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Recent advances on the fungi of Pu-erh ripe tea

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Abstract

Pu-erh tea is one sort of unique Chinese post-fermented tea. Fungi play an important role in forming its special flavor, taste and healthy function During the post-fermentation process of Pu-erh tea, meanwhile, some harmful fungi may bring potential safety hazards to the production of Pu-erh tea. This paper summarizes recent advances on the Fungi of Pu-erh ripe tea,which concerned about fungal colonization, metabolism and safety of fungi.

Introduction

Pu-erh tea is a kind of post-fermented tea, which can be divided into two types according to the difference of fermentation process. One is Pu-erh raw tea which fermented through long-time aging process has nothing to do with the microbial action(Yang, 2006). Another is Pu-erh ripe tea which fermented through pile fermentation concerned a large number of microorganisms including fungi and bacteria(Fang *et al*., 2008; Lei, 2009; Zhao *et al.*, 2010),In recent years, more and more reports are concern on populations, metabolism, safety etc. about Fungi of Pu-erh ripe tea, This paper summarizes recent advances on the Fungi of Pu-erh ripe tea.

Fungal populations in the pile fermentation of Pu-erh ripe tea

As early as in 1957, Hu *et al.* studied microorganisms in four sort reprocessed tea, isolating and identifying main microorganisms such as *Penicillium*, *Aspergillus*, *Mucor*, *Rhizopus* and *Cladosprium* (Hu,1957). But microbial researchs of Pu-erh ripe tea have not been published until the late 1980s after it's process was standardizing in1979. Pile fermentation, is the most critical process for Pu-erh ripe tea . During the pile fermentation process, the environment of warm temperature and humidity is very suitable for the mass growth and reproduction of fungi.

Simulating the pile fermentation process of Pu-erh ripe tea in lab, Chen (1985) *et al*. discovered that there were *Aspergillus niger*, *Penicillium* spp., *Rhizopus*, *Aspergillus glaucus* and *Yeasts* in the pile fermenting tea, During pile fermentation process, *Aspergillus niger, Penicillium* spp. and *Rhizopus* began to multiply at the beginning of fermentation; the fungal quantity reached to the maximum value in the first turning and second turning periods and then reduced gradually; during the whole Pile fermentation process, *Aspergillus niger*, accounting for about 80% of the total number of microorganisms, was dominant in quantity and enjoyed the absolute dominant position in the pile fermentation of Pu-erh tea (Chen *et al.*,1985; Liu *et al*., 1986).

However, there are fungi including *Aspergillus niger*, *Aspergillus gloucus*, *Aspergillus terreus*, *Aspergillus candidus*, *Penicillium*, *Rhizopus* and *Saccharomyces* in the mass pile-fermentation production of Yunnan Pu-erh tea (Zhou *et al*., 2004; Xu *et al*., 2005) . Among these fungi, *Aspergillus terreus* and *Aspergillus candidus* are the fungal populations which have been isolated for the first time. But due to the limitation of morphological identification methods, the specific relationships between some fungal populations such as *Penicillium* and *Rhizopus* are not identified deeply.

After pile fermentation, Pu-erh ripe tea is made into the finished tea after drying and compression processes. The finished tea also contains many fungal populations. Michiharu *et al.* (2008) directly extracted the DNA of fungus in the Pu-erh tea and applied the PCR technology to amplify the DNA fragments. Through identification, it was found that two fungal species widely exist in the commercially available Pu-erh tea products, that is, *Aspergillus niger* and

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blastobotrys adeninivorans of *Saccharomyces* .

Zhao *et al.* (2010) conducted the systematic researches on the fungal populations in the tea samples from 13 medium- and large-sized Pu-erh tea factories in Yunnan province. Results showed that fungi in the Pu-erh tea can be divided into 40 species of 19 genera, including 13 species of *Aspergillus-Aspergillus niger, Aspergillus orgyze, Aspergillus phoenicis, Aspergillus penicilliode, Aspergillus foetidus, Aspergillus awamori, Aspergillus tubingensis, Aspergillus wenti, Aspergillus dimorphicus, Emericella nidulans, Aspergillus sydowii, Gliocladium cibotii,* and *Aspergillus fumigatus*, 7 species of *Penicillium-Penicillium citrinim, Penicillium oxalicum, Penicillium griseofulvum, Penicillium variabile, Penicillium chrysogemm, Penicillium aculeatum* and *Penicillum* sp., and other 20 species of 16 genera.

In conclusion, the common fungi isolated in the fermentation process of Pu-erh tea mainly belong to *Aspergillus, Yeasts, Penicillium, Rhizopus* and *Mucor* (as shown in table 1) (Fang *et al*., 2008; Lei, 2009; Hu, 1957; Hu *et al*., 1979; Chen *et al*., 1985; Liu *et al*., 1986; Zhou, 2004; Xu *et al*., 2005; Michiharu *et al*., 2008; Zhao *et al*., 2010); among them, *Aspergillus* and *Yeasts* are dominant in the fermentation process of Pu-erh tea.

Main characteristics of fungi during the rapid post-fermentation process of Pu-erh tea

The quantity changing with the processing process

The medium-temperature molds like *Aspergillus niger, Penicillium* spp. and *Rhizopus* grow rapidly at the early stage of pile fermentation and gradually drop later; while the low-temperature xerophilous Aspergillus glaucus begins to reproduce at this time. During the whole pile-fermentation process, the enzyme activity of polyphenol oxidase, peroxidase and ascorbic acid oxidase secreted by the related fungi changes irregularly with the growth and reproduction processes of fungi, which is positively correlated with the growth status of fungi. The enzyme activity and the humidity and warmth caused by the growth of microorganisms make it possible the chemical reactions of tea components like oxidation, degradation and condensation (Chen *et al*., 1985; Zhou *et al*., 2004) . He *et al.* studied the pile fermentation process of Guangdong Pu-erh tea and results showed that microbial communities were complex in the pile fermentation with fungi occupying the dominant position especially at the early stage of pile fermentation; molds developed first and then gradually gave place to Yeasts (He *et al*., 1987) in the middle and later periods. Liu *et al.*

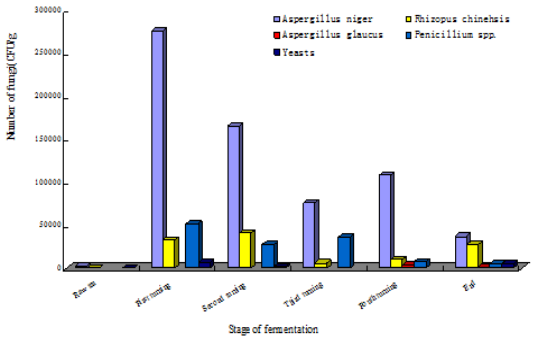

Figure 1. The quantity changes of main fungi during the Pu-erh tea fermentation (Liu *et al.*, 2005)

(2005) also conducted researches on the dynamic changes of fungal populations and concluded the similar research results. The changes in the numbers of fungi during the pile fermentation process are shown in Fig.1 (based on the research results of Liu *et al.* 2005). *Aspergillus* and *Rhizopus* are dominant in these molds. The number of *Yeasts* is small during the first few days and does not grow until the middle of pile fermentation. Gradually, Yeasts become the dominant fungi. Molds can take the polysaccharide as carbon source and the glycometabolism can produce numerous disaccharides and monosaccharides, making Yeasts enough nutrition to ensure the rapid reproduction. At the same time, the mass reproduction of Yeasts and molds suppresses the growth of bacteria (Liu, 2009). *Aspergillus niger* is the dominant fungus in the pile fermentation process of Pu-erh tea. Its number reaches the maximum value of 3×10^5 CFU/g at the second turn, gradually reduces in the following processes and becomes 3.6×10^4 CFU/g at the end of the pile fermentation.

Regional characteristics of fungal populations

The population diversity of microorganisms can be influenced by the growing environment, which gives it obvious regional differences(Shen *et al*., 2000). The fungi in the fermentation process of Pu-erh tea come from the surrounding environment, so the fungal species and population structures of Pu-erh tea of different regions and sources of raw materials may be different. Just due to the differences of fungal populations, Yunnan Pu-erh tea can provide various flavors (Liu, 2009).

Aspergillus niger, Penicillium spp., *Rhizopus, Aspergillus glaucus* and *Yeasts* were isolated in the fermentation process of Pu-erh tea in Chongqing (Chen *et al*., 1985). And fungi including *Aspergillus niger, Aspergillus gloucus, Aspergillus terreus, Aspergillus candidus, Penicllium, Rhizopus* and *Saccharomyces* were isolated and identified in the mass pile-fermentation process of Pu-erh tea in

Table 1. Fungal colonization determined during the post-fermentation of Pu-erh tea

Type of post-fermentation	Fungi		References
	Genus	Species	
Natural post-fermentation	*Aspergillus*	*A. niger, A oryzae, A glaucus, A. Fumigatus, A. Phoenicis,*	Fang *et al*., 2008; Lei, 2009; Hu *et al*., 1979; Zhao *et al*., 2010.
	Penicillium	*P. Chrysogenum, P. Aculeatum, Penicillium sp.*	
	Others	*Fusarium, Stemphypium, Mucor, Rhizopus, Trichoderma spp., Yeast*	
Artificial post-fermentation	*Aspergillus*	*A. niger, A. foetidus, A. Tubingensis, A. Awamori, A. Wenti, A. Fumigatus, A. Phoenicis, A. Penicilliode, A. Nidulans, A. Sydowii, Aoryzae, A glaucus*	Hu *et al*., 1979; Liu *et al*., 1986; Zhou *et al*., 2004; Xu *et al*., 2005; Michiharu *et al*., 2008; Zhao *et al*., 2010; Chen *et al*., 2006.
	Penicillium	*P.citrinum, P. Oxalicum, P. Griseofulvum, P. Variable, P. Chrysogenum, P. Aculeatum, Penicillium sp.*	
	Others	*Fusarium, Rhizopus, Trichoderma spp., Saccharomyces, Blastobotrys adeninivorans*	

Qunming (Zhou *et al*., 2004; Xu *et al*., 2005); among these fungi, *Aspergillus terreus* and *Aspergillus candidus* are fungal populations that have been isolated for the first time.

Chen *et al.* (2006) randomly collected samples in various places of Yunnan province and found that tea samples from different regions had different fungal populations: *Aspergillus wentii* var. *fumeus*, *Aspergillus penicillioides*, *Aspergillus aureolatus* and *Aspergillus japonicus var. japonicus* were isolated from the fermentative stacks of Pu-erh ripe tea collected from Yiwu town, Mengla County; *Aspergillus penicillioides, Aspergillus aureolatus, Aspergillus foetidus, Aspergillus japonicus var. Japonicus* and *Aspergillus restrictus* were isolated from the fermentative stacks of Pu-erh ripe tea collected from Jinuo Mountain, Jinhong City; *Aspergillus egyptiacus* was isolated from the fermentative stacks of Pu-erh ripe tea collected from Kunming. The above-mentioned results show that fungal populations in the fermentation processes of Pu-erh tea in Chongqing and Yunan are different. Even for Pu-erh tea produced in various places of Yunnan province, the fungal populations are quite different. This may explain why the flavors of Pu-erh tea are various (Chen *et al*., 2006). The direct reason of the differences of fungal populations in different places is the microbial diversity in raw material sources and post-fermentation places (Liu, 2009).

Fungi and Pu-erh tea quality

During the pile fermentation process of Pu-erh tea, the growth and metabolism of fungi, playing an important role in the formation of Pu-erh tea quality, ensure the complex changes of Pu-erh tea components like degradation, isomerism, coupling and polymerization.

Fungi involving in the transformation of components of Pu-erh tea

During the pile fermentation of Pu-erh tea, the content of TP (tea polyphenol) drops rapidly. It is generally believed that fungi are involved in the processes of oxidation, degradation and polymerization of TP. And tremendous TPs have transformed into the specific components of Pu-erh ripe tea including gallic acid, theaflavin, thearubigin and theabrownin (Lv *et al*., 2013). Xie *et al*. (2013) took advantage of the culture medium with TP as the sole carbon source to cultivate fungi like *Yeasts, Aspergillus niger, Rhizopus,* and *Penicillium*. Results showed that these fungi could grow well and the content of monosaccharide, polysaccharide, and oligosaccharide also increased in different levels during this process. It can be concluded that TP may have other degradation methods during the pile-fermentation process of Pu-erh tea, that is to say, under the action of fungi, TP may be transformed into other tea components which can improve the quality of Pu-erh tea including monosaccharide, polysaccharide and oligosaccharide.

Caffeine increases during the fermentation process of Pu-erh tea. Wang *et al.* (2013) considered that fungi could change the existing form of caffeine. This would result in the decrease of free caffeine and the increase of the combined caffeine; Wang *et al*. did single-strain fermentation experiments and proposed that theophylline was the precusor substance of caffeine; they thought fungi like *Aspergillus niger* could convert theophylline into caffeine and the involvement of *Aspergillus niger* would increase the content of caffeine during the fermentation process of Pu-erh tea (Wang *et al*., 2008)

To promote the transformation and formation of quality components of Pu-erh tea, fungi involve

Table 2. Toxic eatrolites produced by the common *Penicillium* spp and its effect on the health

Species	Toxic extrolites	Potential effect	References
Penicillium chrysogenum	Penicillins	Antibiotoxic	Pitt *et al*., 1999;Samson *et al*., 2004; Frisvad *et al*., 2004; Thomas *et al*., 2004.
	Roquefortine C	Neurotoxic	
	Meleagrin	Mutagenic	
Penicilllum expansum	Patulin	Neurotoxic	Thomas *et al*., 2004;Raper *et al*.,1949; Mary *et al*., 2005.
	Citrinin	nephrotoxic	
Penicillium citrinum	Citrinin	nephrotoxic	Raper *et al*.,1949.
Penicillium griseofulvum	Patulin	Neurotoxic	Mary *et al*., 2005.
Penicillium verrucosum	OTA	carcinogenic, nephrotoxic	Cabañes *et al*., 2008; Bragulat *et al*., 2008.
	Citrinin	nephrotoxic	
Penicillium spp.	OTA	carcinogenic,nephrotoxic	Cabañes *et al*., 2008; Rosa *et al*., 2006.

Notes: According to research results of Pitt *et al.*

in the decomposition and degradation of protein, amino acid and carbohydrate in Pu-erh tea and takes part in a series of reactions like polymerization and condensation between various products by secreting extracellular enzymes. The common extracellular enzymes include: polyphenol oxidase, glycosidase, pectinase, glucamylase, cellulase, naringinase and lactalase secreted by *Aspergillus niger*; glucose oxidase secreted by *Penicillium*; amylase and pectinase secreted by *Rhizopus*; and amylase, protease and pectinase secreted by *Yeasts*. Tannase can hydrolyze tannin to produce organic acids, conducting further esterification to form aromatic flavor substances. Under the action of extracellular enzymes, the content of flavoring materials of fresh, sweet, sour and bitter tastes in raw tea will decrease and gradually change into fragrant aroma of ripe tea (Dong *et al*., 2013).

Fungal metabolism introducing non-tea substances

During the pile fermentation of Pu-erh tea, the growth and metabolism of fungi can not only promote the transformation of tea components, but also introduce other non-tea substances-the specific metabolites of fungi. The fungal metabolites constitute an important part of Pu-erh tea; for example, dimethoxy - 4 - methyl benzene is the typical substance of musty taste and stale flavor in Pu-erh tea. Researches of Gong *et al*. (1993) showed that it was the specific metabolite during the growth of *Aspergillus niger.* In addition, the fungal metabolites are the key components of healthcare functions of Pu-erh tea. For instance, the metabolisms of *Aspergillus tubingensis, Aspergillus wenti, Aspergillus fumigatus, Penicillium chrysogenum, Trichoderma asperellum,* and *Trichoderma citrinoviride* can produce lovastatin which only exists in the Pu-erh ripe tea (Zhao *et al*., 2013). Besides, there are other non-tea substances produced from the metabolisms of fungi. It is necessary to conduct further studies on this aspect. These research results will play an important role in the improvement of Pu-erh tea quality and the in-depth assessment of safety and healthcare function of Pu-erh tea.

Fungi and Pu-erh tea safety

Although some scholars have carried out isolation and identification researches on microorganisms of Pu-erh tea, no research result of harmful microorganisms and mycotoxins has been published. We can just summarize various research results to speculate the harmful fungi of Pu-erh tea which mainly include *Aspergillus, Penicillium* and other fungi like *Fusarium.*

Aspergillus

Aspergillus niger, generally acknowledged as a comparatively safe industrial fungus in the last century, is the Aspergillus of the largest number in the Pu-erh tea (Chen *et al*., 1985; Liu et al., 1986). But since the 21st century, researchers have found that it had the certain ability to produce mycotoxins. Researches of Schuster *et al*. showed that the metabolism of *Aspergillus niger* could generate Ochratoxin A and 3-10% of *Aspergillus niger* strains could produce toxins (Schuster *et al*., 2002). Giancarlo *et al*. (2005) found that a part of *Aspergillus niger* strains isolated from the vineyard, grape and grape processing could produce Orchatoxin A and they considered that the concentration range of Orchatoxin A produced by *Aspergillus niger* strains in the wine is 250-360 μg/L. *Aspergillus niger* can not only produce OTA on the grape substrate, but also has the ability to generate mycotoxins on other culture media (Tjamos *et al.*, 2004; Da *et al.*, 2002). Kouadio *et al.* (2007) took the coffee beans as the culture medium and discovered that *Aspergillus niger* could generate a certain amount of carcinogenic Ochratoxin A. They also isolated some *Aspergillus niger* strains which can metabolize OTA from grapes and vineyards. Frisvad

et al. (2007) took the extracting solutions of malt, rice, potato and carrot as culture media and tested that *Aspergillus niger* could also produce another mycotoxin through metabolism-Fumonisin B. This mycotoxin is carcinogenic. It is unknown whether *Aspergillus niger* can produce mycotoxins similar to Ochratoxin and Fumonisin.

Aspergillus flavus is a common toxic fungus. James *et al*. (1994) once isolated *Aspergillus flavus* from the fresh green tea leaves, but did not isolate it from Pu-erh tea. Because *Aspergillus flavus* can produce the mycotoxin of strong carcinogenesis-aflatoxin, *Aspergillus flavus* has always been the focus of attention. Test items for *Aspergillus flavus* are specified in the quality standards of Chinese Pu-erh tea exporting to Russia.

Aspergillus versicolor can also exist in Pu-erh tea (James *et al.* 1994). It can produce sterigmatocystin through metabolism. The structure of sterigmatocystin is similar to that of AFB_1 and they can be mutually transformational, so sterigmatocystin will develop toxicity similar to that of AFB_1. James *et al.* (1994) studied the capability of *Aspergillus versicolor* strains to generate sterigmatocystin. They found that *Aspergillus versicolor* could produce sterigmatocystin on the culture medium of oatmeal and failed to produce sterigmatocystin in the pure green tea or the green tea with 10% oatmeal. This indicated that a certain tea component can inhibit the capability of *Aspergillus versicolor* to produce sterigmatocystin. It is supposed that there is no sterigmatocystin produced by *Aspergillus versicolor* through metabolism in Pu-erh tea.

Zhou *et al.* (2004) isolated *Aspergillus terreus* from Pu-erh tea. On the culture media like Czapek's solution, *Aspergillus terreus* can produce clavacin (also called as patulin) which is a kind of pathogenic mycotoxin. *Aspergillus terreus* can also produce statin-like substances (Cai *et al*., 2000; Hendricjson *et al*., 1999). Statin-like substances can treat cardiovascular diseases. But they, together with some drugs, can also cause death because of the dissolution of striated muscles in human hearts and joints (Hanai *et al*., 2007).

Penicillium

Penicillium is very common in Pu-erh tea and widespread in the storage and artificial post-fermentation processes of Pu-erh tea (Zhao *et al.*, 2010; Chen *et al*., 2006). Some researches consider that *Penicillium* is the main reason of the flavor quality of Pu-erh tea; for example, *Penicillium chrysogenum, Deuteromycotina, Hyphomycetes, Hyphomycetales* and *Moniliaceae* can produce a variety of enzymes and organic acids and generate penicillin through metabolism, inhibiting the infectious bacteria and spoilage organisms of Pu-erh tea and having a positive impact on the formation of fragrant aroma and superior quality of Pu-erh tea (Wei, 1979; Fang *et al*., 2008).

There are more than 600 species of *Penicillium* with the similar morphological characteristics (Fallah *et al.*, 2004). Zhao *et al.* (2010) applied the molecular identification to isolate and identify *Penicillium citrinum, Penicillium oxalicum, Penicillium griseofulvum* and *Penicillium acileatum*. Some species of *Penicillium* like *Penicillium citrinum* can produce mycotoxins like patulin which may bring certain harms to human body. Common *penicillium* populations and their toxic products are shown in Table 2.

Other harmful fungi

In addition to *Aspergillus* and *Penicillium,* a small number of *Fusarium* and *Trichoderma* spp. can also be found in Pu-erh tea (Liu *et al.*, 1986; Fang *et al*., 2008; Lei, 2009; Zhao *et al*., 2010). *Fusarium* and *Trichoderma* spp. can produce mycotoxins through metabolism like Trichothecene. Trichothecene can damage and inhibit the body's normal immune system (Vidal,1990; Michael *et al*., 1998; Cynthia, 2004), resulting in the serious damage of body issues of active cell division such as bone marrow, lymph nodes, spleen, thymus and intestinal mucosa. Its mechanism is to affect the protein synthesis through the impacts on the synthesis of DNA and RNA and the blocking-up of the start of the translation. It mainly affects the rapidly growing issues (such as skin and mucous membranes) and immune organs (thymus and bursa of Fabricius) (Richard,1991; Prelusky *et al*., 1994; Rotter *et al.*, 1996). On one hand, the fungi in the Pu-erh tea exert influence on the improvement of the quality of Pu-erh tea, like Yeasts; on the other hand, they can produce some harmful metabolites like some species of *Penicillium* and *Fusarium* which may bring potential safety hazards to the production of Pu-erh tea.

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