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Research Note

Genetic and Biochemical Characterization of Monokaryotic Progeny Strains of Button Mushroom (*Agaricus bisporus*)

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Abstract To promote the selection of promising monokaryotic strains of button mushroom (*Agaricus bisporus*) during breeding, 61 progeny strains derived from basidiospores of two different lines of dikaryotic parental strains, ASI1038 and ASI1346, were analyzed by nucleotide sequencing of the intergenic spacer I (IGS I) region in their rDNA and by extracellular enzyme assays. Nineteen different sizes of IGS I, which ranged from 1,301 to 1,348 bp, were present among twenty ASI1346-derived progeny strains, while 15 different sizes of IGS I, which ranged from 700 to 1,347 bp, were present among twenty ASI1038-derived progeny strains. Phylogenetic analysis of the IGS sequences revealed that different clades were present in both the ASI10388- and ASI1346-derived progeny strains. Plating assays of seven kinds of extracellular enzymes (β-glucosidase, avicelase, CM-cellulase, amylase, pectinase, xylanase, and protease) also revealed apparent variation in the ability to produce extracellular enzymes among the 40 tested progeny strains from both parental *A. bisporus* strains. Overall, this study demonstrates that characterization of IGS I regions and extracellular enzymes is useful for the assessment of the substrate-degrading ability and heterogenicity of *A. bisporus* monokaryotic strains.

Keywords *Agaricus bisporus*, Extracellular enzyme, Monokaryotic strain, Mushroom breeding

Agaricus bisporus (J. Lange) Imbach, commonly known as the button mushroom, is one of the most widely cultivated edible mushrooms in the world. The demand for breeding of button mushrooms has recently increased in Korea to enhance the selective range of domestic cultivars for cultivation and for reviving mushroom exports. The first hybrid strains of white button mushrooms were released in 1981 [1]. In general, to breed new *A. bisporus* strains in the conventional manner, hybridization of two compatible monokaryotic (haploid) strains is needed together with information on their genetic and biochemical properties. Thus, acquisition of monokaryotic strains derived from haploid basidiospores is a prerequisite for genetic analysis and selective breeding of button mushrooms. The formation of basidiospores is mostly bisporic in *A. bisporus* [2]. *A. bisporus* var. *bisporus* exhibits limited heterothallism in conjunction with a low percentage of tetrasporic basidia (1.3% on average) that are haploid [3]. The acquisition of homokaryons is very difficult by conventional basidiospore isolation from *A. bisporus* strains because of their secondary homothallic life cycle [2, 4].

Due to the limited heterothallism of *A. bisporus*, systematic investigations of the variability in its monokaryotic strains have been somewhat limited. Regarding mushroom breeding, a thorough understanding of the strains' genetic and biochemical properties is also essential to discriminate their usefulness as genetic resources when the morphological and physiological features are similar, as is the case with button mushrooms.

In this study, the nucleotide sequences of intergenic spacer I (IGS I) regions and the ability to produce extracellular enzymes were analyzed to characterize the genetic and biochemical variation in monokaryotic progeny strains of *A. bisporus*.

Two different lines of dikaryotic parental strains, ASI1038

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Table 1. Variation of IGS I region size in the progeny strains of ASI1038 and ASI1346

Strain No.	Size of IGS region (bp)	Strain No.	Size of IGS region (bp)
ASI1346 (Parent)	1,312	ASI1038 (Parent)	1,311
ASI1346-1	1,321	ASI1038-26	703
ASI1346-2	1,319	ASI1038-51	1,313
ASI1346-3	1,338	ASI1038-59	1,312
ASI1346-5	1,348	ASI1038-66	1,309
ASI1346-6	1,301	ASI1038-71	1,310
ASI1346-9	1,309	ASI1038-73	1,311
ASI1346-10	1,323	ASI1038-74	1,304
ASI1346-22	1,305	ASI1038-82	1,347
ASI1346-33	1,327	ASI1038-87	1,311
ASI1346-34	1,321	ASI1038-89	1,316
ASI1346-37	1,331	ASI1038-91	1,001
ASI1346-38	1,329	ASI1038-179	1,300
ASI1346-75	1,311	ASI1038-201	1,310
ASI1346-83	1,308	ASI1038-203	1,314
ASI1346-85	1,320	ASI1038-222	1,008
ASI1346-88	1,310	ASI1038-237	1,313
ASI1346-82	1,316	ASI1038-238	1,328
ASI1346-81	1,306	ASI1038-249	1,312
ASI1346-31	1,303	ASI1038-287	1,310
ASI1346-89	1,311	ASI1038-327	1,300

ASI, Agricultural Science Institute; IGS, intergenic spacer. Acronym for mushroom strains used in Mushroom Science Division in Rural Development Administration, Korea.

(white mushroom line of domestic origin) and ASI1346 (brown mushroom line of USA origin), and 61 monokaryotic progeny strains that were derived from the identified haploid basidiospores of the two parental strains, were obtained from the Mushroom Research Division, National Institute of Horticultural and Herbal Sciences, Rural Development Administration (RDA), Eumseong, Korea. According to the results of genetic diversity analysis of 45 dikaryotic *A. bisporus* strains using PCR fingerprinting, ASI1038 and ASI1346 strains are genetically different groups, which were divided into groups A and F, respectively [5].

For the IGS I analysis, ASI1038 and ASI1346 and their 40 monokaryotic progeny strains listed in Table 1 were cultured on cellophane-layered corn meal agar (CMA) at 25°C for 7 days and their mycelia were collected by scraping with a sterile scalpel. Genomic DNA was extracted from the prepared mycelia using the method described by Kim *et al.* [6], and the IGS I region was amplified by PCR using primers LR12R (5′-GAACGCCTCTAAGTCAGAA-TCC-3′) and 5SRNA (5′-ATCAGACGGGATGCGGT-3′) [7, 8]. Sequencing of the PCR amplicons was performed at Macrogen Inc. (Seoul, Korea). Identity of the determined nucleotide sequences was verified as IGS I sequences by BLAST searches of the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Table 1 shows the size of all the determined IGS I nucleotide sequences of 42 *A. bisporus* strains. Strikingly diverse sizes were resolved in the monokaryotic progeny strains from both ASI1038 and ASI1346 lines. Among the 20 ASI1346-derived progeny strains, 19 different IGS I sizes were found, which ranged from 1,303 to 1,348 bp. Furthermore, there were no monokaryotic strains with the 1312-bp size of the parental (ASI1346) IGS I. In contrast, 15 different IGS I sizes, which ranged from 700 to 1,347 bp, were present among the 20 ASI1038-derived progeny strains. Unlike the ASI1346-derived progeny strains, two ASI1038-derived progeny strains (ASI1038-73 and -87) exhibited the 1,311-bp size of the parental (ASI1038) IGS I, and three ASI1038-derived progeny strains (ASI1038-26, -91, and -222) had IGS I sequences that were less than 1,300 bp. Overall, Table 1 indicates that size variation in the IGS I region is high in *A. bisporus* monokaryotic strains. This variability in size could be a useful tool for discrimination of individual monokaryotic strains.

To further analyze genetic variation among the monokaryotic strains listed in Table 1, phylogenetic analysis based on the IGS I sequences was performed with the MEGA 6.06 program [9]. For phylogenetic tree construction, the maximum likelihood method was used with 1,000 bootstrap replicates [9]. Two divergent groups were revealed among the ASI1346-derived progeny strains in the phylogenetic tree (Fig. 1). The parental ASI1346 strain grouped with eighteen out of twenty progeny strains. Only two progeny strains were separated from the others. This result indicates that most of the ASI1346-derived progeny strains are genetically closely related. As opposed to the phylogenetic tree in Fig. 1, several divergent groups were revealed among the ASI1038-derived progeny strains in the phylogenetic tree in Fig. 2. The parental strain ASI1038

Fig. 1. Phylogenetic tree based on IGS I sequences of brown mushroom line ASI1346-derived monokaryotic progeny strains of *Agaricus bisporus*. Parental strain ASI1346 is underlined. IGS, intergenic spacer; ASI, Agricultural Science Institute.

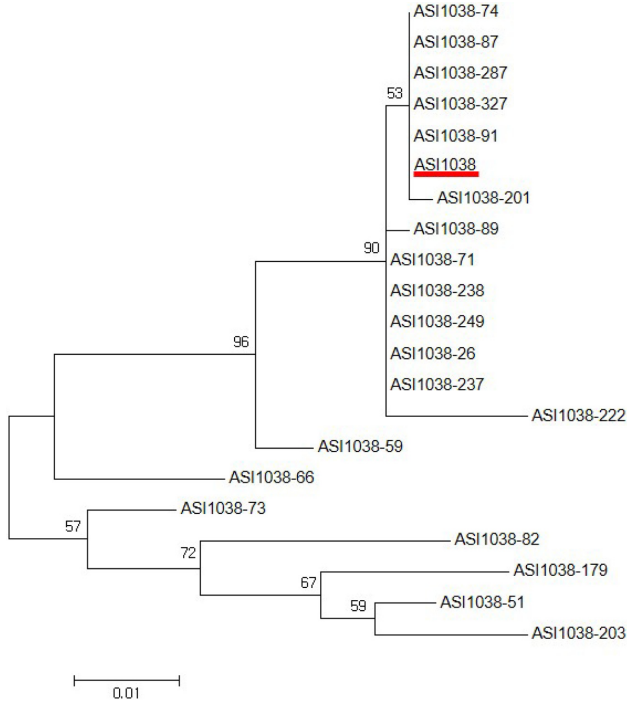

Fig. 2. Phylogenetic tree based on IGS I sequences of white mushroom line ASI1038-derived monokaryotic progeny strains of *Agaricus bisporus*. Parental strain ASI1038 is underlined. IGS, intergenic spacer; ASI, Agricultural Science Institute.

grouped with six out of the twenty progeny strains. The results in Fig. 2 indicate that the ASI1038-derived progeny strains are genetically diverse. It is interesting that progeny strains from different parental lines showed distinct relationship patterns. Based on the results in Table 1 and Figs. 1 and 2, it is assumed that the IGS I region is predisposed to change during the process of basidiospore formation in *A. bisporus*. Heterogeneity in the length of the IGS has been reported in fungi [10, 11]. In addition, the possibility of using IGS regions in discriminatory analysis among intra-specific individuals of mushroom species such as *Auricularia auricula-judae, Laccaria bicolor,* and *Lentinula edodes* has been suggested [12-14]. Thus, it is thought that IGS region I information is also useful for the characterization of the heterogeneity of monokaryotic strains of *A. bisporus*. So far, there have been no sequence data reported for the *A. bisporus* IGS region in any extended pool of monokaryotic strains derived from a parental strain. Therefore, the IGS I data generated in the present study is valuable for future work on strain breeding using the analyzed monokaryotic strains.

In a previous study, we found that a plating method-based assay for extracellular enzymes is useful for biochemical characterization of dikaryotic *A. bisporus* strains from different origins [15]. Thus, we used the same method to characterize *A. bisporus* strains ASI1038 and ASI1346 and their 40 monokaryotic progeny strains described in the legends of Figs. 3 and 4. The 42 strains were precultured on CMA and transferred onto chromogenic media containing 0.5% of one of the following carbon sources as enzymatic substrate (D-cellobiose for β-glucosidase; CM-cellulose and avicel for CM-cellulase and avicelase, respectively; potato starch for amylase; skim milk for protease; xylan from oat spelts for xylanase [all from Sigma-Aldrich, St. Louis, MO, USA]; and polygalacturonic acid [MP Biomedical, Strasbourg, France] for pectinase), 0.1% yeast nitrogen base (BD, Franklin Lakes, NJ, USA) as a fundamental nitrogen source, 0.5% Congo Red (Sigma-Aldrich) for the chromogenic reaction, and 1.5% agar powder [16]. After incubation at 25°C for 14 days, the size (radial diameter, in mm) of clear zones on the chromogenic media formed by the reaction between enzymes produced by the tested strains and substrates was measured for extracellular enzyme evaluation. These tests were carried out with five replicates.

The results of the extracellular enzyme test are shown in Figs. 3 and 4. The degree of extracellular enzyme production varied considerably among the monokaryotic strains. In Fig. 3, among the progeny strains of the white ASI1038 line, those showing significantly better enzyme production than the parental strain were identified in the β-glucosidase, avicelase, amylase, and pectinase assays. Regarding CM-cellulase, no progeny strains exhibited better enzyme production than the parental strain. In contrast, among the progeny strains of the brown ASI1346 line, strains showing significantly better enzyme production than the parental strain were found in the β-glucosidase, avicelase, and protease

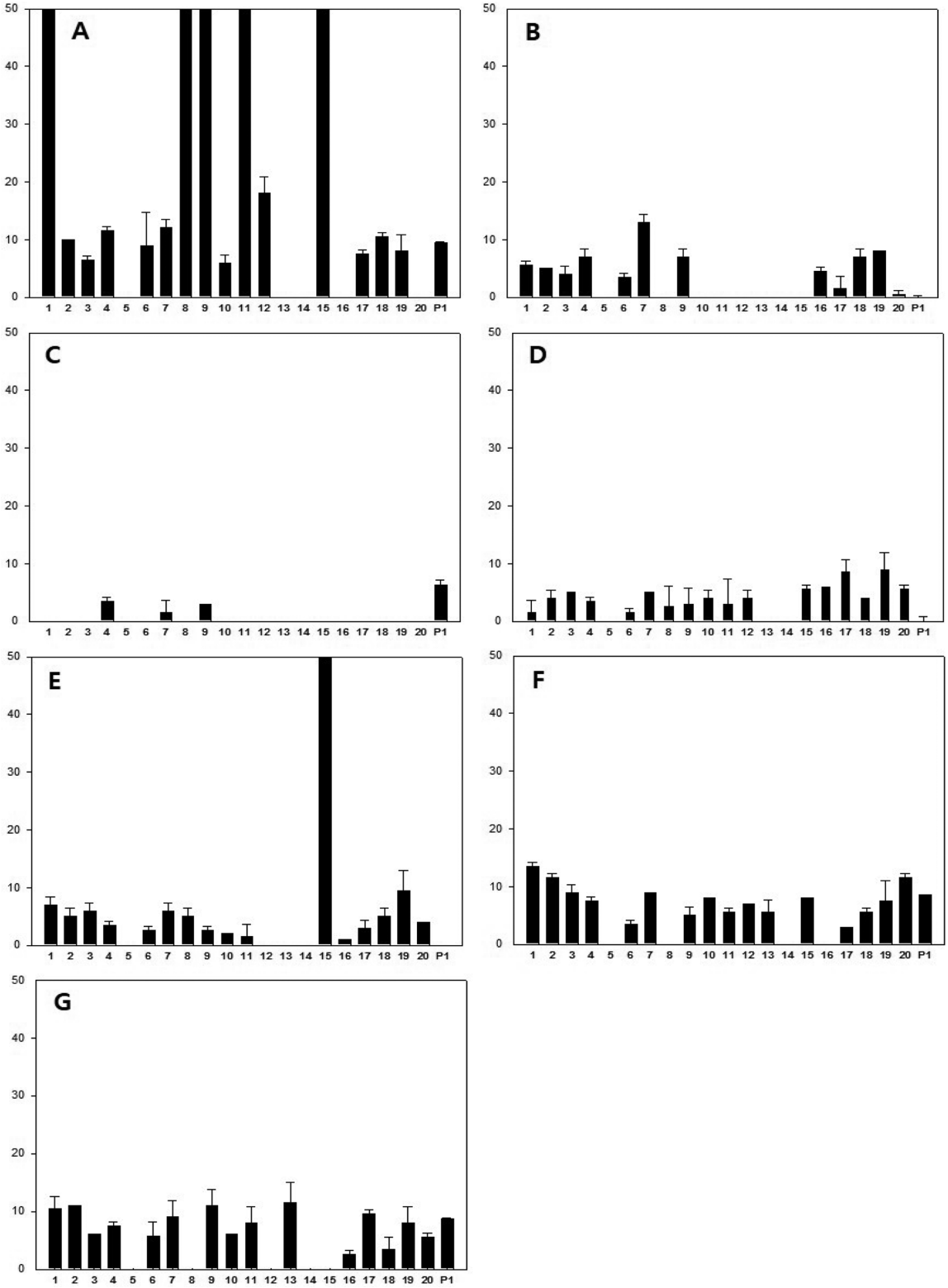

Fig. 3. Extracellular enzyme production of white mushroom line ASI1038-derived monokaryotic progeny strains of *Agaricus bisporus*. A, β-Glucosidase; B, Avicelase; C, CM-cellulase; D, Amylase; E, Pectinase; F, Xylanase; G, Protease. The *y*-axis is the size (mm) of clear zones formed on chromogenic media by enzymes produced by the ASI1038-derived strains. On the *x*-axis are ASI11038-derived monokaryotic progeny strains (1, ASI1038-32; 2, ASI1038-61; 3, ASI1038-66; 4, ASI1038-74; 5, ASI1038-86; 6, ASI1038-89; 7, ASI1038-97; 8, ASI1038-128; 9, ASI1038-129; 10, ASI1038-177; 11, ASI1038-179; 12, ASI1038-181; 13, ASI1038-182; 14, ASI1038-240; 15, ASI1038-241; 16, ASI1038-263; 17, ASI1038-266; 18, ASI1038-344; 19, ASI1038-347; 20, ASI1038-3480) and parental strain (P1, ASI1038). ASI, Agricultural Science Institute.

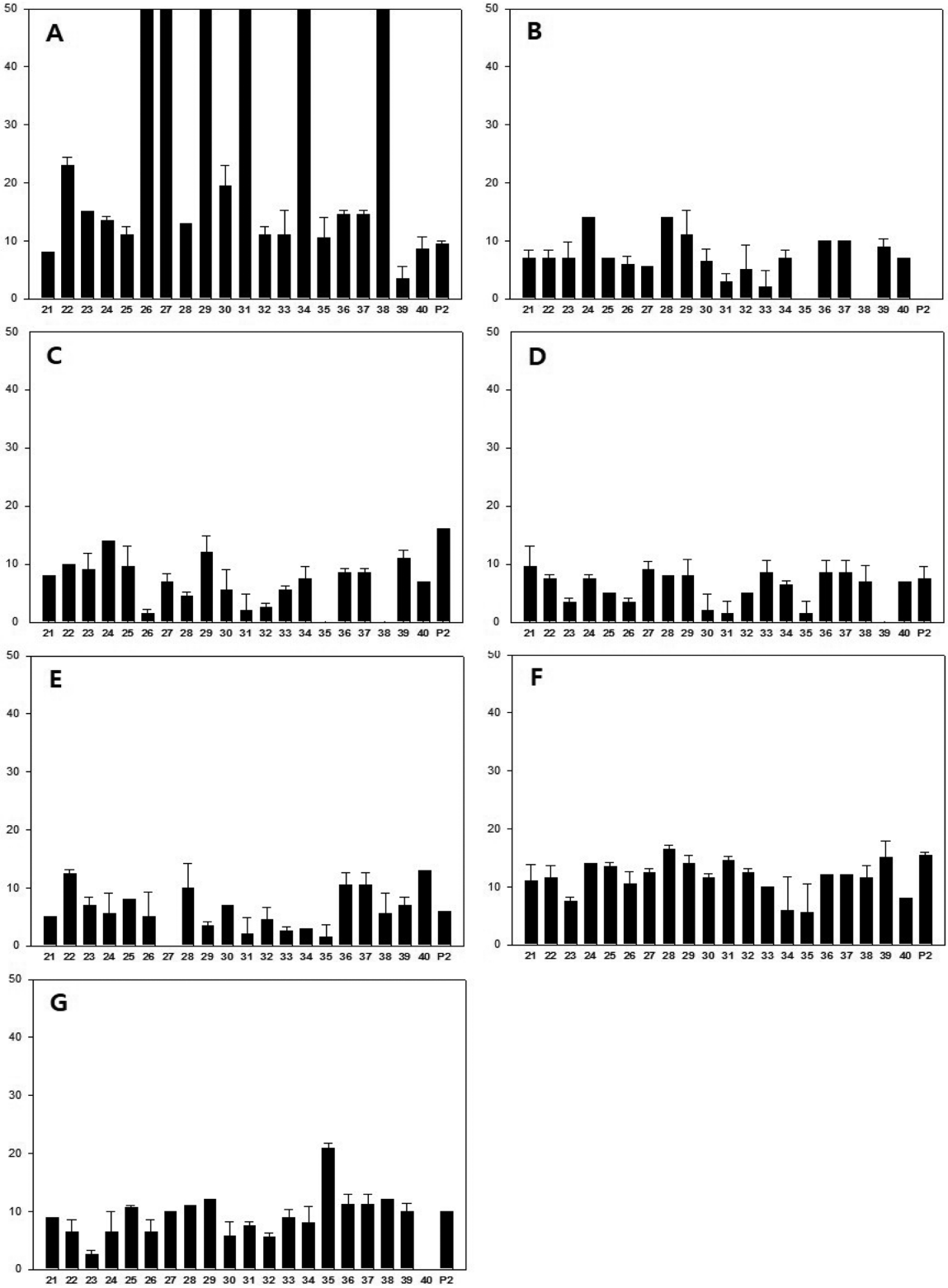

Fig. 4. Extracellular enzyme production of brown mushroom line ASI1346-derived monokaryotic progeny strains of *Agaricus bisporus*. A, β-Glucosidase; B, Avicelase; C, CM-cellulase; D, Amylase; E, Pectinase; F, Xylanase; G, Protease. The *y*-axis is the size (mm) of clear zones formed on chromogenic media by enzymes produced by the ASI1346-derived strains. On the *x*-axis are ASI1346-derived monokaryotic progeny strains (21, ASI1346-1; 22, ASI1346-2; 23, ASI1346-3; 24, ASI1346-5; 25, ASI1346-9; 26, ASI1346-10; 27, ASI1346-11; 28, ASI1346-22; 29, ASI1346-23; 30, ASI1346-29; 31, ASI1346-31; 32, ASI1346-32; 33, ASI1346-33; 34, ASI1346-34; 35, ASI1346-37; 36, ASI1346-38; 37, ASI1346-60; 38, ASI1346-75; 39, ASI1346-83; 40, ASI1346-85) and parental strain (P2, ASI1346). ASI, Agricultural Science Institute.

assays (Fig. 4). Among the seven kinds of extracellular enzymes that were assayed, the least variation among the progeny strains was found in the xylanase assay. When we compared ASI1038- and ASI1346-derived progeny strains, we found that the ASI1346-derived progeny strains were slightly better enzyme producers than the ASI1038-derived progeny strains. Thus, comparisons of extracellular enzyme assay data for monokaryotic progeny pools would be useful for a better understanding of the biochemical properties of their dikaryotic parental strain.

In conclusion, we genetically and biochemically characterized 61 monokaryotic progeny strains from two different parental lines of *A. bisporus*. This is the first report of a combinatorial analysis of *A. bisporus* monokaryotic strains derived from dikaryotic strains of domestic and foreign origin. Considering that monokaryotic progeny strains are potentially valuable genetic resources for the breeding of noble hybrid cultivars, their characterization is indispensable for *A. bisporus* breeders. Our assessment of extracellular enzyme production, based on the ability of *A. bisporus* to degrade seven different substrates, along with our identification of a genetically discriminating region within the IGS I region of rDNA, should enhance our basic knowledge of monokaryotic strains derived from different lines of dikaryotic strains. We expect that our analytical method and data will be applicable to the selection of promising monokaryotic strains for button mushroom breeding.

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REFERENCES

1. Fritsche G. Breeding mushrooms. Mushroom J 1986;157:4-17.
2. Raper CA, Raper JR, Miller RE. Genetic analysis of the life cycle of *Agaricus bisporus*. Mycologia 1972;64:1088-117.
3. Callac P, Imbernon M, Kerrigan RW, Olivier JM. The two life cycles of *Agaricus bisporus*. In: Royse DJ, editor. Mushroom Biology and Mushroom Products: Proceedings of the 2nd International Conference; 1996 Jun 9-12; University Park, PA, USA. University Park: The Pennsylvania State University; 1996. p. 57-66.
4. Kerrigan RW, Royer JC, Baller LM, Kohli Y, Horgen PA, Anderson JB. Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. Genetics 1993;133:225-36.
5. Min KJ, Kim JK, Kwak AM, Kong WS, Oh YH, Kang HW. Genetic diversity of *Agaricus bisporus* strains by PCR polymorphism. Kor J Mycol 2014;42:1-8.
6. Kim SH, Uzunovic A, Breuil C. Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood by PCR. Appl Environ Microbiol 1999;65:287-90.
7. Vilgalys R, Gonzalez D. Organization of ribosomal DNA in the basidiomycete *Thanatephorus praticola*. Curr Genet 1990; 18:277-80.
8. Vilgalys R, Hopple JS Jr, Hibbett DS. Phylogenetic implications of generic concepts in fungal taxonomy: the impact of molecular systematic studies. Mycol Helv 1994;6:73-91.
9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725-9.
10. Morton A, Tabrett AM, Carder JH, Barbara DJ. Sub-repeat sequences in the ribosomal RNA intergenic regions of *Verticillium alboatrum* and *V. dahliae*. Mycol Res 1995;99: 257-66.
11. Ganley AR, Scott B. Extraordinary ribosomal spacer length heterogeneity in a *Neotyphodium* endophyte hybrid: implications for concerted evolution. Genetics 1998;150:1625-37.
12. Li L, Zhong CH, Bian YB. The molecular diversity analysis of *Auricularia auricula-judae* in China by nuclear ribosomal DNA intergenic spacer. Electron J Biotechnol 2014;17:27-33.
13. Selosse MA, Costa G, Di Battista C, Le Tacon F, Martin F. Meiotic segregation and recombination of the intergenic spacer of the ribosomal DNA in the ectomycorrhizal basidiomycete *Laccaria bicolor*. Curr Genet 1996;30:332-7.
14. Saito T, Tanaka N, Shinozawa T. Characterization of subrepeat regions within rDNA intergenic spacers of the edible basidiomycete *Lentinula edodes*. Biosci Biotechnol Biochem 2002;66:2125-33.
15. Kwon HW, Kim JY, Min SH, Choi MA, Oh YL, Kong WS, Kim SH. Biochemical characterization of *Agaricus bisporus* dikaryon strains. Kor J Mycol 2014;42:86-90.
16. Kwon HW, Back IJ, Ko HG, You CH, Kim SH. Extracellular enzyme activities of the monokaryotic strains generated from basidiospores of shiitake mushroom. Mycobiology 2008;36: 74-6.