P-2 かつお節かび付け工程で働く好乾性糸状菌の分類と菌叢解析に関する研究

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Introduction

日本の伝統食品である鰹節は、荒節と枯節に大別できる。カツオ肉を煮熟・焙乾ものが荒節であり、さらに *Aspergillus* 属糸状菌（鰹節カビ）にてかび付け発酵・熟成させたものが枯節である。かび付け工程は、約 6 か月間かけて「かび付け⇒天日干し⇒かびの払落し」を 4 回ほど繰り返す作業であり（Fig. 1 (A)）、脂肪の分解、香味の付与、色相の変化などをもたらすとともにまろやかな風味や上品な色合いを有する節に変えてくれる。鰹節カビは、*A. glaucus* およびその類縁種と言われてきたが、かび付け工程で、どのような *Aspergillus* 属糸状菌がこれを担っているか明らかではない。本研究では、かび付け工程やサンプルの違いによる好乾性糸状菌の優占種やそれらの加水分解酵素の生産特性の解明を目的とした。

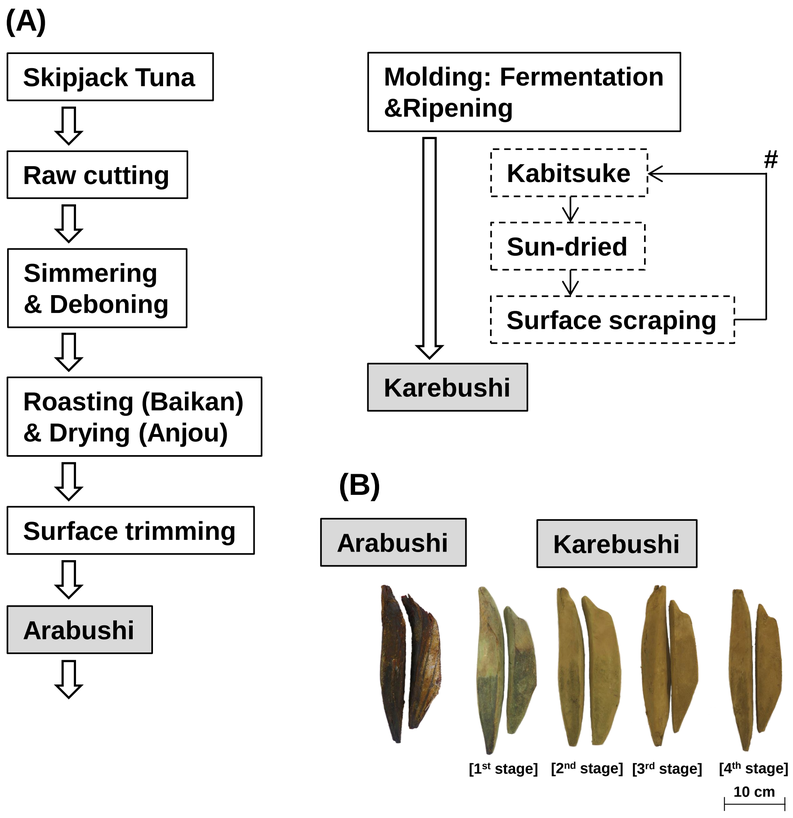


Fig. 1. Katuobushi processing. (A) Flow chart of katsuobushi (arabushi and karebushi) processing. Steps in the manufacturing process were as in reference (Dimichi & Wada, 1994). #1, Boiling at 85° C for 70–90 min. #2, Smoking at 80–90° C for 5–6 h. This step is repeated eight to ten times over 10 days. #3, Molding step (fermentation and ripening), consisting of kabitsuke (incubating in the fermentation room and cultivating molds), sun-drying (leaving outdoors), with surface scraping (removing molds from the katsuobushi surface) usually repeated two to four times; in traditional manufacturing, this step is repeated four times over 6 months. Generally, the 1st and 2nd fermentation-stages and 3rd and 4th fermentation-stages are performed in fermentation rooms at 27° C at 85% to 88% and 50% to 60% humidity, respectively. **(B)** Photographs of arabushi and karebushi during the repeated mold fermentation (1st to 4th stages). **(C)** The surfaces of 1st fermentation-stage fillet (left) and 4th fermentation-stage fillet (right).

Materials & Methods

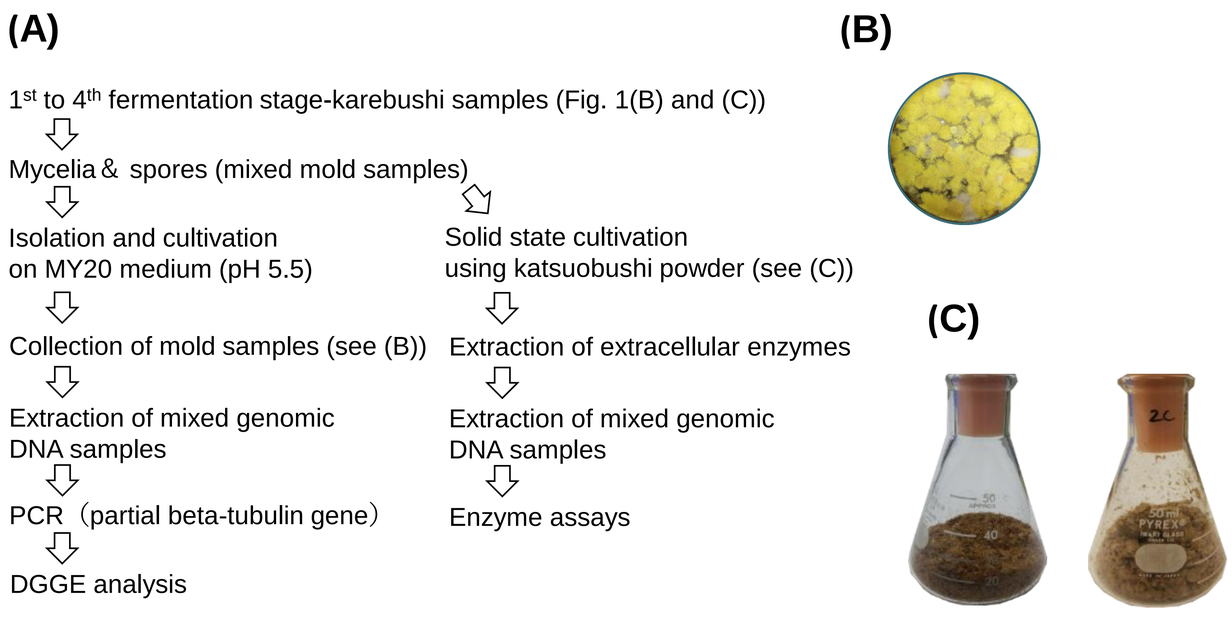


Fig. 2. Cultivation of mixed *Aspergillus* spp. (A) Flow chart of isolation and cultivation of *Aspergillus* spp. derived from karebushi samples. **(B)** Photograph of mixed culture sample on MY20 agar plate. **(C)** Photographs of solid-state cultivation samples.

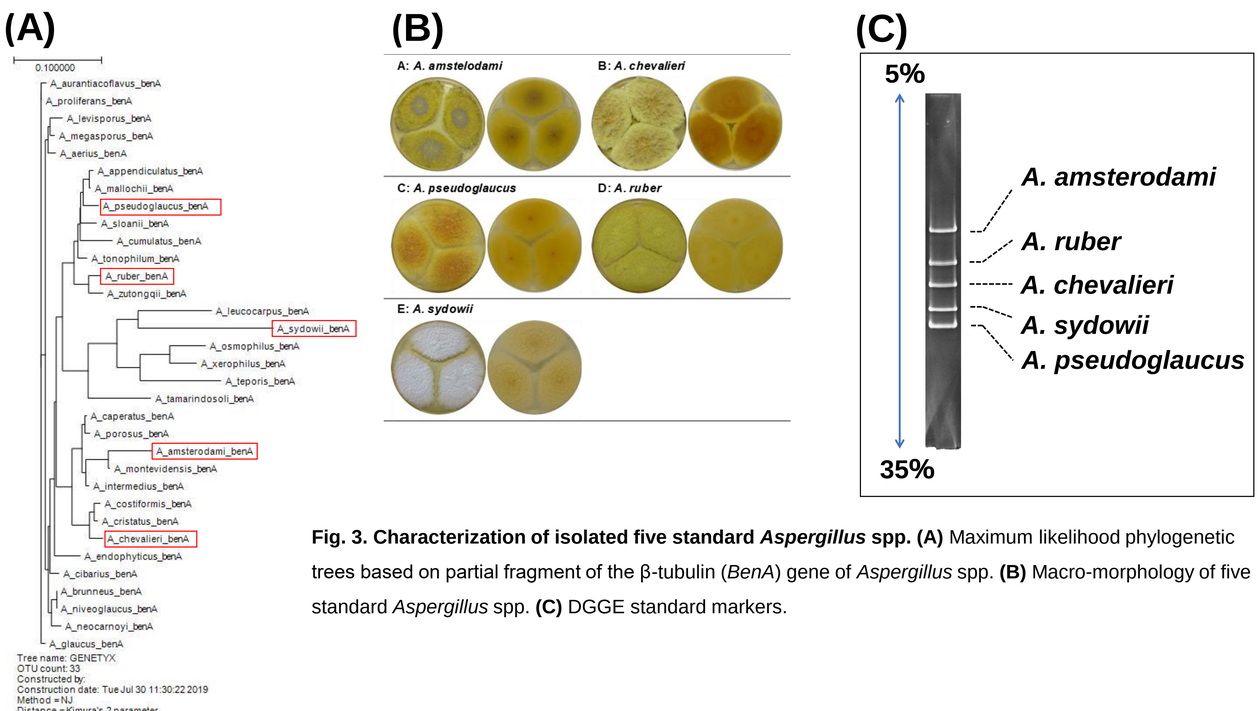
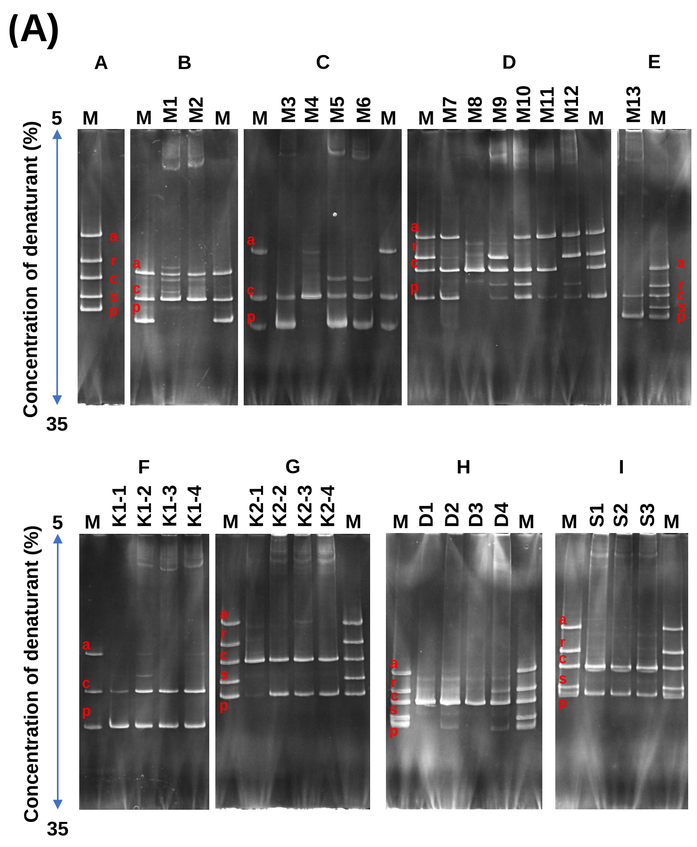


Fig. 3. Characterization of isolated five standard *Aspergillus* spp. (A) Maximum likelihood phylogenetic trees based on partial fragment of the β-tubulin (*BenA*) gene of *Aspergillus* spp. **(B)** Macro-morphology of five standard *Aspergillus* spp. **(C)** DGGE standard markers.

Results & Discussion



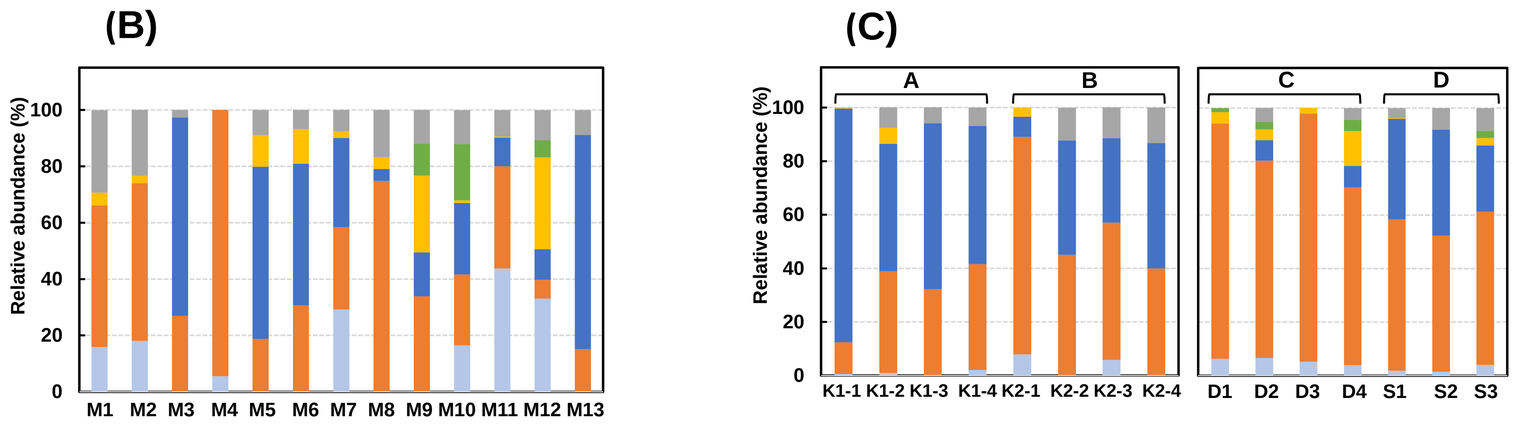


Fig. 4. Denaturing gradient gel electrophoresis (DGGE) profiles of the partial *β-tubulin* (*BenA*) gene amplicons obtained from samples of karebushi obtained from different manufacturers and at different molding stages. **(A)** Five *Aspergillus* spp. were selected as standards. Mixed molds from the surface of karebushi samples obtained from 16 different manufacturers (M1 to M13, and K, D, and S) were cultivated on MY20 agar plates. **A.** Standard amplified fragments from strains *Aspergillus amstelodami* (a), *A. chevalieri* (c), *A. pseudoglaucus* (p), *A. ruber* (r), and *A. sydowii* (s) were loaded in lane M. **B–E.** Mixed genomic DNAs that were prepared from mixed culture samples derived from the 3rd or 4th molding stage by different manufacturers (manufacturers M1 to M13) were used for PCR-DGGE. **F–I.** Mixed genomic DNAs prepared from mixed culture samples derived from the 1st to the 4th molding stages by the same manufacturer (manufacturer K) in spring (K1-1 to K1-4) and autumn (K2-1 to K2-4), and from the 1st to the 4th molding stages by manufacturers D and S (1st–4th, D-1 to D-4) and (1st–3rd, S-1 to S-3). **(B)** Relative quantification of *Aspergillus* spp. derived from the 3rd or 4th molding stage products from different manufacturers. Mixed genomic DNAs that were prepared from mixed culture samples derived from the 3rd or 4th molding stage products of different manufacturers (manufacturers M1 to M13) were used for PCR-DGGE. **(C)** Relative quantification of *Aspergillus* spp. derived from the 1st to the 4th molding stage products of the same manufacturer. Mixed genomic DNAs were prepared from mixed culture samples derived from the 1st to the 4th molding stage products of the same manufacturer (manufacturer K) in spring (A: K1-1 to K1-4) and autumn (B: K2-1 to K2-4), and of different manufacturers (D and S) (C: 1st–4th, D-1 to D-4) and (D: 1st–3rd, S-1 to S-3). Separated partial β-tubulin (*BenA*) DNA bands were densitometrically analyzed and relative compositions were calculated.

Table 1. Production of extracellular hydrolytic enzymes in mixed culture at 0.85 a_w.

Sample	Enzyme activity (U/ml)					
	Protease (×10^{-2})		Amino-peptidase (×10^{-3})	Carboxy-peptidase (×10^{-3})	Lipase (×10^{-2})	
	pH 3.0	pH 7.5			C4	C16
M1	<1.00	<1.00	5.5±0.03	<1.00	17.1±0.40	3.1±0.02
M2	<1.00	1.3±0.01	8.2±0.02	<1.00	19.0±0.88	3.1±0.03
M3	<1.00	<1.00	3.7±0.01	<1.00	11.9±1.10	0.5±0.06
M4	<1.00	1.7±0.01	6.3±0.01	1.5±0.03	19.0±1.30	3.5±0.01
M5	<1.00	<1.00	4.0±0.03	<1.00	11.9±0.08	N.D.
M6	<1.00	1.2±0.01	1.1±0.05	1.9±0.01	22.0±1.45	4.8±0.05
M7	2.4±0.16	2.4±0.01	1.1±0.05	1.3±0.01	20.0±0.38	7.4±0.01
M8	2.0±0.02	1.7±0.03	1.2±0.01	1.2±0.02	22.2±1.40	9.8±0.09
M9	N.D.	<1.00	5.7±0.03	1.6±0.01	19.6±0.36	8.1±0.05
M10	<1.00	<1.00	8.1±0.03	1.3±0.01	18.5±0.41	6.2±0.01
M11	1.2±0.13	1.0±0.01	1.2±0.01	2.1±0.01	12.8±0.07	4.0±0.03
M12	1.1±0.19	<1.00	1.4±0.01	1.6±0.01	17.7±0.07	5.1±0.01
M13	<1.00	1.9±0.02	3.6±0.01	2.4±0.01	21.8±0.06	6.4±0.03

枯節から*A. amstelodami*（水色）、*A. chevalieri*（橙）、*A. pseudoglaucus*（青）、*A. ruber*（黄）、*A. sydowii*（緑）の5種を分離・同定し、基準株とした（Fig. 3 (A)&(B)）。次に、混合培養・ゲノムDNA調製法を検討後、*BenA*遺伝子の増幅とDGGEで菌叢解析した。「異なる製造元から入手した4番枯節」について優占種の推移を調べた結果（Fig. 4 (A)&(B)）、5種の基準株の中でも*A. pseudoglaucus* および*A. chevalieri* が優占種であり、節の水分や脂質含量の違いにより*A. amstelodami*、*A. ruber*、*A. sydowii* も優占種となっていた。「異なる製造元から入手した4番枯節」から回収した胞子（カビ混合サンプル）を水分含量の異なる鰹節固体培地で培養し（0.85および0.95 aw）、得られた抽出液（粗酵素液）について加水分解酵素活性を調べた。その結果、低水分下（0.85 aw）で培養して得られた抽出液において測定した加水分解酵素活性値が高い傾向が見られた（Table 1）。特に、脂質の加水分解に関わるリパーゼの活性だけでなく、筆者が提唱した鰹肉の脱色に関わるアスパルティックプロテアーゼの活性は、低水分活性下で培養したサンプルにおいてそのほとんどが高活性であった。さらに、「同じ製造元から入手した１番から4 番枯節」について優占種の推移を調べた結果（Fig. 4 (A)&(C)）、*A. pseudoglaucus* および*A. chevalieri* が優占種であり、特にサンプルKではかび付け発酵が進むとともに2種の構成比がほぼ１：１に収束した。

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