Effect of Exogenous Neutral Protease Fermentation on Cigar Leaf Quality

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Abstract: In order to reveal the effect of exogenous neutral protease addition on the quality of cigar tobacco leaves during fermentation. Taking Sichuan Shifang cigar tobacco leaves as the research object, the changes of conventional chemical composition, amino acid content, organic acids and other substances in tobacco leaves after natural fermentation and fermentation with exogenous enzyme preparations were investigated, and the changes of sensory quality of cigar tobacco leaves before and after fermentation were also investigated. The results show that: 1) After fermentation, the content of total sugar, reducing sugar, total nitrogen, chlorogenic acid and rutin in tobacco leaves decreased, and the content of reducing sugar fermented with enzyme preparation decreased more. 2) After fermentation, the total amount of free amino acids in tobacco leaves increased. With the increase of exogenous neutral protease, the content of amino acids first increased and then decreased. 3) After fermentation, the content of organic acids in tobacco leaves increased. When the amount of enzyme added was 2.5g/kg leaves, the increase of organic acid content was greater than that of natural fermentation, and the other additions of enzyme were close to that of natural fermentation. 4) After fermentation, the content of plastid pigment in tobacco leaves decreased slightly. With the increase of neutral protease, the content of plastid pigment increased. 5) After the fermentation of exogenous enzyme preparation, the sensory quality of tobacco leaves is improved, but too much protease will produce bitter taste.

1 INTRODUCTION

In recent years, the sales of domestic cigars have increased in China. High-quality cigar tobacco leaves are the basis for making high-quality cigars. However, at present, the quality of domestic cigar tobacco leaves is generally low, and there are certain differences with foreign imported raw materials. The fermentation process of cigar tobacco leaves can improve the intrinsic quality of tobacco leaves to a certain extent by using the microbial degradation of tobacco leaves and the chemical transformation of substances, but relying on these processes alone cannot fully degrade the bad components in tobacco leaves (Wang, 2014). In addition, natural fermentation has obvious shortcomings such as long storage cycle, large warehouse occupation, and large backlog of funds. Therefore, further research and improvement of tobacco fermentation technology has extensive and practical significance for improving the quality of cigar leaves, saving production costs and improving economic benefits (Peng, 2009).

Protein is an important component of tobacco cell structure, and hydrolysates and further transformation products are the original substances of fragrance in some tobacco. Generally speaking, the protein content in tobacco leaves is 7%~10% is more suitable. If the protein content in tobacco leaves is too high, the smoke intensity is too large, the aroma and deterioration, resulting in spicy, bitterness and irritation, but also reduce its flammability (Sun, 2011; Yan, 2002; Tao, 2008), at the same time, protein is also a precursor of many harmful substances in the smoke, such as quinoline and pyridine (Schmelts, 1972; Higmane, 1970; Ruan, 2005; Johnson, 1971), HCN and 2-aminonaphthalene (Torikaiu, 2005), etc., seriously affecting the quality and safety of tobacco aroma.

In recent years, more and more studies have shown that the hydrolases produced by microorganisms can effectively degrade the proteins in tobacco leaves, so that the intrinsic chemical components in tobacco leaves tend to be coordinated, so as to achieve the purpose of improving the quality of tobacco leaves. Therefore, the use of biological enzyme technology to treat tobacco leaves can make the macromolecular substances in tobacco leaves moderately converted, thereby improving the intrinsic quality of tobacco leaves. *Luo* (Luo, 2020) et al. analyzed the fragrance-causing components of different enzyme-treated stems by setting protease, papain and polyphenol oxidase, and the results showed that among the eight major fragrance-causing components such as alcohols, aldehydes and ketones, except for the content of ester fragrance-causing components, the content of papain-acid protease and papain-polyphenol oxidase treated stems in other

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categories was increased compared with that without enzyme treatment. *Xu* (Xu, 2014) et al. optimized the dosage of complex cellulase and papain by single-factor method, and finally determined that the optimal dosage of compound cellulase and papain in tobacco end was 1000 U/g and 5500 U/g, respectively, and the reaction products had obvious effects on reducing cigarette gas irritation and harmonizing aroma. *Sun* (Sun, 2014) et al treated low-grade tobacco leaves with polysaccharide complex enzyme, neutral protease, pectinase and amylase, and found that polysaccharide complex enzyme and neutral protease had moderate degradation effects on low-grade tobacco cell wall substances. These studies all used tobacco-baked tobacco leaves as subjects, while cigar tobacco leaves were less studied as subjects.

Therefore, Sichuan Shifang cigar tobacco leaves was utilized as the research object in this research, focusing on the effect of exogenous neutral protease addition on the quality of cigar tobacco leaves during the fermentation process of cigar leaves. By analyzing the changes of conventional chemical composition, amino acid content, organic acids and other substances before and after natural fermentation and exogenous enzyme preparations, and combining the results of sensory analysis, the quality improvement effect of neutral proteases on cigar leaves was revealed. This study can provide a theoretical basis for the application of enzyme preparations in the cigar tobacco industry.

2 METHODOLOGY

2.1 Materials and Reagents

Material: Cigar tobacco leaves were produced in Shifang, Deyang, Sichuan.

Reagent: neutral protease was purchased from Novozymes, the enzyme activity test is (1694U/g), and the transport and storage conditions of the enzyme preparation are -4 ℃.

2.2 Instruments and Equipment

HPP750eco constant temperature and humidity incubator (Memmert, Germany) was used to provide a temperature and humidity environment for the fermentation process of cigar tobacco leaves. AA3 continuous flow analyzer (SEAL, Germany) was utilized for the detection of conventional chemical components in tobacco leaves. 6890B gas chromatography (Agilent, USA) was utilized for the detection of organic acid content in tobacco leaves. 1260 high performance liquid chromatography (Agilent, China) was utilized for the detection of polyphenol and pigment content in tobacco leaves. ICS1100 ion chromatograph (Thermo, USA) was used for the detection of carbohydrates and free amino acids in tobacco leaves.

2.3 Method

2.3.1.Fermentation test

The addition amount of neutral protease is shown in Table 1. The experimental group (adding exogenous enzyme preparations) added enzyme preparations by spraying enzyme preparation solution while stirring, and the moisture of tobacco leaves after adding enzyme preparations was 25%. While the control group (natural fermentation) was sprayed only with purified water that met the experimental requirements. After the pretreatment was completed, the experimental group and the control group samples were placed together in a 50°C, 80% constant temperature and humidity box for fermentation for 96 h, and immediately after the end of fermentation, they were placed in an oven at 85°C and inactivated for 15 min.

Table 1. Experimental design of control and experimental groups.

	Enzyme addition/(g/kg leaves)		
Control group	0	0	0
Neutral proteases	1.25	2.50	5.00

2.3.2.Determination of chemical composition

The determination of water-soluble sugar, total plant alkaloids, total nitrogen and starch contents in tobacco leaves was referred to YC/T 159-2019, YC/T 160-2002, YC/T 161-2002 and YC/T 216-2007, respectively. The determination of free amino acid content refers to the tobacco industry standard YC/T 282-2009. The test of polyphenol content refers to YC/T 202-2006. For the test of non-volatile organic acid content, refer to YC/T 288-2009. For the test of plastid pigment content, refer to YC/T 382-2010.

2.3.3.Sensory quality evaluation

The sensory quality evaluation of cigar leaves before and after fermentation was scored from three aspects: aroma characteristics, smoke characteristics and taste characteristics, with reference to GB 15269.4-2001 method.

3 Data and results

3.1 Changes in the Content of Conventional Chemical Components

The results of the content of conventional chemical components in tobacco leaves after natural fermentation and added enzyme preparation fermentation were shown in Figure 1. After fermentation, the total sugar content in tobacco leaves decreased, regardless of whether exogenous enzyme preparations were added. The content of reducing sugars was reduced, and the fermentation of added enzyme preparations was much lower than that of

natural fermentation, which may be due to the Maillard reaction between free amino acids, the degradation product of neutral proteases, and sugars in leaves. But there was no significant change in increasing the amount of enzyme. The total nitrogen content decreased slightly, and the decrease was slightly obvious when the enzyme addition was 1.25g/kg leaves. The total plant alkaloid content decreased significantly except for the enzyme addition amount of 1.25g/kg leaves, and there was no obvious change in the others.

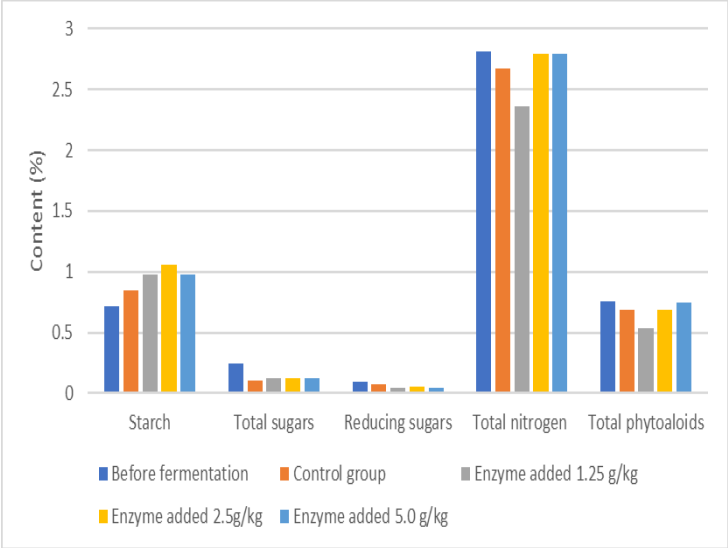

Figure 1. Content of conventional chemical components after fermentation with natural fermentation and added enzyme preparations fermentation.

3.2 Changes in Free Amino Acid Content

Amino acids are the basic building blocks of proteins and are the products of neutral protease action. Free amino acids play a bidirectional role in flue gas quality. On the one hand, the free amino acids of tobacco leaves are decomposed to produce ammonia under the action of acids, alkalis and enzymes, especially during the combustion process in tobacco leaves. On the other hand, amino acids react with other substances, and the substances formed generally have a flavor that harmonizes with the tobacco aroma and increases the fullness of the smoke. The results of the total amount of free amino acids in tobacco leaves after natural fermentation and added enzyme preparation fermentation were shown in Figure 2. After the end of fermentation, the sum of amino acids increases slightly. With the increase of exogenous neutral protease addition, the total amino acid content first increased and then decreased.

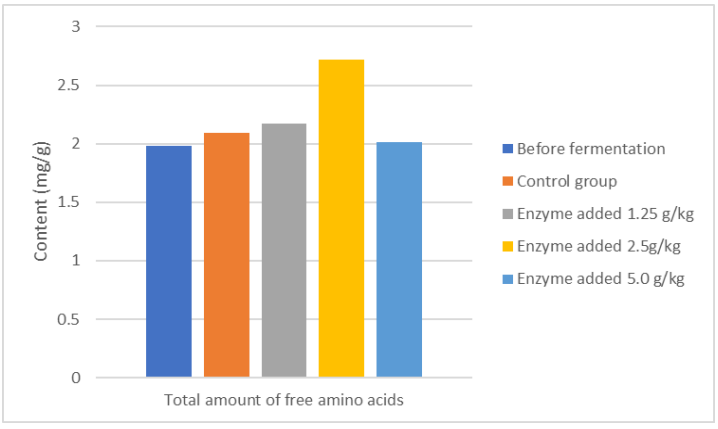

Figure 2. Total amount of free amino acids in tobacco leaves after natural fermentation and added enzyme preparations fermentation.

3.3 Changes in Polyphenol Content

During combustion, polyphenolic compounds produce many substances that have a positive effect on the smoke and aroma due to cracking. The results of the polyphenol content in tobacco leaves after natural fermentation and enzyme preparation fermentation were shown in Figure 3. The chlorogenic acid and rutin in tobacco leaves decreased slightly after fermentation, regardless of whether neutral proteases were added; There is no change in the scopolamine before and after fermentation.

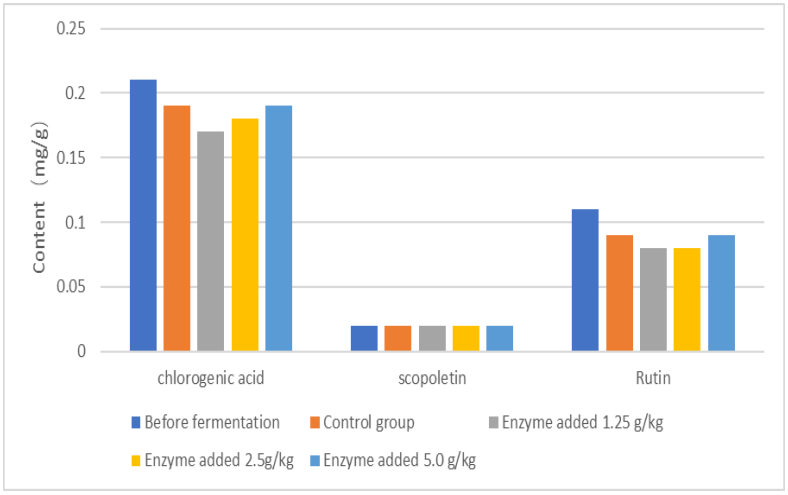

Figure 3. Polyphenol content in tobacco leaves after natural fermentation and added enzyme preparations fermentation.

3.4 Changes in Organic Acid Content

The organic acids in tobacco leaves balance the pH of the flue gas, while also cracking the aroma-producing substances. The results of organic acid content in tobacco leaves after natural fermentation and added enzyme preparation fermentation were shown in Figure 4. After the end of natural fermentation, the contents of oxalic acid, malic acid and citric acid in tobacco leaves increased compared with those before fermentation, and the content of citric acid increased the highest, about 5 times the initial content. When the amount of single enzyme was 2.5g/kg leaves, the contents of oxalic acid, malic acid and citric acid in tobacco leaves were higher than those of natural fermentation, and the content of each substance in the other groups was close to or slightly lower than that of natural fermentation.

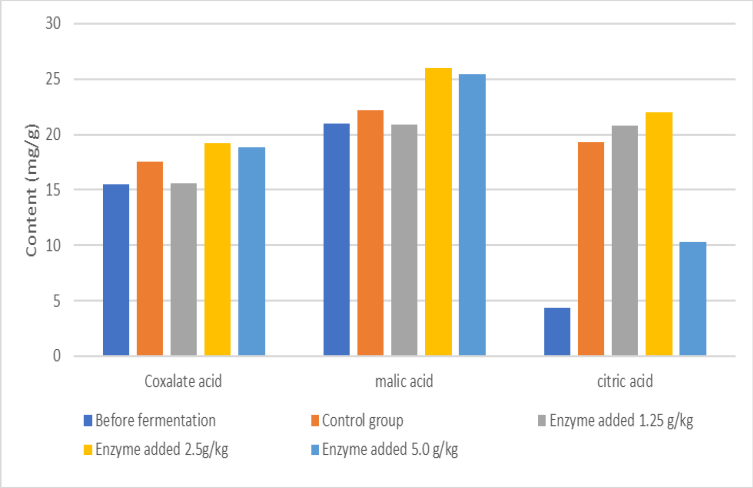

Figure 4. Organic acid content in tobacco leaves after fermentation by natural fermentation and added enzyme preparations.

3.5 Changes in the Content of Plastid Pigment Substances

Lutein and β-carotene are important flavor precursors in cigar leaves. The results of plastid pigment content in tobacco leaves after natural fermentation and added enzyme preparations fermentation were shown in Figure 5. Compared with unfermented tobacco leaves, due to the degradation of pigments in tobacco leaves during fermentation and the reduction of dry matter content, the lutein content in tobacco leaves was slightly reduced after natural fermentation, while the content of β-carotene in tobacco leaves was slightly increased. When the enzyme preparation was added to ferment, the fermentation process of tobacco leaf was accelerated, and the content of lutein and β-carotene in tobacco leaf showed a trend of first decreasing and then increasing with the increase of neutral protease addition.

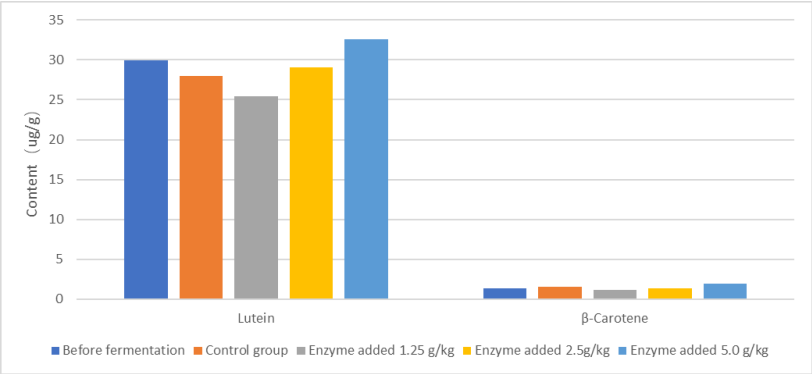

Figure 5. Plastid pigment content in tobacco leaves after natural fermentation and added enzyme preparations fermentation.

3.6 Changes in Sensory Quality

After adding exogenous neutral protease fermentation of cigar tobacco leaves, the delicate feeling of smoke is enhanced, the smoke is clean and the taste is comfortable. However, as the concentration of neutral protease is added, the overall sweetness is weakened and slightly bitter, which may be caused by excessive protease addition and the bitter taste produced when the protein is burned.

4 CONCLUSIONS

In this work, the changes of conventional chemical composition, amino acid content, organic acids and other substances in tobacco leaves natural fermentation and fermentation with exogenous enzyme preparations were investigated. In addition, the changes of sensory quality of tobacco leaves after fermentation were also investigated. The results show that: 1) After fermentation, the content of sugar, total nitrogen, chlorogenic acid and rutin in tobacco leaves decreased, and the content of reducing sugar fermented with enzyme preparation decreased more. 2) After fermentation, the total amount of free amino acids in tobacco leaves increased. With the increase of exogenous neutral protease, the content of amino acids first increased and then decreased. 3) After fermentation, the content of oxalic acid, citric acid and malic acid in tobacco leaves increased. When the amount of enzyme added was 2.5g/kg leaves, the increase of organic acid content was greater than that of natural fermentation, and other conditions were close to that of natural fermentation. 4) After fermentation, the content of lutein and β-carotene in tobacco leaves decreased slightly. With the increase of neutral protease, the content of plastid pigment increased. 5) After the fermentation of exogenous enzyme preparation, the sensory quality of tobacco leaves is improved, but too much protease will produce bitter taste.

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There are no conflicts to declare.

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