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Rapid and Accurate Screening of Lysine-Producing Edible Mushrooms via the Homocitrate Synthase Gene as a Universal Molecular Marker

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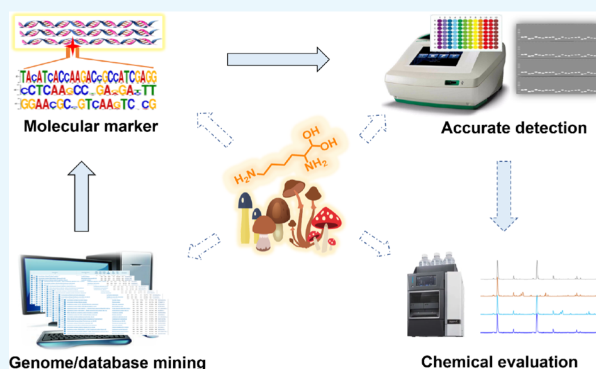
ABSTRACT: Edible mushrooms are important nutraceutical sources of foods and drugs, which can produce various nutritional ingredients including all essential amino acids. The method of rapid screening for the strains producing specific functional components is very indispensable. Homocitrate synthase is one of the key enzymes in the α-aminoadipate pathway for lysine biosynthesis and has preferable sequence conservation in Agaricales. Based on the blast of homocitrate synthase homologous genes of strains of Agaricales, we achieved combinations of degenerate primers as molecular markers to rapidly screen the lysine-producing edible mushrooms. The experimental results revealed that the consistency between PCR amplification and HPLC analysis attained 82 and 75% in strains of Agaricales and Polyporales, respectively. The finding showed that the molecular marker has higher universality for screening edible mushroom resources of Agaricales. This PCR-based approach shows excellent potential in evaluating and discriminating edible wild-grown mushrooms with high lysine content in Agaricales.



INTRODUCTION

Mushrooms have long been considered a source of food and medicine. Edible mushrooms are traditionally gathered and used as valued nutritional supplies globally. They contain carbohydrate and fibers, proteins and amino acids, lipids, vitamins, flavors and taste compounds, antioxidants, and other various beneficial constituents.[1] Basidiomycetes, including the majority of familiar mushrooms and toadstools, contain various ingredients suitable for human consumption and play a crucial role in food and medicine industry.[2] Thus, the high nutritional value of mushrooms has improved both consumption and commercialization.[3] Recently, mushroom mycelium has been recognized as one of the important nutrient sources beneficial to humans.[4] In addition to fresh and dried mushrooms, mycelial exudates, a substitute for mushroom products, have been used as a food or flavor material in functional foods and nutraceuticals. Among the various nutritional components of mushrooms, their amino acid composition, an important indicator of the nutritional value, flavor, and taste, is close to that of soy proteins.[5] The types and contents of amino acids lead to the unique flavor of mushrooms. Among the different amino acids, four are sweet (alanine, glycine, serine, and threonine), seven are bitter (arginine, leucine, isoleucine, histidine, methionine, phenylalanine, and valine), and both lysine and tyrosine are tasteless amino acids as essential amino acids.[6] Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are the nine essential amino acids found in common edible mushrooms such as *Pleurotus* spp. and *Lentinula* spp. There is an obvious difference in the compositions of amino acids in different edible mushrooms. For example, threonine and lysine are the main amino acids produced in *Cantharellus cibarius*.[7] Therefore, gaining a proper understanding of the amino acid content and differences among varieties and species will improve utilization of edible mushrooms.

Lysine is an essential amino acid involved in the assembly of proteins as a structural component of the cell, and lysine also participates in the formation of other cell components. Lack of lysine will seriously affect the growth and development, immunity, and central nervous system functions in humans. Lysine is the hub for the formation and utilization of other amino acids. Therefore, insufficient lysine supply will hinder

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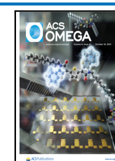

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Table 1. Edible Mushroom Strains Used in This Study

family	species	strains	family	species	strains
Tricholomataceae[a]	*Lepista sordida*	SZL-003		*Ganoderma tropicum*	SZL-166
	L. sordida	SZL-026		*Ganoderma australe*	SZL-182
	L. sordida	SZL-072	Omphalotaceae[a]	*Lentinula edodes*	SZL-043
	L. sordida	SZL-097		*L. edodes*	SZL-178
Physalacriaceae[a]	*Flammulina velutipes*	SZL-109	Bolbitiaceae[a]	*Panaeolus retirugis*	SZL-100
	F. velutipes	SZL-118	Lyophyllaceae[a]	*Hypsizygus marmoreus*	SZL-112
	Oudemansiella raphanipes	SZL-117		*H. marmoreus*	SZL-114
Agaricaceae[a]	*Coprinus comatus*	SZL-089		*H. marmoreus*	SZL-121
	C. comatus	SZL-174	Pleurotaceae[a]	*Pleurotus geesteranus*	SZL-113
	Agaricus sp.	SZL-015		*Pleurotus sajor-caju*	SZL-116
	Agaricus sp.	SZL-130		*Pleurotus eryngii*	SZL-122
	Agaricus sp.	SZL-079		*Pleurotus ostreatus*	SZL-127
	Agaricus blazei	SZL-110	Psathyrellaceae[a]	*Psathyrella bivelata*	SZL-004
	Agaricus bisporus	SZL-025		*P. bivelata*	SZL-028
	A. bisporus	SZL-125		*P. bivelata*	SZL-030
	A. bitorquis	SZL-059		*P. bivelata*	SZL-054
	A. bitorquis	SZL-046		*P. subsingeri*	SZL-021
	A. bresadolanus	SZL-052		*P. subsingeri*	SZL-061
	A. bresadolanus	SZL-051		*Psathyrella candolleana*	SZL-058
	A. subrufescens	SZL-053		*P. candolleana*	SZL-147
	A. subrufescens	SZL-074		*P. candolleana*	SZL-148
	A. subrufescens	SZL-099		*Coprinellus radians*	SZL-020
	Macrolepiota sp.	SZL-168		*C. radians*	SZL-087
	Agaricus xanthodermus	SZL-038		*C. radians*	SZL-108
	A. xanthodermus	SZL-047		*C. radians*	SZL-179
	A. xanthodermus	SZL-040		*Coprinopsis atramentaria*	SZL-014
	A. xanthodermus	SZL-029		*C. atramentaria*	SZL-036
	A. xanthodermus	SZL-044		*C. atramentaria*	SZL-049
	A. xanthodermus	SZL-081		*C. atramentaria*	SZL-060
	A. parvibrunneus	SZL-105	Amanitaceae[a]	*Amanita* sp.	SZL-157
Hymenogastraceae[a]	*Hebeloma* sp.	SZL-010	Lycoperdaceae[a]	*Lycoperdon* sp.	SZL-180
	Hebeloma sp.	SZL-033	Nidulariaceae[a]	*Cyathus* sp.	SZL-181
Strophariaceae[a]	*Stropharia rugosoannulata*	SZL-119	Hymenochaetaceae[b]	*Phellinus* sp.	SZL-128
	Agrocybe pediades	SZL-154		*Phellinus* sp.	SZL-129
	Agrocybe aegirit	SZL-111		*Phellinus* sp.	SZL-183
Polyporaceae[b]	*Trametes versicolor*	SZL-137	Phanerochaetaceae[b]	*Phlebiopsis gigantea*	SZL-155
	T. versicolor	SZL-151			

[a]Represents the families in Agaricales. [b]Represents the families in Polyporales.

the utilization of other amino acids.[8,9] Thus, lysine has gained application prospects in pharmaceutical industry to formulate an amino acid balanced diet and in amino acid infusions.[10] Adding lysine to mushroom products as food may be one effective way to get sufficient lysine supply. Several studies have evaluated the amino acid production abilities including lysine in mushrooms such as *Pleurotus* spp., *Boletus* spp., and *Lentinus* spp.[5,11,12] Numerous methods have been developed to quantify amino acids in edible wild-grown mushrooms. The traditional detection approach is derived from photometric or fluorimetric detection. Reagent interference, instability of derivatives, long preparation time, and difficulties in derivatization to specific amino acids are the disadvantages of an ordinary HPLC method.[13] Therefore, reversed-phase high-performance liquid chromatography (HPLC) and high-throughput UPLC−MS/MS were established for the detection of amino acids with adequate separation and high sensitivity.[14,15] The use of LC−MS/MS eliminates the derivatization step and allows for overlapping amino acid retention times, thereby shortening the analysis time.[16] However, no method has been developed to quantify amino acids rapidly and accurately with molecular biological techniques in edible mushrooms.

Degenerate primers are designed based on the genomic DNA sequences of related and already sequenced gene homologs and are used in the so-called degenerate PCR to amplify DNA sequences of unknown genes. This method has effectively identified new members of the gene family or orthologous genes from different organisms with unavailable genomic information. The approach is widely used to screen genetically modified food with lower cost and higher throughput. Rapid screening via PCR with degenerate primers was applied to identify targeted genes and proteins[17] and identify and amplify homologs.[18,19] Degenerate primers also improved the efficiency and specificity of PCR amplification.[20] Moreover, several degenerate PCR primer design tools, such as DPPrimer and CEMAsuite, have been developed,[21,22] which boosted the various application of degenerate primers for PCR screening.

In the lysine biosynthetic progress, homocitrate synthase (HCS) that catalyzes the first step in the α-aminoadipate pathway (AAA pathway) is considered the rate-limiting

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enzyme in higher fungi.[23,24] The lysine biosynthetic pathway and its related genes have been analyzed based on the genome of *Flammulina velutipes*, where the *Fvhcs* gene expression is closely associated with the lysine content in *F. velutipes*.[25] The Fvhcs protein has homologs in fungi and is closely related to other homocitrate synthases in Agricomycotina.[25] Moreover, orthologous protein sequences as DNA bar codes have been used to assist in phylogenic research in fungi. These research advances provide great possibility for high-throughput rapid screening of edible wild-grown mushrooms with related molecular markers. In this study, degenerate primers were designed based on the homocitrate synthase homolog genes as molecular markers to rapidly and accurately screen edible mushrooms with higher lysine content. Further, lysine produced by these mushroom strains via fermentation was evaluated. This work will provide valuable guidelines for rapid and accurate screening of edible wild-grown mushrooms with lysine-producing activity.

■ MATERIALS AND METHODS

Edible Mushroom Strains and Chemical Reagents. The 73 edible mushroom strains of 16 families in Agaricales and Polyporales, namely, Tricholomataceae (4), Physalacriaceae (3), Agaricaceae (23), Hymenogastraceae (2), Strophariaceae (3), Omphalotaceae (2), Bolbitiaceae (1), Lyophyllaceae (3), Pleurotaceae (4), Psathyrellaceae (17), Amanitaceae (1), Lycoperdaceae (1), Nidulariaceae (1), Hymenochaetaceae (3), Polyporaceae (4), and Phanerochaetaceae (1), were used in this study. These strains were stored in the State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences (Beijing, China), and are listed in Table 1. Chemical reagents L-lysine (PubChem CID: 5962, 99%, Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China), phenyl isothiocyanate (PubChem CID: 7673, Aladdin Biochemical Technology Co., Ltd., Shanghai, China), triethylamine (PubChem CID: 8471, TEA), methanol (PubChem CID: 887), and acetonitrile (PubChem CID: 6342, HPLC grade, Fisher, Waltham, USA) were used to determine the lysine yield in edible mushrooms. All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Media for Culturing Edible Mushrooms. Potato dextrose agar (PDA; Becton, Dickinson and Company, USA) was used to cultivate and activate the mushroom strains in this study. The liquid minimum medium (LMM; 10 g/L glucose, 50 g/L nitrate salts, 1 g/L trace elements, 5 g/L yeast extract, pH = 6.5) was used to cultivate the mushroom strains for genomic DNA extraction and fermentation. The potato dextrose broth (PDB; Becton, Dickinson and Company, USA) and the cornmeal soy yeast extract medium (CSYM; 25 g/L cornmeal, 10 g/L soy flour, 2 g/L yeast extract, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L KH_2PO_4, 0.25 g/L $K_2HPO_4{\cdot}7H_2O$, pH = 5.5) were also used for fermentation and product analysis.

Genomic DNA Extraction. Genomic DNA was extracted from mushroom mycelial tissues following the modified sodium dodecyl sulfate method.[26] Among the different strains, 73 strains were developed under the same culture conditions, consistent with that reported by Kala et al.[4] (Table 1). The strains were cultured for genomic DNA extraction at 28 °C for 5−7 days. Approximately 10 mg of mushroom mycelium was mixed with 700 μL of LETS buffer (20 mM EDTA, 10 mM Tris−HCl, 100 mM $LiCl{\cdot}H_2O$, 5 g/L sodium dodecyl sulfate, pH = 8.0) and ground with a high-throughput tissue grinder (Ningbo Xinzhi Bio-Technology Co., Ltd., Ningbo, China). Then, each sample was mixed with 700 μL of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), vigorously shaken, and centrifuged at 4 °C and 13,000 rpm for 10 min. The supernatant was transferred into another new 1.5 mL microtube. To this step, 1 mL of 95% ethanol was added and carefully mixed. The mixture was centrifuged at 4 °C and 13,000 rpm for 10 min, and the supernatant was removed entirely. Each DNA pellet was dissolved in TE buffer (1 mM EDTA, 10 mM Tris−HCl). Finally, the concentration and quality of extracted genomic DNA were evaluated by 10 g/L agarose gel electrophoresis and by a NanoDrop.

Degenerate Primers Design. Degenerate primers were designed based on the conservation of the nucleotide sequence of homocitrate synthase homologous gene (hcs) in edible mushroom strains in Agaricales. The reported hcs nucleotide sequence in the F. velutipes (KC250020.1) was submitted to NCBI (http://www.ncbi.nlm.nih.gov) for homology alignment.[25] The nucleotide sequences with higher homology to the hcs gene in *F. velutipes* were downloaded. The conserved regions of nucleotide sequences of hcs gene in different strains of Agaricales were selected and the degenerate primers were designed by DNAMAN software.[17] The downloaded information of multiple homologous strains was listed in Table S2. The degenerate primers used in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

PCR Amplification with Degenerate Primers. The two pairs of degenerate primers were used to amplify on the conserved region on the homologous hcs genes using genomic DNA of 73 edible mushroom strains. The PCR was performed in 20 μL reaction volume, containing 10 μL of 2×San Taq PCR Mix (Sangon Biotech Co., Ltd. Shanghai, China), 10 ng of genomic DNA, 0.2 μM of each primer, and 9.2 μL of sterilized ddH2O. The PCR were performed under the following conditions: 94 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were separated using electrophoresis in 1% agarose gel. The PCR amplification and products analysis followed the procedures as described previously.

Strains Activation and Fermentation. All of the mushroom strains in this study were inoculated on PDA and cultured at 28 °C for 5 days. Subsequently, each strain was re-inoculated on fresh PDA and cultured at 28 °C for 4 days. Different fungal strains' mycelial cakes (1 cm diameter) were obtained using a sterile puncher from the similar positions of PDA plates. After mycelia was broken by a high-throughput tissue grinder, equal quantities were added to 20 mL of both LMM medium and CSYM medium fermented at 26 °C for 2−4 days, respectively.[27]

Lysine Derivatization. The L-lysine content in the fermentation broth was determined following a previously reported method with slight modification.[13] The L-lysine standard was dissolved and diluted to the required concentration with ultrapure water (Millipore Simplicity 165, Millipore, USA). Phenyl isothiocyanate (PITC) and triethylamine solution used in the lysine derivatization was prepared 1 h earlier. Pipette 100 μL of 0.1 M PITC (1.2 mL of PITC was dissolved in 98.8 mL acetonitrile) and 100 μL of 1 M triethylamine (13.5 mL of triethylamine was dissolved in 86.5 mL acetonitrile) were mixed into 200 μL of L-lysine solution, and the mixture was incubated at room temperature for 1 h. The reaction was terminated with 400 μL of hexane. The lower

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	Strains	Conservative sequence (5'-3')		
		560 570 580	1140 1150	1200 1210
I	*Armillaria solidipes*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGACTT	GGAACGCCGTCAAGTCGCG
I	*Armillaria ostoyae*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGACTT	GGAACGCCGTCAAGTCGCG
I	*Flammulina velutipes*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCGGAGGACTT	GGAACGCTGTCAAGTCGCG
I	*Cylindrobasidium torrendii*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGATTT	GGAATGCGGTCAAGTCGCG
II	*Hebeloma cylindrosporum*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGACTT	GGAACGCCGTCAAGTCCCG
II	*Gymnopilus dilepis*	TACATCACCAAGACCGCTATCGAGG	CCTCAAGCCTGAAGATTT	GGAACGCTGTCAAGTCGCG
II	*Galerina marginata*	TATATCACCAAGACTGCCATCGAGG	TCTCAAACCCGAAGATTT	GGAACGCTGTTAAGTCTCG
II	*Psilocybe cyanescens*	TACATCACCAAGACTGCCATCGAGG	CCTCAAGCCCGAAGATTT	GGAACGCCGTCAAGTCCCG
III	*Hypholoma sublateritium*	TACATCACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGACTT	GGAATGCCGTCAAGTCGCG
IV	*Gymnopus luxurians*	TACATCACCAAGACCGCCATCGAGG	TCTCAAGCCTGAGGACTT	GGAACGCTGTCAAGTCTCG
V	*Amanita muscaria*	TACATCACCAAGACTGCCATCGAGG	CCTCAAACCGGAAGATTT	GGAACGCAGTCAAGTCCAG
VI	*Hypsizygus marmoreus*	TACATCACTAAGACCGCCATCGAGG	CCTCAAGCCTGAAGATTT	GGAACGCCGTCAAATCACG
VII	*Coprinellus micaceus*	TATATTACCAAGACCGCCATCGAGG	CCTCAAGCCTGAGGACTT	GGAACGCCGTCAAGTCTCG
VII	*Coprinopsis marcescibilis*	TACATCACCAAGACAGCCATTGAAG	CCTCAAGCCAGAAGATTT	GGAACGCCGTCAAGTCCCG
VIII	*Laccaria amethystina*	TACATCACCAAAACCGCCATTGAAG	CCTCAAGCCAGAGGATTT	GGAACGCCGTCAAATCACG
IX	HCS Ag_les F_1	TACATCACCAAGACYGCCATC		
IX	HCS Ag_les F_2	ACCAAGACCGCCATCGAGG		
X	HCS Ag_les R_1		AARTCYTCSGGCTTGAGG	
X	HCS Ag_les R_2			CGMGACTTGACRGCGTTCC

Figure 1. Sequence alignment of the homocitrate synthase gene (*hcs*) in edible mushroom strains and the designed primers for screening as molecular markers. I. Physalacriaceae; II. Hymenogastraceae; III. Strophariaceae; IV. Omphalotaceae; V. Amanitaceae; VI. Lyophyllaceae; VII. Psathyrellaceae; VIII. Hydnangiaceae. IX. HCS Ag_les F_1 and HCS Ag_les F_2 mean the forward degenerate primers; X. HCS Ag_les R_1 and HCS Ag_les R_2 mean the reverse degenerate primers.

layer solution was taken into a 1.5 mL microtube and centrifuged at 13,000 rpm for 10 min. Approximately 200 μL of the supernatant was transferred into a sample vial for HPLC and LCMS analyses. Further, the fermentation broth of mushroom samples was collected by centrifugation at 13,000 rpm for 10 min. The lysine content in the fermentation broth was determined to be similar to the derivatization method described above.

Lysine Quantitation by HPLC. A Waters e2695-2998 HPLC system equipped with a UV detector (Waters Corporation, USA) was used to determine the L-lysine content in different mushroom samples. Chromatographic analysis was performed using an XTERRA MS C18 column (4.6 mm × 250 mm × 5 μm) at a flow rate of 1 mL/min, a detection wavelength of 254 nm, and an injection volume of 10 μL. The HPLC method was evaluated and optimized following the previously reported method with modification.[13] The HPLC mobile phase includes solvent A (100% methanol) and solvent B (0.1% formic acid aqueous solution), and the solvents were filtered through a 0.2 μm microporous membrane before use. The HPLC gradient elution program was set as follows: 0−2 min, solvent A 10−28%; 2−13 min, solvent A 28−28%; 13−28 min, solvent A 28−85%; 28−28.1 min, solvent A 85−100%; 28.1−33 min, solvent A 100−100%; 33−33.1 min, solvent A 100−10%; 33.1−38 min, solvent A 10−10%. Lysine quantitation was determined as the derivatization of lysine.

Standard Curve Generation for Lysine Quantification. The standard curve used to determine the concentration of lysine was established as follows. Lysine was accurately weighted and dissolved in water to prepare a 1 mg/mL standard stock solution. Then, the standard stock solution was diluted with water to 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 mg/mL concentrations. The diluted solutions were derivatized and analyzed by HPLC following the method described above. Finally, the lysine standard curve was generated by plotting the lysine concentration as the abscissa and HPLC peak area as the ordinate using the chart option function in a Microsoft Excel 2016 software package.

LC−MS Analysis. Agilent 1200 LC/MSD SL (Santa-Clara, USA) was used to analyze the molecular weight of L-lysine derivatives in the fermentation products of *Agaricus blazei* SZL-110, and data were analyzed using LC/MSD software Agilent ChemStation Rev. B.04.01 Sp1. The LC−MS gradient elution program was set as follows: 0−30 min, 5−100% acetonitrile; 30−35 min, 100% acetonitrile; 35−35.1 min, 100−5% acetonitrile; 35.1−40 min, 5% acetonitrile. Other program settings were similar to the protocol reported earlier.[13] The molecular weight of L-lysine and its derivatives were verified by LC−MS following the above protocol.

Statistical Analysis. All data were represented as the mean ± standard deviation of three replicates. The lysine content of samples was analyzed using statistical software GraphPad Prism 8.

■ RESULTS AND DISCUSSION

DNA Extraction from Mushroom Strains. The quality of genomic DNA used as a template is an important factor in establishing a PCR model to screen edible mushroom strains with high lysine content. All strains were activated and freshly cultivated under suitable temperature conditions to achieve the goal. The genomic DNA was extracted from every strain with concentrations ranging from 132.77 ± 33.12 to 4893.80 ± 13.89 ng/μL from the mycelium of strains. The yield and purity of DNA extracted from several samples were low, probably due to the presence of polysaccharide, polyphenol, lipid, protein, and RNA contaminants interfering with the absorbance measurements (A260/A230 and A260/A280) (Table S1). The DNA of these strains were extracted several times with the same DNA yield and purity. Therefore, it maybe because the DNA extract protocol was not the optimal method or condition for genomic DNA extraction of all these strains.

Designing Degenerate Primers for Amplifying the *hcs* Gene. Here, the *hcs* gene was chosen as the molecular marker to develop a reliable assay based on PCR. Homocitrate synthase is the first rate-limiting enzyme in the AAA pathway of lysine biosynthesis in higher fungi.[23,24] Also, it was confirmed that the *Fvhcs* gene expression is closely associated

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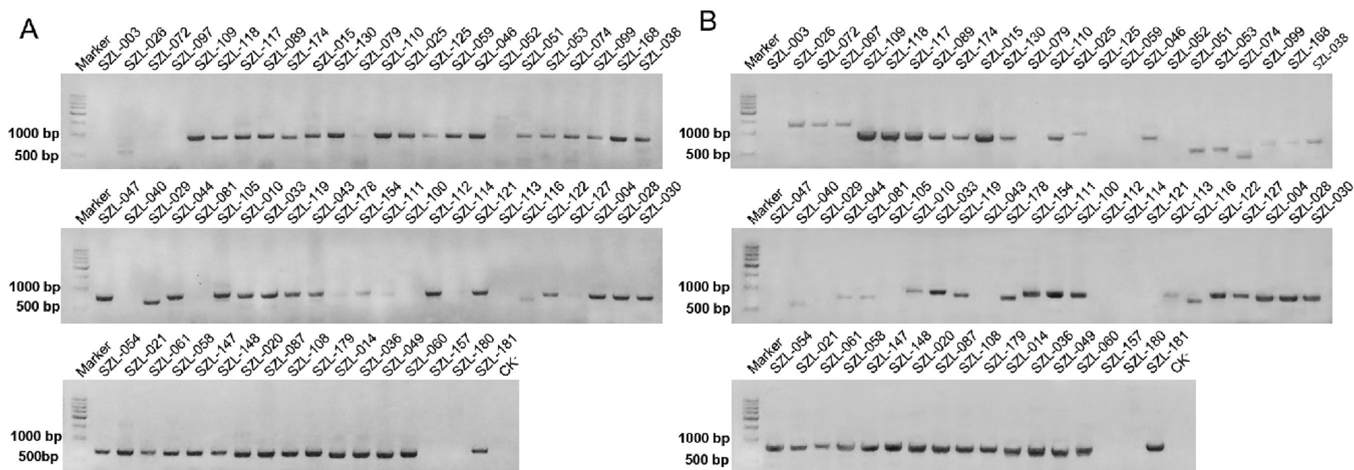

Figure 2. Diagnostic PCR with degenerate primers for the edible mushroom strains of Agaricales. (A) PCR with HCS Ag-les F_1/R_1; (B) PCR with HCS Ag-les F_2/R_2.

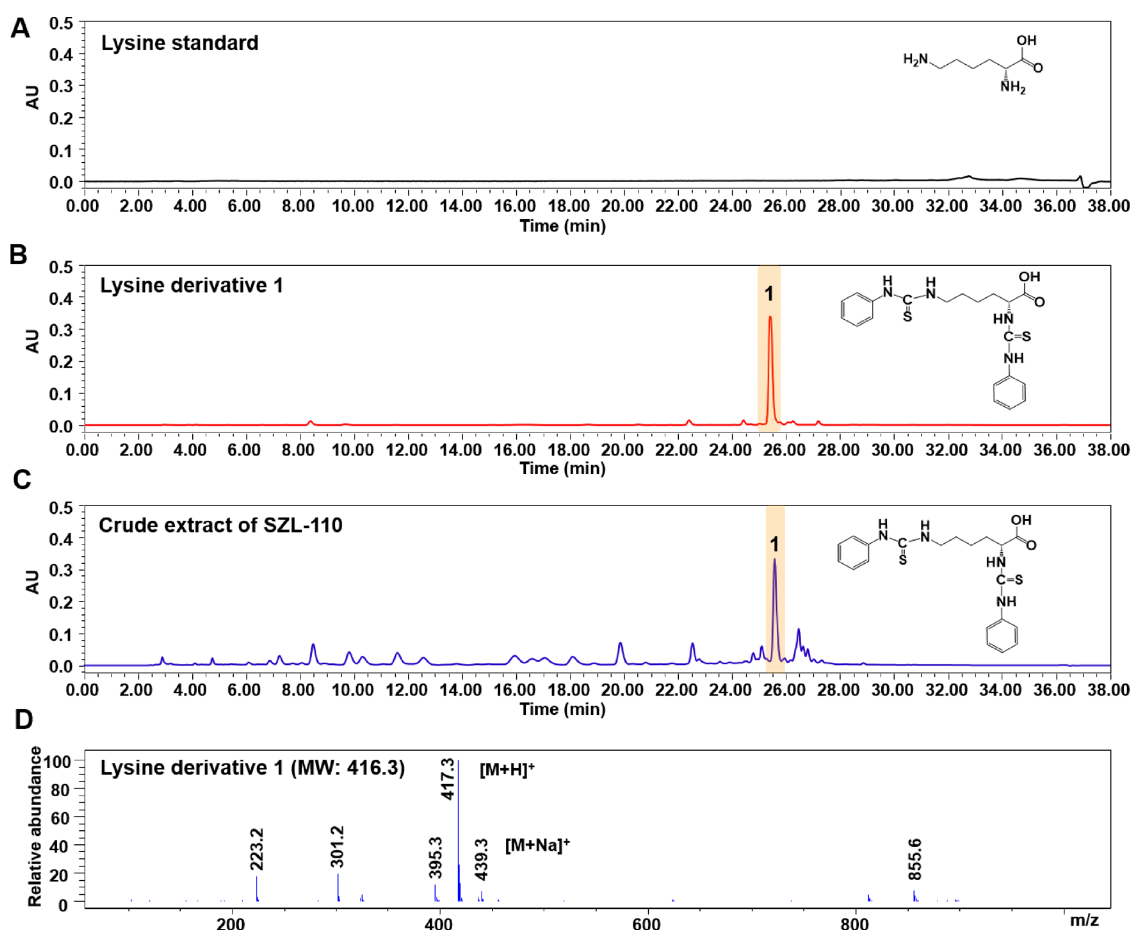

Figure 3. Establishment of the analytical method for lysine produced in the fermentation of edible mushrooms. (A) HPLC analysis of the L-lysine standard; (B) HPLC analysis of the L-lysine derivative standard; (C) HPLC analysis of crude extracts of SZL110; (D) LC–MS analysis of L-lysine derivative **1**.

with the lysine content in *F. velutipes*. Moreover, the Fvhcs protein is closely related to the other homologous homocitrate synthase proteins in the fungal strains of Agaricomycotina according to phylogenetic analysis.[25] In this study, two pairs of degenerate primers were designed based on the conserved sequences for rapidly screening the 15 mushroom strains in eight families of Agaricales (Table S2). The sequences of primers including HCS Ag_lesF$_1$ (TACATCACCAAGA-CYGCCATC) and HCS Ag_lesR$_1$ (AARTCYCSGGCTT-GAGG), HCS Ag_lesF$_2$ (ACCAAGACCGCCATCGAGG) and HCS Ag_lesR$_2$ (CGMGACTTGACRGCGTTCC) are shown in Figure 1.

PCR Amplification of the *hcs* Gene. To detect the effect of the *hcs* gene as a molecular marker, 65 mushroom strains

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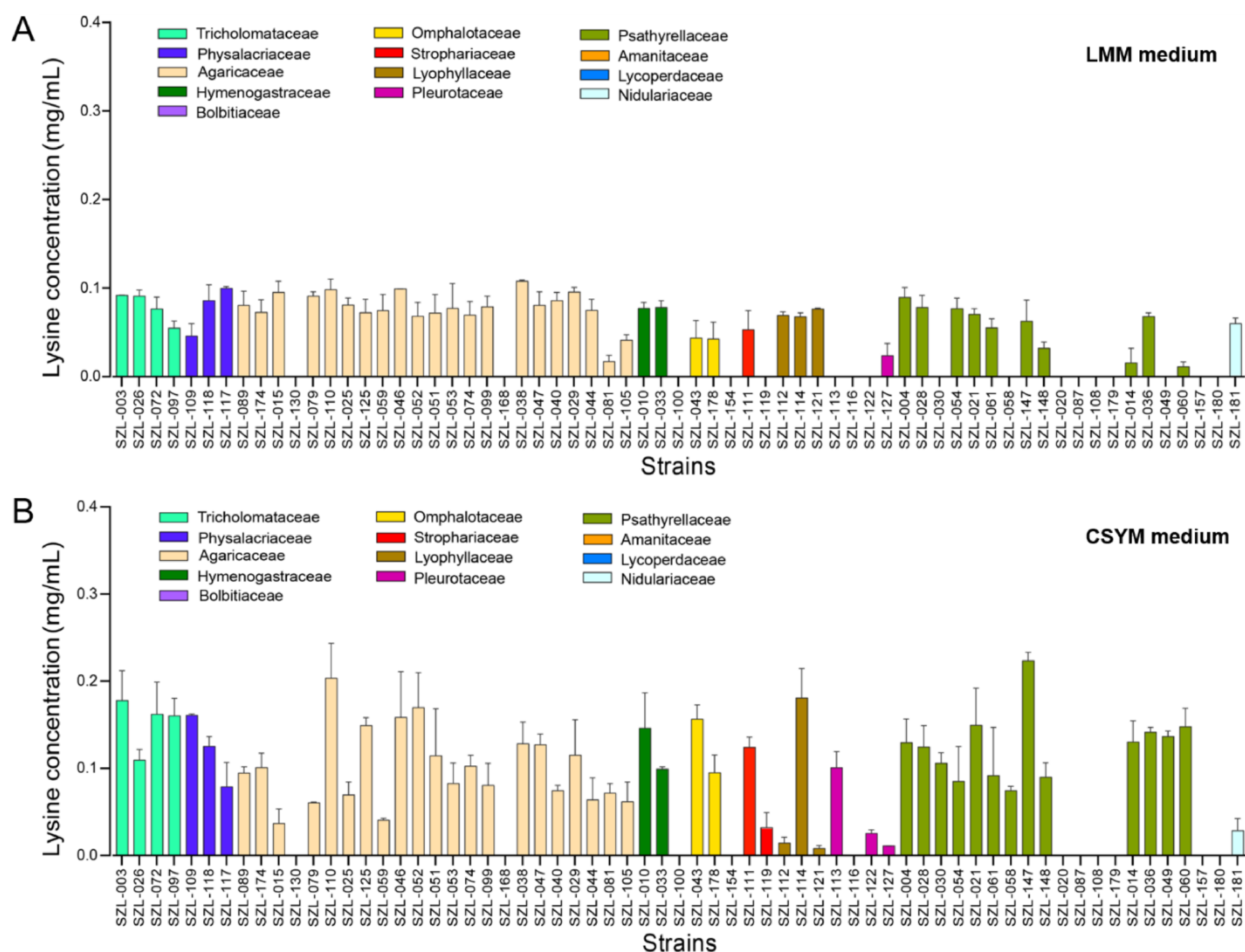

Figure 4. Analysis of lysine yield for the edible mushroom strains of Agaricales. (A) Culture in the LMM medium; (B) Culture in the CSYM medium. The lysine concentration values were achieved with calculation according to the peak area of lysine derivative **1** by HPLC analysis.

belonging to 32 species, 19 genus, and 13 families of Agaricales, including the known edible mushrooms *Agaricus bisporus*, *Lentinula edodes*, and *Pleurotus ostreatus*, were chosen (Table 1). The special PCR amplicons using HCS Ag_lesF$_1$/HCS Ag_lesR$_1$ and HCS Ag_lesF$_2$/HCS Ag_lesR$_2$ primers were achieved from the DNA samples of 49 strains (about 75.4%) and 51 strains (about 78.5%), respectively (Figure 2A,B). Meanwhile, the specific amplicons in 59 strains (about 90.8%) were achieved based on combining the results with the two pair primers. The number of degenerate bases in the primers slightly affected the PCR efficiency according to our results. Taken together, these observations indicate the need to assess all strains for screening with combination of the two pair primers based on the *hcs* gene as a molecular marker.

Verification of Quantitative Lysine Analysis. For statistical analysis, mushrooms can be distinguished based on their amino acid profiles. The amino acid contents of the studied mushroom species have been presented. For example, the total free amino acid content varied between 14.93 and 29.54 mg/g among the different strains; it was the lowest in *C. cibarius* and the highest in *Boletus edulis* mushrooms.[28] Lysine was detected in relatively high concentrations in some species. Measured concentrations are in a lower level than the reported contents. This difference can be explained both by the differences in samples and by the differences in preprocessing methods.[13] L-Lysine was not directly detected by HPLC analysis. To achieve a credible detection method of amino acids and to assess if the mushroom strains produced lysine or not, we determine the lysine derivative content according to the method of phenyl isothiocyanate (PITC) derivatization.[13] In the reaction system of lysine and PITC, three lysine derivatives **1**−**3** were detected by HPLC, in which derivative **1** with a molecular weight of 417.3 $[M + H]^+$ accounted for 97.3% of the total output as the major outcome and derivatives **2** and **3** with a molecular weight of 281.2 $[M + H]^+$ accounted for 2.7% of the total output (Figure S1). This phenomenon of three products is probably due to the fact that excess PITC is enough to combine with the two amino groups in lysine to produce derivative **1** in large amounts.[13]

Subsequently, the crude extract obtained after the fermentation of the *A. blazei* SZL-110 strain was used to verify the dependability of the lysine derivatization method. As expected, the lysine derivative **1** was detected at 25.5 min by HPLC analysis, and constituents with molecular weights of 417.3 $[M + H]^+$ and 439.3 $[M + Na]^+$ were obtained by LC−MS analysis (Figure 3). The standard curve of lysine determined by HPLC analysis was achieved, and the linearity of the method was evaluated (Figure S2). It was used to obtain the lysine concentration after fermentation of the edible mushroom strains.

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Family		Tricholomataceae				Physalacriaceae			Agaricaceae															
Strains		SZL -003	SZL -026	SZL -072	SZL -097	SZL -109	SZL -118	SZL -117	SZL -089	SZL -174	SZL -015	SZL -130	SZL -079	SZL -110	SZL -025	SZL -125	SZL -059	SZL -046	SZL -052	SZL -051	SZL -053	SZL -074	SZL -099	SZL -168
PCR	P_F1/R1																							
	P_F2/R2																							
HPLC	LMM																							
	CSYM																							
Analysis		—	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+	+	+	—

Family		Agaricaceae							Hymenogastraceae, Bolbitiaceae			Omphalotaceae		Strophariaceae			Lyophyllaceae			Pleurotaceae Psathyrellaceae				
Strains		SZL-038	SZL -047	SZL -040	SZL -029	SZL -044	SZL -081	SZL -105	SZL -010	SZL -033	SZL -100	SZL -043	SZL -178	SZL -154	SZL -111	SZL -119	SZL -112	SZL -114	SZL -121	SZL -113	SZL -116	SZL -122	SZL -127	SZL -004
PCR	P_F1/R1																							
	P_F2/R2																							
HPLC	LMM																							
	CSYM																							
Analysis		+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	+	—	+	+	—	+	+	+

Family		Psathyrellaceae																Amanitaceae Lycoperdaceae Nidulariaceae			Efficiency
Strains		SZL-028	SZL -030	SZL -054	SZL -021	SZL -061	SZL -058	SZL -147	SZL -148	SZL -020	SZL -087	SZL -108	SZL -179	SZL -014	SZL -036	SZL -049	SZL -060	SZL -157	SZL -180	SZL -181	
PCR	P_F1/R1																				82%
	P_F2/R2																				
HPLC	LMM																				
	CSYM																				
Analysis		+	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+	+	+	

Figure 5. Consistency evaluation of the rapid screening method of lysine production in Agaricales strains. The light red color means that there is PCR amplicon and lysine production in PCR and HPLC analysis, respectively. Also, the light blue color means that there is no PCR amplicon and lysine production in PCR and HPLC analysis, respectively.

Optimization of Fermentation for Lysine Production. Amino acids are the primary metabolites in fungi. The growth time and carbon-to-nitrogen ratio influence the formation and accumulation of amino acids to a certain extent.[5,27,29] To standardize the fermentation media and time, LMM, PDB, and CSYM were chosen for cultivation for up to 10 days using five strains of Agaricaceae, Physalacriaceae, Psathyrellaceae, Tricholomataceae, and Omphalotaceae. The results revealed that the lysine yield was higher in the eutrophic CSYM medium than in other two oligotrophic media (LMM and PDB) for all these five strains (Figure S3). Moreover, the lysine yield attained the peak for the strains of Agaricaceae, Physalacriaceae, Psathyrellaceae, and Tricholomataceae in 4 days. In the case of the strain of Omphalotaceae, the lysine yield decreased after 2 days in all of the three media (Figure S3A−E). Generally, lysine is consumed to synthesize polypeptides and secondary metabolites after reaching a certain level.[8] Based on these observations, the detection of lysine was performed after growing in both LMM and CSYM media in 4 days for all the mushroom strains of families, except for the strain of Omphalotaceae in 2 days.

Quantification of Lysine Produced by Edible Mushrooms. The 65 mushroom strains of 13 families were cultured in both the oligotrophic medium (LMM) and eutrophic medium (CSYM), and the lysine yield of each strain was determined by HPLC analysis according to the data in Table S3. Obvious differences were detected in the lysine content among the mushroom strains. Among these strains, 17 strains in the LMM medium and 11 strains in the CSYM medium did not produce lysine (Figure 4A,B). Six strains produced lysine only in the CSYM medium, probably due to higher carbon and nitrogen levels in the CSYM medium than in the LMM medium. Only *Agaricus* sp. SZL-130 and *Macrolepiota* sp. SZL-168 did not contain lysine among the 23 strains of Agaricaceae in both media. The four *Coprinellus radians* strains among the 23 strains of Psathyrellaceae did not produce lysine. *Panaeolus retirugis* SZL-100 of Bolbitiaceae, *Amanita* sp. SZL-157 of Amanitaceae, and *Lycoperdon* sp. SZL180 of Lycoperdaceae did not produce lysine in both media, too. In addition, obvious differences were detected in lysine yield between the two media for most of the strains used in this work. These strains, such as *Stropharia rugosoannulata* SZL-119 (0.031 mg/mL), *Pleurotus geesteranus* SZL-113 (0.101 mg/mL), *Pleurotus eryngii* SZL-122 (0.025 mg/mL), *Psathyrella bivelata* SZL-030 (0.106 mg/mL), *Psathyrella candolleana* SZL-058 (0.074 mg/mL), and *Coprinopsis atramentaria* SZL-049 (0.136 mg/mL), yielded

lysine only in the CSYM medium (Figure 4B). Moreover, significant differences were detected in lysine yield among the different strains of the same species such as *Lepista sordida*, *A. bisporus*, *Agaricus bresadolanus*, *Agaricus xanthodermus*, *Psathyrella bivelata*, *P. candolleana*, *C. radians*, and *C. atramentaria*. The yield of lysine in different strains of the same species was different, which was possibly related to the specific growth climate environment of the strain and the difference of the unique breeding ground and collection site.[28]

Consistency Evaluation of the *hcs* Gene as a Molecular Marker for Lysine Yield in Mushrooms. The PCR and HPLC results of 65 edible mushroom strains of Agaricales were evaluated and compared to determine the efficiency of PCR using the *hcs* gene as a molecular marker to screen the edible mushrooms with high lysine yield (Figure 5). The PCR results were inconsistent with lysine production for 12 strains. For the three strains of *L. sordida* SZL-003, *A. bresadolanus* SZL-052, and *Hypsizygus marmoreus* SZL-114, PCR amplicons were not achieved by PCR with lysine production by HPLC analysis. For the nine strains, namely, *Agaricus* sp. SZL-130, *Macrolepiota* sp. SZL-168, *Agrocybe pediades* SZL-154, *P. retirugis* SZL-100, *Pleurotus sajor-caju*. SZL-116, and *C. radians* SZL-020, SZL-087, SZL-108, and SZL-179, PCR amplicons were obtained by PCR with no lysine production by HPLC analysis (Figure 5). It was not probably suited for *C. radians* strains to produce lysine in both media. Meanwhile, there was no PCR amplicons or lysine production in the two strains of *Lycoperdon* sp. SZL-180 and *Amanita* sp. SZL-157. To summarize, an efficiency of 82% was achieved for lysine production by these edible mushrooms of Agaricales with the *hcs* gene as a molecular marker. These findings indicated that the *hcs* gene as a molecular marker could be widely used to screen lysine-producing strains of Agaricales.

Further, eight strains from four genera in Polyporales were used to evaluate the universality of the *hcs* gene as the molecular marker in other fungal group. Among the taxonomic group, both PCR amplicons and lysine production were checked in six strains, and PCR amplicons but without lysine production were displayed in two strains (Figure S4). Comparison of both the PCR detection and HPLC analysis revealed an efficiency of approximately 75% by PCR screening according to the consistency of the results between PCR and HPLC analysis. Moreover, the phylogenetic analysis of Agaricomycetes showed a close genetic correlation between Agaricales and Polyporales, which indicated the possibility of using the same screening degenerate primers to select the lysine-producing strains of Polyporales, too.[30] On the other hand, due to fewer strains of Polyporales used in this study, the suitability of the combination of degenerate primers needs to be further verified with more number of strains of Polyporales.

In this study, a PCR-based strategy based on the homocitrate synthase gene as the molecular marker proposed is a valuable tool for rapid and accurate screening of edible mushrooms of Agaricales with higher lysine yield. The consistency values between PCR and HPLC results were approximately 82% in 65 strains of 13 families of Agaricales and 75% in eight strains of three families of Polyporales. Correlation between PCR amplification and HPLC analysis proved that the degenerate primers was efficient for 65 strains of Agaricales. Further, the results suggested that the two degenerate primers may be used in combination to screen Basidiomycetes also. Taken together, this PCR-based approach shows excellent potential in evaluating and discriminating the edible wild-grown mushrooms with high lysine content. However, whether there are other important restrictive factors for the detection efficiency or none is unknown. Any shortcomings of this method mentioned here will be optimized based on more *hcs* gene homologs in Basidiomycetes in the future. Sensory testing with more sensitive molecular markers should be conducted to expand the application of the proposed method with more homocitrate synthase homologous genes or other functional proteins in edible mushrooms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03175.

> Detection of concentration and quality for genomic DNA of the edible mushroom strains used in this study as determined by a NanoDrop (Table S1); homologous sequence alignments of the *hcs* gene in *F. velutipes* with the homologs in 14 strains of Agaricales in NCBI (Table S2); peak areas of lysine concentration in both LMM and CSYM media for all strains by HPLC analysis in this study (Table S3); principle of lysine derivatization (Figure S1); standard curve of lysine determined by HPLC analysis (Figure S2); optimization of fermentation media and time for lysine production in different strains of Agaricales (Figure S3); and PCR screening and evaluation of lysine production of Polyporales strains (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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