-hexadecanoic acid (M32), oleic Acid (M46), linolenic acid (M47), and octadecanoic acid (M48), as well as terpenoids and their derivatives including phytol, acetate (M25) and 4,8,13-duvatriene-1,3-diol (M49). It was previously reported that nicotine (M4) and scopoletin (M35) might enhance the physiological strength and pungent odor brought by smoke (Fan *et al*., 2021, Shen *et al*., 2021), and that nicotine (M4) was also likely to induce the nicotinic acetylcholine receptors (nAChRs) to produce

bitterness (Carstens & Carstens, 2022). Therefore, the physiological strength and pungent odor brought by the tobacco leaves in the TAE group were evidently reduced and the sweetness was substantially increased. Moreover, fatty acid ethyl esters could make the tobacco leaves more aromatic (Xu *et al*., 2021); long-chain fatty acids could regulate the pH levels of the smoke (Kou *et al*., 2016) endow the smoke with fatty and cured flavors, and increase taste softness (Zhang, 2012, Wang *et al*., 2018); terpenoids and their derivatives improved the aroma quality and volume of the smoke (Li *et al*., 2022). Hence, the tobacco leaves in the TAE group produced smooth smoke and boasted enhanced aroma volume and quality.

Potential mechanisms underlying the effects of enzyme preparation on upper tobacco leaves: The enzyme preparation was prepared by spray-drying the extracellular macromolecular concentrate of *Paenibacillus amylolyticus*, which might carry aromatic substances. The total ion flow diagram of GC-MS (Supplementary Fig. 1) illustrated that the small molecular substances in the enzyme preparation were all strongly volatile with low boiling points, so they were prone to volatilize in the shred drying stage. Furthermore, as only a small quantity of enzyme preparation (0.1%) was added, it was difficult for the re-dried tobacco leaves to absorb the flavor substances in the enzyme preparation.

The component analysis demonstrated that proteins (52.2%), reducing sugars (19.96%) and polysaccharides (8.93%) were the primary components in the enzyme preparation (Fig. 4A). In the detection of macromolecule-degrading enzymes, it was discovered that there was amylase, pectinase and cellulase and protease in the enzyme preparation (Fig. 4B). Besides, these enzymes were all capable of degrading the starch, pectin, cellulose, and proteins in tobacco leaves while elevating the content of reducing sugars (Fig. 4C). The sugar/nicotine ratio was lifted from 14.08 to 21.35 (TIE vs. TAE). Combined with the analysis in Fig. 2, 3, and 4, we speculate that the enzyme preparation's pectinase and cellulase can disrupt the cell wall of tobacco leaves. This disruption results in a partial removal of the offensive scent and nicotine content during the drying phase of the shredded tobacco, subsequently leading to a reduction in both the physiological strength and the pungent odor caused by the flue-cured tobacco. Amylase could transform the intracellular starch in tobacco leaves into reducing sugars. With the increased content of reducing sugars and declined level of nicotine, the sugar/nicotine ratio was elevated. Hence, the sweetness of tobacco leaves was evidently enhanced. In addition, due to the intensified damage to the tobacco leaf structure, the dissolution volume of higher fatty acids and terpenoids was increased, contributing to soft and smooth smoke as well as increased aroma quality and volume.

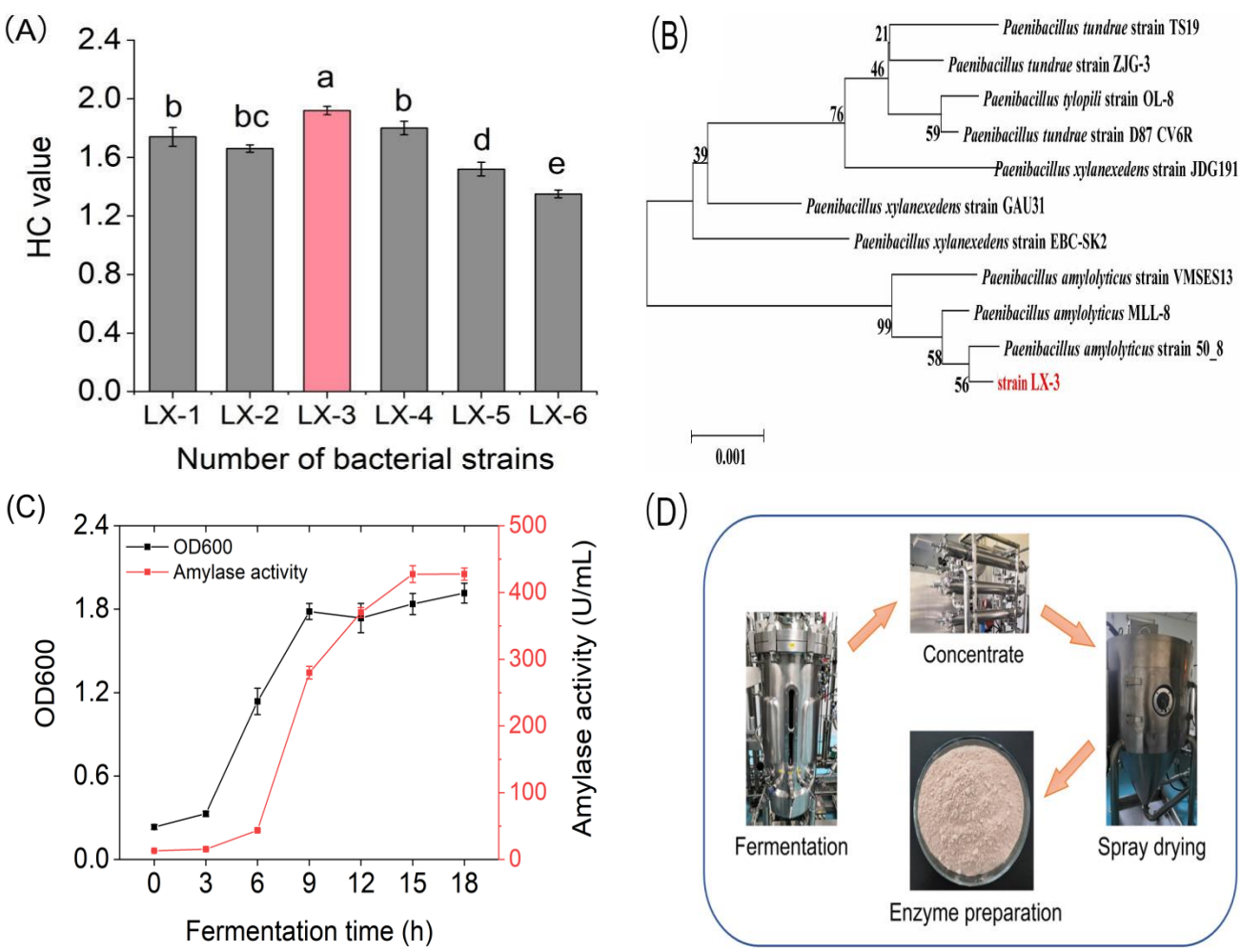


Fig. 1. Screening (A), identification (B), fermentation of high-yield amylase strains (C), and preparation of enzyme preparation (D).

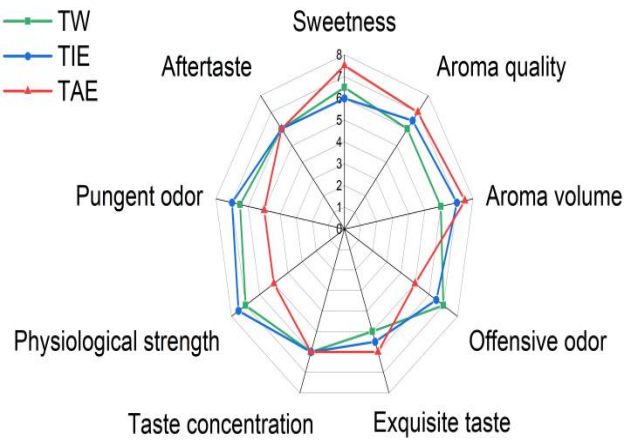


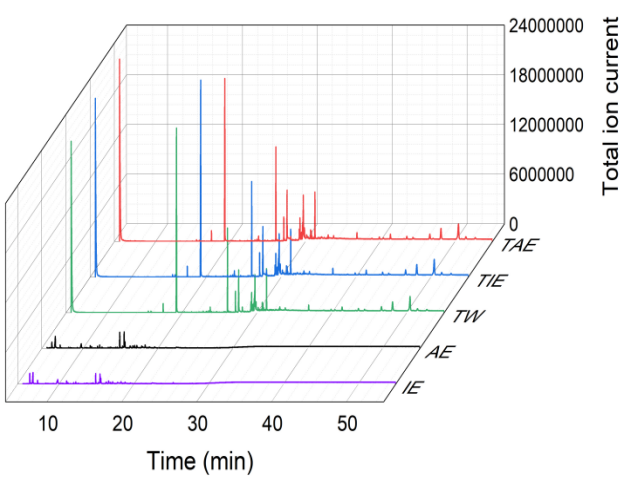
Fig. 2. Effects of enzyme preparation on the sensory quality of upper flue-cured tobacco leaves.

Discussion

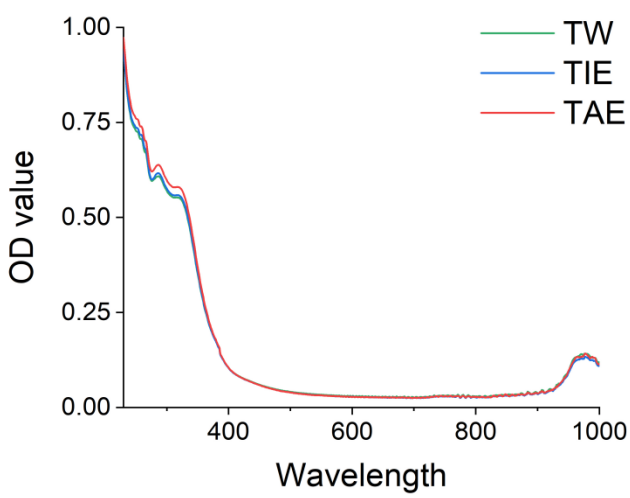
Filamentous fungi tend to tangle into balls during liquid-state fermentation and thereby are associated with unstable fermentation product yield (Chen *et al*., 2017). This problem can be avoided through bacterial fermentation. Moreover, with the advantage of a short cycle, bacterial fermentation is better in tune with the demands of industrial production. As multiple kinds of enzymes function together, the levels of macromolecular substances including starch, pectin, cellulose, and proteins all are dropped. Besides, the nicotine content in the upper tobacco leaves declined while the content of reducing sugars rose, contributing to a higher sugar/nicotine ratio. In this way, the deficiencies of high nicotine content and low sugar/nicotine ratio can be overcome. Besides, through the proper degradation of macromolecular substances in tobacco leaves, their combustion characteristics are improved and the unpleasant odor brought by incomplete combustion is diminished (Sha *et al*., 2018). Hence, the objectives of damage reduction and quality improvement in the upper tobacco leaves are realized.

Many bacteria are able to produce nicotine-degrading enzymes (Su *et al*., 2015, Desta *et al*., 2019, Wang *et al*., 2019). But pitifully, the enzyme preparation used in this study did not reveal the presence of these enzymes, possibly due to the absence of nicotinic induction in the medium. The likely cause for the decrease in nicotine levels in tobacco leaves following the application of the enzyme preparation was the action of enzymes that degrade macromolecules, resulting in shorter chain lengths and enhanced porosity of the cell wall in substances like pectin and cellulose. As a result, channels for the material exchange inside and outside cells in tobacco leaves were widened even though no final degradation products were generated. This explained why the full-wavelength absorbance value in the extracts of tobacco leaves in the TAE group was high before shred drying (Supplementary Fig. 2). In this circumstance, nicotine of lower boiling point was likely to be partially volatilized during the short shred drying phase, while higher fatty acids, terpenoids, and their derivatives of higher boiling points were retained.

In the future, we will isolate and purify the amylase, pectinase, cellulase, and protease in *Paenibacillus amylolyticus*, and investigate the process of breaking down macromolecules in tobacco leaves by examining restriction enzyme sites and depolymerization mechanisms. On the other hand, we will combine fermentation and genetic engineering to enhance the nicotine degradation capacity of *Paenibacillus amylolyticus* and develop enzyme preparations more suitable for quality improvement among tobacco leaves. Additionally, many unknown proteins are present in our enzyme preparation (Supplementary Fig. 3), which may also affect the material transformation in tobacco leaves. Henceforth, we will employ proteomic techniques to analyze their components in detail and utilize metabolomic techniques to conclude the intracellular material transformation patterns of each enzyme component in the enzyme preparation.



Supplementary Fig. 1. GC-MS total ion flow diagram of inactive enzymes (IE), active enzymes (AE), and intracellular extracts of different treatment groups (TW, TIE, and TAE) of upper flue-cured tobacco leaves.



Supplementary Fig. 2. Full wavelength scanning images of intracellular extracts from freshly cured tobacco leaves of different treatment groups before redrying.

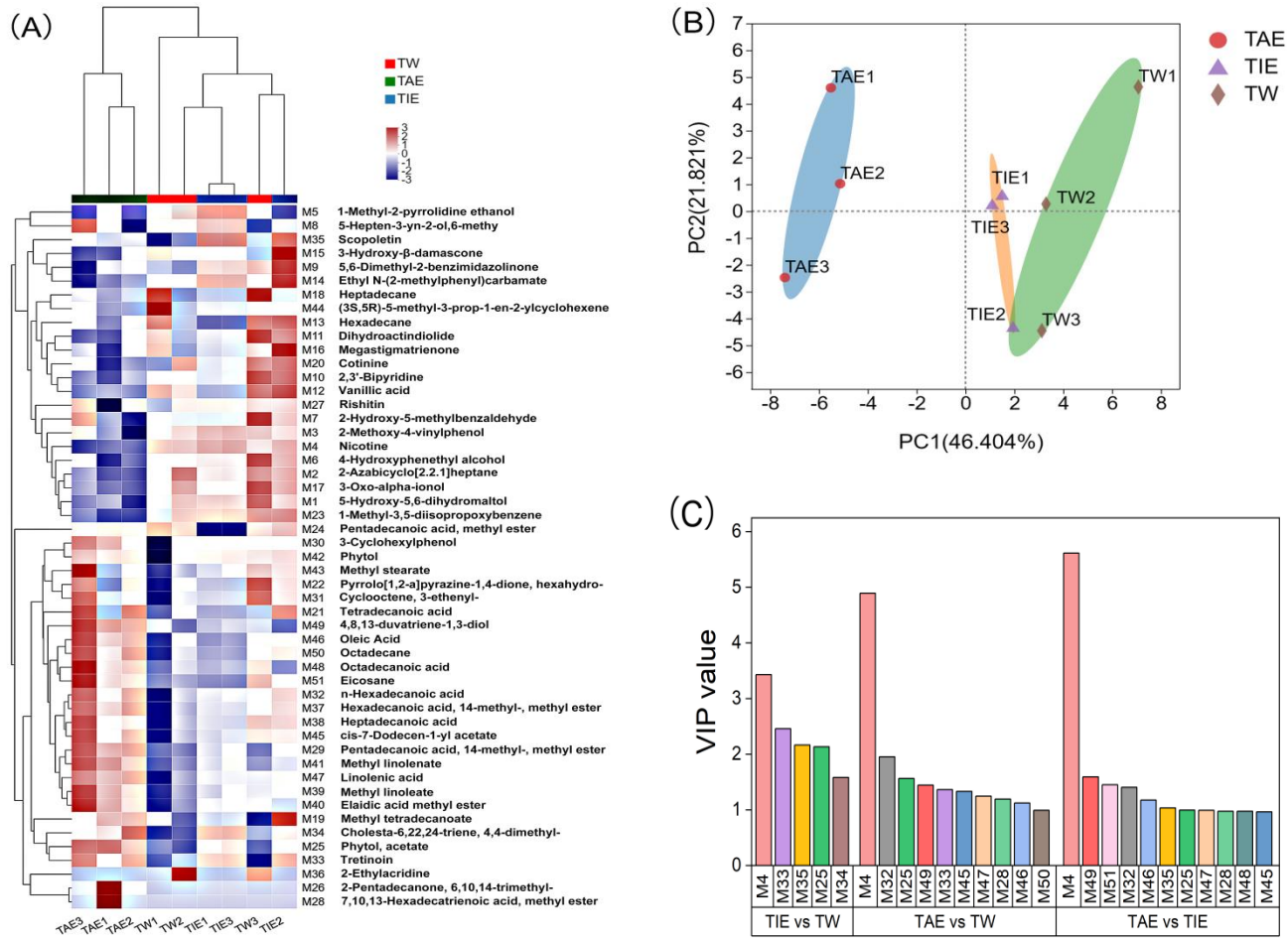


Fig. 3. Cluster thermal analysis (A), principal component analysis (B), and partial least squares analysis (C) of extracts from upper flue-cured tobacco leaves.

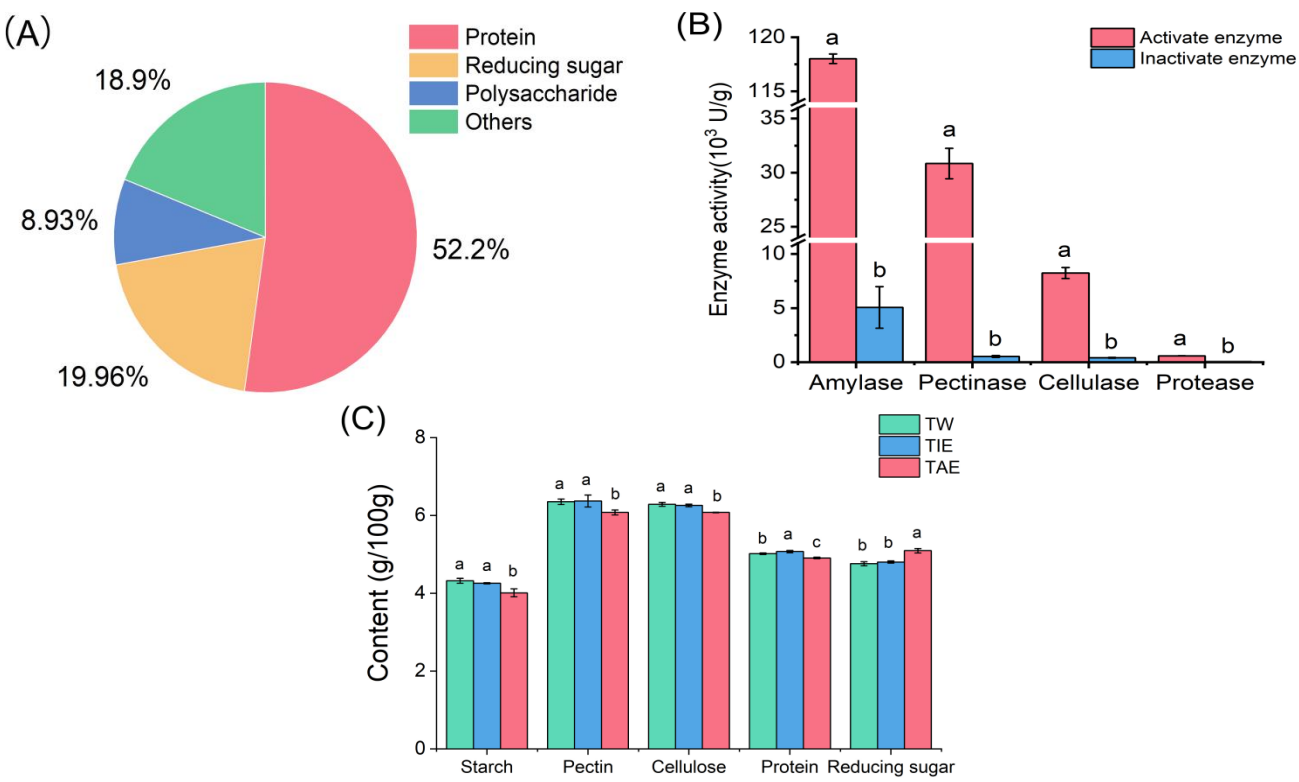
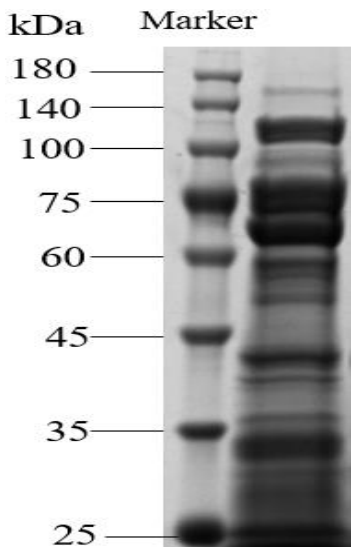


Fig. 4. Primary chemical components in the enzyme preparation (A); Activity of functional enzymes (B); Degradation of macromolecular substances in tobacco leaves by the enzyme preparation (C).



Supplementary Fig. 3. SDS-PAGE electrophoresis of enzyme preparations.

Conclusion

The enzyme preparation prepared from extracellular macromolecular concentrates of *Paenibacillus amylolyticus* plays a damage-reducing and quality-improving part in the upper flue-cured tobacco leaves. The specific manifestations include decreased nicotine levels, increased levels of higher fatty acids, terpenoids, and their derivatives, diminished physiological strength and pungent odor, and enhanced aroma quality and volume. Meanwhile, the macromolecule-degrading enzymes in the enzyme preparation degrade the starch, pectin, and cellulose in flue-cured tobacco, leading to the increased level of reducing sugars and thus a higher sugar/nicotine ratio.

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