Short title: *Morchella* mushroom strain aging assessment

Activity Assay of Amylase and Xylanase Is Available for Quantitative Assessment of Strain Aging in Cultivated Culinary-Medicinal *Morchella* Mushrooms (Ascomycotina)

Peixin He,[a] Zhuo Chen,[a] Ying Men,[a] Miaomiao Wang,[a] Wensheng Wang,[b] & Wei Liu[c,*]

[a]College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou 450001, P.R. China; [b]Henan Junsheng Agricultural Science and Technology Co., Ltd., Zhengzhou 450001, P.R. China; [c]Germplasm Bank of Wild Species, Yunnan Key Laboratory for Fungal Diversity and Green Development, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P.R. China

*Address all correspondence to: Wei Liu, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P.R. China; E-mail: zhenpingliuwei@163.com.

ABSTRACT: Strain aging has been mainly contributing to the “uncertainty” of *Morchella* farming. The situation calls for urgent quantitative assessment of strain aging in cultivated *Morchella* mushrooms. In this paper, systemic senescence of the productive strains of *M. eximia*, *M. importuna* and *M. sextelata* was achieved through successive subculturing to provide subcultures with different degree of aging for further studies. Then the quantitative assessment of morel strain aging was conducted by activity assay of amylase and xylanase using dinitrosalicylic acid (DNS) method. The results suggested that both activity of amylase and xylanase decreased along with the rise of subculture times. Meanwhile, the correlation between xylanase activity and time of subculturing in the tested morel strains was higher than that of amylase assay. Consequently, assay of amylase and xylanase activity by DNS method can be used in the quantitative assessment of morel strain aging, and assay of xylanase activity is the

better alternative. The work will improve the settlement of “uncertainty” in morel industry and thus be beneficial for stable development of morel farming.

KEY WORDS: Ascomycota, *Morchella,* senescence, successive culturing, medicinal mushrooms

ABBREVIATIONS: CYM, yeast extracts medium; **DNS,** 3,5-dinitrosalicylic acid

I. INTRODUCTION

Mushrooms have been used in folk medicine throughout the world since ancient times. Medicinal mushrooms that contain biologically active compounds in fruit bodies, cultured mycelium, and cultured broth are thought to possess approximately 130 medicinal functions.[1-3] True *Morchella* (Pezizales, Ascomycota) are world famous culinary and medicinal mushrooms for their delicate taste and outstanding medicinal values.[4-10] The prominent economic values inspired eager fundamental and technological studies for the domestication of *Morchella* mushrooms to meet the still-increasing market demands for more than a century.[4,6,11] On the basis of Ower’s innovations,[12] field soil cultivation of *Morchella* mushrooms characterized by the use of cultivable morel strains and exogenous nutrition aiding has been realized in China since 2012.[4,6] In production season of 2021-2022, the total cultivation area exceeded 17000 ha in most areas of China. Some strains of *M. importuna*, *M. sextelata* and *M. eximia* have been widely applied in scale outdoor production.

Unfortunately, along with the rapid expansion of artificial cultivation, the prominent problem of “uncertainty” has been frustrating morel industry.[11,13] The uncertainty may be mainly imputable to the knowledge gaps on many aspects of the genetics and biology of *Morchella* spp. As ascomycetous mushrooms, morels are liable to strain aging in comparison to Basidiomycetes.[11,14] The evaluation of morel strain aging is mainly based on cultural

characteristics in practical production.[13] However, the degree of aging in most cultures of *Morchella* spp. was difficult to estimate by cultural characteristics, which may bring about large amounts of aging cultures being used in morel cultivation, and thus bring remarkable losses for morel production and mainly attributes to the "uncertainty" of morel farming.[11] The situation calls for urgent quantitative assessment of strain aging in cultivated *Morchella* mushrooms.

In mushroom biology, senescence can be defined as a time-related process of progressive decline or loss of physiological functions.[15] Production of some extracellular enzymes which degrade various biomolecules in compost, such as cellulose, hemi-cellulose, starch, lipids, and lignin, is closely related with mushroom cultivation. Therefore, assay of the activity of certain extracellular enzymes, e.g. amylase, xylanase, cellulase and laccase of different subcultures may be used to assess the strain aging of some edible and medicinal mushrooms.[16-19] Considering the huge losses annually brought about by misuse of senescent cultivated strains and the urgency of reliable technology for quantitative assessment of strain aging, relevance of the activity of certain extracellular enzymes with strain aging of *Morchella* mushrooms was conducted to select the feasible methods for evaluation of morel strain aging. The results showed that assay of the activity of amylase and xylanase was suitable for quantitative assessment of strain aging in three cultivated *Morchella* mushrooms. The work will be beneficial for stable development of morel farming.

II. MATERIALS AND METHODS

A. Fungal Strains and Activation of Culture

Morchella eximia YL-9 is one of the isolates from fresh ascocarps of cultivated variety of YL, which was introduced from Yulin Service Station of Land-reclaimable Agriculture, Shaanxi Province, P. R. China. Strains of *M. importuna* 4-21 and *M. sextelata* 13-21 are productive ones

domesticated from wild morels occurred in Sichuan Province, China. All strains were productive ones and thus they were not preserved in any National Culture Collection. Nevertheless, they are available from Peixin He in Zhengzhou University of Light Industry. A mycelial plug from stock culture was inoculated on the surface of complete yeast extracts medium (CYM) (glucose 20 g/L, yeast extracts 2 g/L, peptone 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.46 g/L, and agar 20 g/L) plates and incubated at 24°C in the dark for 3-4 d until the mycelia were fully grown.

B. Successive Subculturing

The strain obtained via tissue separation from fresh ascocarp represents subculture 0 (4-21-0, 13-21-0 or YL-9-0). Successive subculturing was done by taking inoculum from the subculture 0 to produce the 1st subculture (4-21-1, 13-21-1 or YL-9-1), repeated for the 2nd one (4-21-2, 13-21-2 or YL-9-2) from the 1st and so on.[11,14] Briefly, a 2-mm-diameter mycelial plug from stock culture was inoculated on one side of a race tube (glass tube with 42 cm length, 2.2 cm diameter) containing 60 mL CYM and incubated at 24 ^{o}C in the dark. Race tubes allow the growth of colonies for a longer period of time (e.g., 25 d) under defined conditions. Before the mycelia reaching the other side of a race tube, mycelial plugs containing the whole hyphal tips excised from colony edges were transferred to fresh race tubes with growth tips toward the margin of medium and continued to cultivate until growth cessation. Meanwhile, the adjacent plugs close to the hyphal tips of race tube cultures were transferred to CYM slant, and then incubated at 24 ^{o}C in the dark. The slant cultures were preserved at 4 ^{o}C for short-term use or –80 ^{o}C under the protection of 20% (w/v) trehalose solution for long-term use. Each subculture was repeated three times with inocula of the same age and from the same race tube.

C. Assay of Amylase and Xylanase Activity

1. Selection of Tested Strains

In successive subculturing, mycelial growth ceased in the 3rd subculture of *M. eximia* YL-9 and the 4th subculture of *M. sextelata* 13-21, while the mycelial growth still proceeded in the 4th subculture of *M. importuna* 4-21. Therefore, subcultures of YL-9-0, -1 and -2, with time of subculturing of 0, 600, and 984 h, respectively, subcultures of 13-21-0, -1, -2 and -3, with time of subculturing of 0, 456, 948 and 1436 h, respectively, and subcultures of 4-21-0, -1, -2, -3 and -4, with time of subculturing of 0, 600, 912, 1325 and 1757 h, respectively, were selected for further study. The tested strains were cultured for activation on CYM plates before use.

2. Preparation of Crude Enzyme Solution

Twenty grams of compost (w/w) (wheat grain 60%, sawdust 28%, rice hull 10%, gypsum 1%, calcium carbonate 1%, water content 50%) in 100 mL of Erlenmeyer flask was sterilized at 121 ^{o}C for 120 min. After cooling, an agar disk (2 cm diameter) was inoculated on the surface of compost. The flasks were incubated at 24 ^{o}C in the dark for 7 – 10 d until the mycelia were fully grown. Four grams of cultures were soaked in 40 mL distilled water for at least 3 h. The extract solution was centrifuged at 5000 *g* and 4 ^{o}C for 15 min, and the supernatant was used as crude enzyme solution. Aliquots (12 mL) of the supernatant were boiled for 15 min, which obtained the inactivated enzyme solution as blank in assay of enzyme activities.

3. Determination of Amylase Activity

The amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method.[20] Briefly, 0.5 mL of crude enzyme solution, 1.5 mL of substrate (0.5% w/v soluble starch) and 1.0 mL of acetate acid-sodium acetate buffer (0.2 M, pH 4.8) were mixed thoroughly in the test tubes, and then incubated at 37 ^{o}C for 30 min in a water bath. The reaction was stopped by adding 2.0 mL of DNS reagent, and the tubes were kept in a boiling water bath for 5 min. The mixture was cooled at room temperature, diluted with distilled water to volume of 25 mL, and the absorbance

was measured at 540 nm against inactivated enzyme blank. One unit (U) of amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar as D-glucose per minute under the assay conditions.

4. Detection of Xylanase Activity

The xylanase activity was measured using the DNS method.[20] Briefly, 0.5 mL of crude enzyme solution and 1.5 mL of substrate [1% w/v xylan, in acetate acid-sodium acetate buffer (0.2 M, pH 4.8)] were mixed thoroughly in test tubes, and then incubated at 50 ^{o}C for 30 min in a water bath. The reaction was stopped by adding 2.0 mL of DNS reagent, and the tubes were kept in a boiling water bath for 10 min. The mixture was cooled at room temperature, diluted with distilled water to volume of 25 mL, and the absorbance was measured at 540 nm against inactivated enzyme blank. One unit (U) of xylanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar as D-xylose per minute under the assay conditions.

D. Statistical Analysis

Unless otherwise specified in “Successive subculturing”, the data were expressed as mean $\pm$ standard deviation (S.D.) (n = 3). Data were subjected to one-way ANOVA followed by the Duncan's multiple range test. Probability values < 0.05 and < 0.01 were regarded as statistically significant and highly significant, respectively. All analyses were carried out using IBM SPSS Statistics. The figures were drawn using Excel 2013, Microsoft, U.S.A.

III. RESULTS

A. Systemic Senescence through Successive Subculturing

After 4 times of successive subculturing, the mycelial growth of *M. importuna* 4-21 still proceeded. The main subculture characteristics were summarized in Table 1. The results suggested that the lifespan and total growth length of the tested morels are strain-dependent. *M.*

eximia YL-9 showed the shortest lifespan (58 d) and total growth length (94.6 cm), while the total growth length of *M. importuna* 4-21 and *M. sextelata* 13-21 both exceeded 130 cm. The results again highlight the necessity of strain vitality determination by successive subculturing in evaluation of cultivating suitability for the productive strains of *Morchella* mushrooms.[13] In morel artificial cultivation, the total growth length of an ideal strain should be greater than 120 cm, which imply that *M. eximia* YL-9 is not suitable for application in practice.

B. Assay of Amylase Activity Can Be Used in Quantitative Assessment of Morel Strain Aging

The amylase activity of tested morel strains showed the same tendency to subculture times, i.e., the enzyme activity decreased with the rise of subculture times. Meanwhile, the correlation between amylase activity and time of subculturing in the tested morel strains was all high (R^2 = 0.8475 – 0.9981), which suggested that the assay of amylase activity by DNS method can be used in quantitative assessment of morel strain aging (Fig. 1). Furthermore, compared to that of *M. sextelata* 13-21 and *M. eximia* YL-9, the curve between time of subculturing and amylase activity in *M. importuna* 4-21 was of flat type. The amylase activities decreased gradually along with the rise of subculturing period, which was consistent with the tendency of culture and production traits in different aging isolates of *M. importuna* T4.[11]

C. Assay of Xylanase Activity Is the Best Alternative for Quantitative Assessment of *Morchella* Strain Aging

The xylanase activity of tested morel strains showed the same tendency to subculture times, i.e., the enzyme activity decreased with the rise of subculture times. Moreover, compared to the assay of dehydrogenase activity, lipid peroxidation and amylase activity, the correlation between xylanase activity and time of subculturing in the tested morel strains was consistently high (R^2 =

0.9274 – 0.9949), which suggested that assay of the xylanase activity by DNS method is the best alternative for quantitative assessment of morel strain aging (Fig. 2).

IV. DISCUSSION

A. Application of Assay of Amylase Activity in Assessment of *Morchella* Strain Aging

The amylase enzyme activity of the tested strains decreased with the rise of subculture times with high regression coefficients, which suggested the applicability of assay of amylase activity by DNS method in quantitative assessment of morel strain aging. In the current field soil morel cultivation popularized in China, wheat grain is the uppermost component in exogenous nutrition bags. Most amylopectin and amylose in wheats were hydrolyzed by extracellular amylases and then consumed in the process of morel cultivation.[21,22] Amylases are broadly classified into α, β, and γ subtypes.[23] Multi-omic analyses revealed high γ-amylase activity, increased α-amylase activity and none β-amylase gene in cultivable *M. importuna* SCYDJ1-A1, which suggested that γ-amylase and α-amylase activity were mainly involved in the rapid degradation of starch of wheat grain in the exogenous nutrition bags.[21] The growth potential and secretome data also demonstrated that *M. importuna* is a good starch decomposer and starch-rich grains in exogenous nutrition bags should be the main substrate for morel growth and development.[22] Aging and metabolism are tightly linked. Metabolism provides energy and metabolites for all cellular processes including senescence.[24] The poor amylase activity of senescent morel strains may partially attribute to their distinctive culture characteristics and declined yield in cultivation.[11] Additionally, amylase activity was also negatively correlated with subculturing times in two Basidiomycetes *Hypsizygus marmoreus* and *Phlebopus portentosus*.[17,18]

B. Application of Assay of Xylanase Activity in Assessment of *Morchella* Strain Aging

Our work also suggested that assay of xylanase activity by DNS method is the best alternative

for quantitative assessment of morel strain aging. Xylanase acts on the main chain of hemicellulose, causes decreasing in degree of polymerization, hence releasing oligomers such as xylobiose and xylose.[25] The xylanolytic enzyme system is normally composed of a repertoire of hydrolytic enzymes, including endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and acetylxylan esterase, which act cooperatively to convert xylan into its constituent sugars.[26] In *M. importuna* M04 cultivable strain, two endoxylanase were identified and might be the key proteins for hemicellulose degradation.[22] In the genome of *M. importuna* SCYDJ1-A1, hemicellulose-hydrolyzing enzymes are encoded by over a dozen of GH genes.[21] Although the observed xylanase activities were not as high as amylases, *M. importuna* had a strong ability to decompose various hemicellulose mainly through extracellular xylanase.[21,22,27] The reduced xylanase activity of senescent strains may exhibit great significance on morel growth and development in production. The assay of amylase and xylanase activity can be used in morel breeding and cultivation for the quantitative assessment of strain aging, which will promote the health advancement of morel industry.

C. Potential of Assay of Endoglucanase and Laccase Activity in Assessment of Morel Strain Aging

Besides amylase and xylanase, potential of the assay of endoglucanase and laccase activity in assessment of morel strain aging was also studied (data not shown). However, the correlation between enzyme activity and time of subculturing of most tested strains was low, which did not support the feasibility of assay of the two-enzyme activity in assessment of morel strain aging. Compared with biodegradation of starch and hemicellulose, *M. importuna* was weak in lignocellulose degradation. Its ability to degrade cellulose into oligosaccharides (mainly the action of endoglucanase) was weaker compared with further degradation into monosaccharides.[22]

Moreover, only a very few lignin-degradation-related proteins were detected with low abundance, which was consistent with the presence of weak lignin degradation ability.[21,22] Furthermore, besides the roles in lignin degradation, fungal laccases have also been assigned several other physiological functions relevant to mushroom cultivation, including the detoxification of phenolic compounds and sporophore development.[28]

V. CONCLUSION

Misuse of senescent strains in morel artificial cultivation annually brought about huge losses, which calls for urgent quantitative assessment of strain aging in cultivated *Morchella* mushrooms. Using the cultivated strains of *M. sextelata* 13-21, *M. importuna* 4-21 and *M. eximia* YL-9, aging was achieved by successive subculturing. Then the relevance of activity of amylase and xylanase with strain aging of *Morchella* mushrooms was studied to select the feasible methods for scientific evaluation of morel strain aging. The results showed that activity assay of amylase and xylanase was suitable for quantitative assessment of strain aging in three cultivated *Morchella* mushrooms, and assay of the xylanase activity is the best alternative. Potential of assay of endoglucanase and laccase activity in assessment of morel strain aging was also discussed. Further illumination of morel aging mechanism will promote the emergence of new method and modification of current used technologies for quantitative assessment of morel strain aging. Meanwhile, wide assessment of morel strain aging will effectively reduce the misuse of senescent morel strains in production, and thus will be beneficial for stable development of morel farming.

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TABLE 1: Subcultural characteristics of 3 tested *Morchella* strains

Strain	**Taxonomic**	**Subculture**	**Lifespan**	**Total growth**

number	status	times	(d)	length (cm)
4-21	*M. importuna*	4	> 73	> 144.9
13-21	*M. sextelata*	4	81	138.2
YL-9	*M. eximia*	3	58	94.6

FIG. 1: Amylase activity of different subcultures in 3 tested morel strains determined by DNS method. Figures labeled with the same letter are not significantly different according to Duncan's multiple range tests at a 5% probability level.

FIG. 2: Xylanase activity of different subcultures in 3 tested morel strains determined by DNS method. Figures labeled with the same letter are not significantly different according to Duncan's multiple range tests at a 5% probability level.

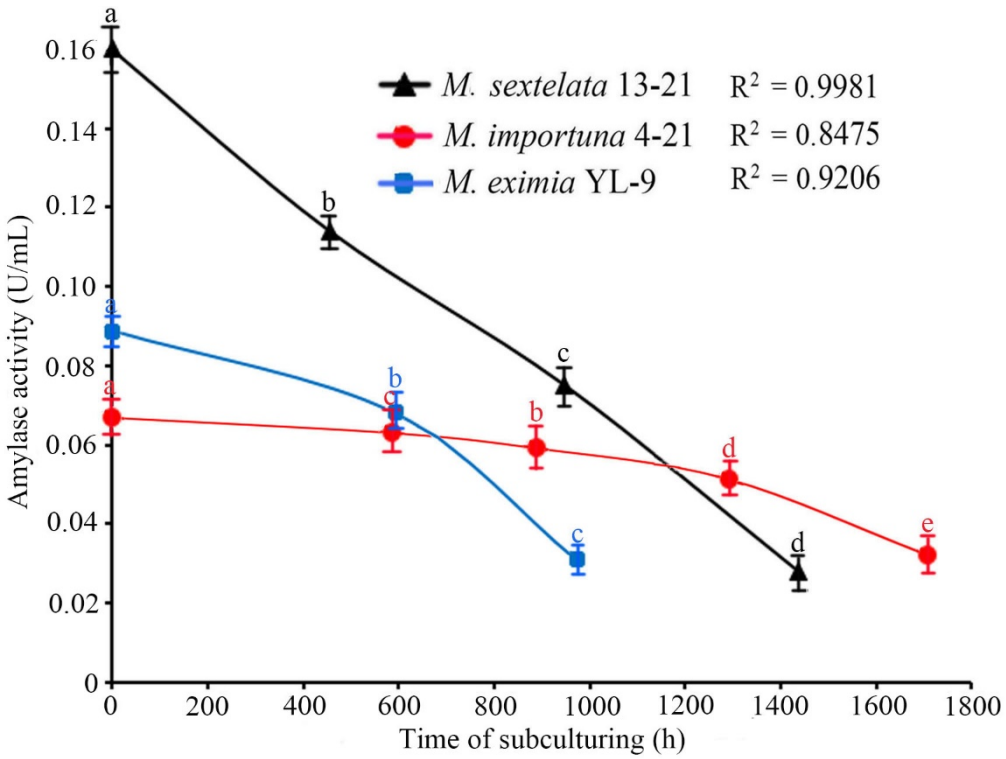
M. sextelata 13-21 R² = 0.9981
M. importuna 4-21 R² = 0.8475
M. eximia YL-9 R² = 0.9206
Amylase activity (U/mL)
Time of subculturing (h)
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0
200
400
600
800
1000
1200
1400
1600
1800

FIGURE 1

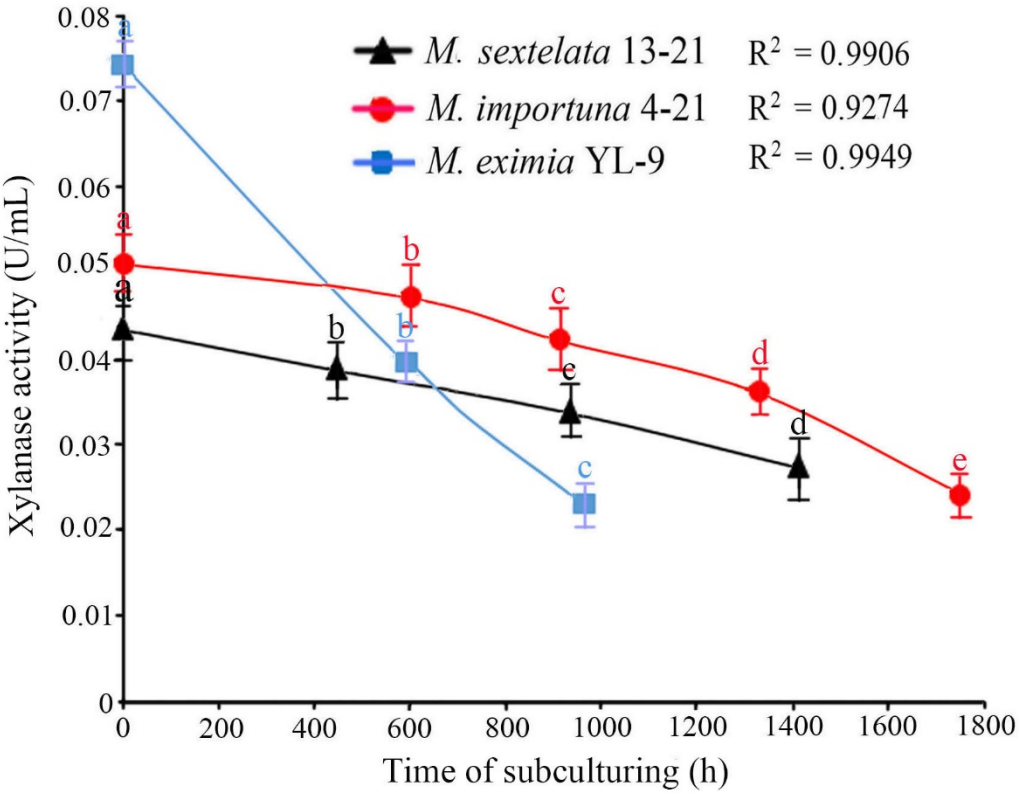
M. sextelata 13-21 R² = 0.9906
M. importuna 4-21 R² = 0.9274
M. eximia YL-9 R² = 0.9949
Xylanase activity (U/mL)
Time of subculturing (h)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
200
400
600
800
1000
1200
1400
1600
1800

FIGURE 2