INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–184/2016/18–6–1159–1168
DOI: 10.17957/IJAB/15.0220
http://www.fspublishers.org

Full Length Article

Evaluation of Nutritive Value and Identification of Fungi in Silage from New Kenaf (*Hibiscus cannabinus*) Cultivars

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Abstract

The effects of harvesting stage of new Kenaf cultivars on the nutritive values, amino acid contents and fungal abundance were investigated. The late and mid-late maturing cultivars offered more DM than did early-maturing cultivars. The DM was highest at 100 DAS in all Kenaf silage. For all cultivars, the crude protein content (7.4–18.4%) of silage was reduced if harvesting was delayed. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were not significantly different among the cultivars; however NDF and ADF contents increased significantly upon late harvesting. All silages had pH values under 4.0, ensuring stable storage. The optimum harvesting time for silage production, as evidenced by yield, fermentation characteristics and nutritive value, was 100 DAS. The concentrations of amino acid in the silage were lower than that in the hay. Silage produced by all Kenaf cultivars exhibited low yeast biodiversity; only one species (*Pichia fermentans*) was detected when the harvesting date was optimal. © 2016 Friends Science Publishers

Keywords: Kenaf silage; Nutritive value; Optimal harvest date; Identification of fungi

Introduction

Kenaf (*Hibiscus cannabinus*) is a short-day annual herbaceous plant that tolerates a broad range of soil types and climates (Dempsey, 1975). Kenaf is normally produced for its fiber. However, the plant, especially in silage form, can also serve as a forage crop for ruminant (Xiccato *et al.*, 1998). Kenaf is a good source of fodder protein; the protein content is high at early growth stages, when the nutrient profile is comparable to that of alfalfa (Suriyajantratong *et al.*, 1973; Phillips *et al.*, 1996).

Korean Kenaf cultivars are divided into three maturation groups depending on flowering date; early-maturing, mid-late maturing, and late maturing. Early-maturing varieties mature in 70–80 day. Such varieties allow high seed yields, but the brief vegetative growth period produces shorter plants of lower biomass (Webber and Bledsoe, 2002; Kang *et al.*, 2004). Late-maturing groups grow vegetatively for 130–140 days and yield significantly higher biomass. However, late maturation increases the risk of seed shattering and reduces seed quality. Therefore, it is necessary to develop domestic mid-late maturing groups yielding high biomass and seed yields (Kang *et al.*, 2004).

Palatability has often been considered to compromise the utility of Kenaf as forage, although high-yield, high-quality Kenaf can ameliorate feedstuff shortages. Silage of Kenaf may overcome the palatability problem (Hancock *et al.*, 1993; Xiccato *et al.*, 1998).

The extent of protein degradation in the silage was influenced by species, pH, dry-matter (DM) content and storage temperature (Scalet *et al.*, 1984; Muck, 1988). Ammonia-N in silage is produced by protein breakdown through either plant proteolysis enzymes or microorganisms (Muck, 1988). The effects of feed preservation on nutritional value have been principally studied in terms of protein contents and digestibility. However, the amino acid composition of Kenaf silage is poorly understood. The nutritive value of such silage has been studied in terms of chemical composition and fermentation quality, but not the perspective of amino acid levels (Chantiratkul *et al.*, 2009).

Fungi are eukaryotic heterotrophic organisms that actively or passively take up nutrients. The fungi that are characteristically present in aerobically deteriorating silages are principally yeasts (Muck, 2013). Research on silage microbiology has been greatly aided by extraction and

To cite this paper: Ryu, J., S.J. Kwon, J.W. Ahn, B.K. Ha, S.W. Jeong, S.B. Im, J.B. Kim, S.H. Kim Y.K. Lee and S.Y. Kang, 2016. Evaluation of nutritive value and identification of fungi in silage from new Kenaf (*Hibiscus cannabinus*) cultivars. *Int. J. Agric. Biol.*, 18: 1159–1168

amplification of specific regions of genomic DNA, followed by electrophoretic separation of amplified fragments. Fungal classifications employ various molecular criteria (Avila *et al.*, 2010; Muck, 2013). Advances in DNA sequencing have facilitated phylogenetic classifications allowing rapid and reliable identification. The internal transcribed spacer (ITS) region of 26S nuclear ribosomal DNA (rDNA) includes two distinct spacer regions (ITS1 and ITS2) and an intervening 5.8S coding sequence; this ITS has been well characterised in terms of interspecific and intergeneric divergence (Hillis *et al.*, 1991; Baldwin *et al.*, 1995).

In the present study, we defined the optimum harvesting dates, in terms of yield and nutritive value, of novel Kenaf (*Hibiscus cannabinus*) cultivars used to prepare silage, and explored silage mineral and amino acid compositions and the microorganisms present.

Materials and Methods

Kenaf Growth and Harvest

Six Kenaf cultivars were used (Table 1; Fig. 1); all were grown in Jeong-eup, Jeollabuk-do, Korea (35˚30′ S, 126˚50′ W). The cultivars were of three different maturation groups: late maturing (Auxu and Jinju), mid-late maturing (Jangdae), and early maturing (Bakma, Jeokbong, and C14). Two morphological mutants exhibiting changes in flower (Bakma) and stem (Jeokbong) colours were derived from the same introduction (C14) of Kenaf cultivar from Italy. The Bakma cultivar was generated by selective breeding, and the Jeokbong and Jangdae cultivars were mutated by gamma-ray irradiation. The Auxu cultivar was introduction of Kenaf cultivar from China. The Kenaf seeds were planted in plots (3x4.2 m) and row spacing of 20 and 60 cm, respectively. Fertiliser (N:P:K 12:7:9 w/w/w) was applied at 550 kg/ha shortly after seeding. Manure was spread before planting, but the plants were not fertilised after planting.

Kenaf was collected from a 3 x 3 m quadrant and biological three replicates were used for each sample. All varieties were planted on 14 May 2014 and harvested at intervals of 20 days (thus, four harvests) from 15 July to 16 September.

Silage Production

Six plants harvested at each growth stage were chopped to lengths of 2–5 cm using manually-feed cutter (DY-4500, Dongyangtechtool, Daegu, Korea) and each sample (ca. 4 kg) was separately ensiled on the same day in a 4 L polyvinyl chloride (PVC) laboratory silos. To simulate compaction in silos, a pressure of 1200 kPa was applied to the ensiled forage with a hydraulic cylinder and airtight closing devices. The silos were stored for 60 days at room temperature (ca. 20–30°C); fermentation was terminated by freezing to –20°C until silage analysis.

Chemical Analysis

Dry matter (DM) levels were determined by drying 100 g amounts of material at 60°C in a forced-air oven for 90 h (AOAC, 1995). Dried samples were ground using a Wiley mill (Thomas Scientific, Inc.) until the particulate matter could pass through a 1 mm diameter screen, and subjected to laboratory tests of nutritional value. DM levels of ground samples were determined by drying 1 g amounts at 104°C in a forced-air oven for 6 h; this permitted moisture corrections to be made upon fibre fraction analyses. NDF and ADF levels were calculated using an ANKOM fibre analyser (Ankom Technology Corp., Fairport, NY, USA) and crude protein (CP) levels and ammonia-N were determined employing AOAC methods (AOAC, 1990). A 20 g sample of each silages were diluted 10 fold (on a mass basis) with distilled water, macerated for 30 s in a high-speed blender, and filtered through four layers of cheesecloth; then, the pH was immediately measured using a pH meter. Twenty millilitre aliquots were placed in 50 mL polypropylene centrifuge tubes and centrifuged for 20 min at 12,000× rpm at 4°C. The supernatants transferred to scintillation vials and frozen until subsequent analysis of fermentation products. Fermentation characteristics (lactic, acetic and butyric acid levels) were determined via high-performance liquid chromatography (Waters 1260, USA). The acids were separated on an Aminex HPX-87H ion-exclusion column running a linear elution gradient; the mobile phase was a mix of solvents A (0.4% v/v formic acid in distilled deionised water) and B (20 mM H_2SO_4).

Amino Acid Analysis

Amino acid levels were determined using an automatic amino acid analyzer (HITACHI L-8900, Hitachi, Japan). Hydrolysis was achieved by exposure to hydrochloride acid (6 mol dm^{-3}) for 23 h at 110°C under an N_2 atmosphere. After filtering, all hydrolysates were neutralised with sodium hydroxide solution, normalised to a volume of 100 mL with sodium citrate buffer (pH 2.2), and stored at 4°C for 24 h. Sample hydrolysates were subjected to chromatographic separation in sodium citrate buffer followed by reaction.

Identification of Fungi

Silage samples (20 g) were 60 days of fermentation, mixed with 30 mL amounts of sterile water, and shaken for 20 min; sequential 10-fold dilutions were then prepared. Yeasts were cultured on two media: Escherichia coli medium (EC: peptone 2%, lactose 0.5%, bile salt mixture 0.15%, dipotassium phosphate 0.4%, monopotassium phosphate 0.15%, sodium chloride 0.5%, and agar 1.5% [all w/w]) and potato dextrose agar medium (PDA: potato starch 4 g/L, dextrose 20 g/L and agar 15 g/L). The incubate temperature were EC and PDA medium of 28ºC and 35ºC, respectively.

Table 1: The origin of Kenaf cultivars used in this study

Cultivar	Blooming period	Maturity type	Flower color	Stem color	Leaf shape	Type	Origin
Jangdae	Aug. 22 (97 DAS*)	Mid-late maturity	Ivory	Green	Palmate	New cultivar (No. 4560**).	Jinju
Jinju	Sep. 15 (120 DAS)	Late-maturity	Ivory	Green	Entire	Accession	Korea
Auxu	Sep. 10 (115 DAS)	Late maturity	Ivory	Green	Palmate	Introduced Cultivar	China
Baekma	Aug. 4 (80 DAS)	Early maturity	White	Green	Entire	New cultivar (No. 5285**)	C14
Jeokbong	Jul. 24 (70 DAS)	Early maturity	Ivory	Dark purple	Entire	New cultivar (No. 5286**)	C14
C14	Jul. 24 (70 DAS)	Early maturity	Ivory	Green	Palmate	Introduced cultivar	Italy

*DAS: Day after seeding, **Korea seed and verity service grant number

After 36 h, three colonies were harvested from each medium for genomic DNA extraction, and the 26S rRNA genes of clones growing on EC medium were partially sequenced using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and LR3R (5′-GGTCCGTGTTTCAAGAC3′). The ITS region of each clone growing on PDA medium was sequenced using the primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′). All sequencing reactions were performed in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad) using an ABI BigDye Terminator version 3.1 Cycle Sequencing Kit as suggested by the manufacturer. Fluorescently labelled fragments were purified by the recommended method to remove unincorporated terminators and dNTPs. All samples were next subjected to electrophoresis using an ABI 3730xl DNA Analyzer. Sequences were compared in terms of the separate nuclear DNA relatedness values of 26S rDNA, and the ITS sequences. Nucleotide sequences used in comparisons were obtained from the FASTA program (Pearson and Lipman, 1988).

Statistical Analysis

ANOVA was performed using the multiple-comparisons method in the statistical package SPSS version 12. A p-value <0.05 was considered to reflect statistical significance. If a treatment effect was shown to be significant, the means were differentiated using Duncan's multiple range test.

Results

Forage Yield and Chemical Composition

The yield and chemical compositions of the various silages are shown in Table 2. Fresh matter (FM) and DM yields increased as the plants became more mature. The DM content was lower (<7 ton·ha^{-1}) at 60–80 DAS, but at 100 DAS (DM 13.7-15 ton·ha^{-1}) and 120 DAS (DM 14.7-24.4 ton·ha^{-1}). The late and mid-late maturing cultivars offered more DM than did early-maturing cultivars, when samples were collected at 100 DAS. The DM was highest at 100 DAS in all Kenaf silage. The CP levels of all silages were

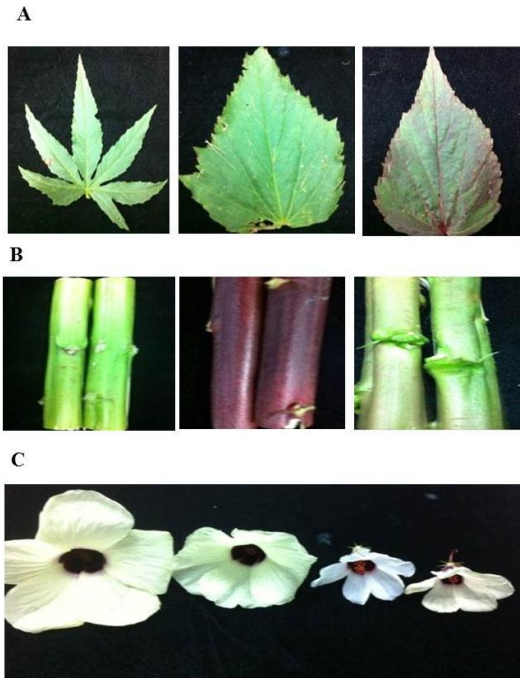


Fig. 1: Morphological characteristics of Kenaf cultivars used in this study. A: Leaf shape and color, B: Stem color, C: flower color

7.4–18.4%, and the value declined significantly as growth advanced; the highest values were observed at 60 DAS. The mid-late maturing and late-maturing cultivars had higher CP contents than did the early-maturing cultivars at 100 DAS. Interestingly, the NDF and ADF contents of the six cultivars did not differ significantly at any harvesting date. However, both levels increased significantly as harvesting was delayed. The highest protein DM yields were recorded at 100 DAS for all cultivars.

Fermentation Characteristics

The fermentation characteristics of the silage are shown in Table 3. Neither the organoleptic characteristics nor overall silage quality differed by cultivar. We found no

Table 2: Change of yield and chemical composition of the silage in the six Kenaf cultivars with different date of harvesting date

Cultivar	Harvest date (DAS*)	CP (%)	CF (%)	NDF (%)	ADF (%)	Protein yield (kg/ha)	Fresh yield (ton/ha)	Dry Yield (ton/ha)	Dry matter (%)
Jangdae	60	18.1a	26.2f	45.6c	32.5c	74.2l	20.5g	4.1h	20.0c
	80	15.0c	25.7f	44.9d	33.4c	81.0k	28.5e	5.4f	18.9d
	100	11.9d	29.2d	46.4c	34.2b	217.8b	76.5c	18.3c	23.9b
	120	9.1f	37.9b	45.2c	34.6a	202.0d	111.0b	22.2b	20.0c
Jinju	60	18.3a	26.7f	45.6c	35.2a	71.4l	19.4g	3.9h	20.1c
	80	15.2c	25.5f	44.8d	33.2c	76.0l	26.4f	5.0g	18.9
	100	11.4e	37.6b	46.7b	34.7b	224.6a	78.3c	19.7	25.2a
	120	8.0	36.5b	46.6b	35.4a	195.2d	122.0a	24.4a	20.0c
Auxu	60	18.4a	26.1f	45.4c	34.0b	77.3l	20.9g	4.2h	20.1c
	80	16.7b	26.3f	45.2c	33.4c	96.9j	29.5e	5.8f	19.7c
	100	10.9e	36.6b	45.7c	33.3c	207.1c	75.0c	19.0	25.3a
	120	8.6f	37.1b	45.5c	35.4a	196.1d	113.7b	22.8b	20.1c
Bakma	60	16.1b	28.5e	47.5b	35.6a	64.4m	20.5g	4.0h	19.5c
	80	14.6c	25.5f	43.8d	32.4c	84.7k	29.9e	5.8f	19.4c
	100	9.6f	36.1b	45.1c	33.3c	144.0e	75.0c	15.0e	20.0c
	120	8.3g	36.3b	47.6b	34.6a	133.6f	80.5c	16.1d	20.0c
Jukbong	60	18.3a	26.1f	42.5e	34.5b	76.9l	20.8g	4.2h	20.2c
	80	16.6b	26.1f	44.2d	34.2b	102.9i	32.6e	6.2f	19.0d
	100	9.0f	35.3b	48.3a	35.1a	123.3g	68.3d	13.7e	20.1c
	120	7.9g	37.6b	48.3a	34.6b	110.6h	75.0c	14.0e	18.7d
C14	60	14.9c	30.0d	47.2b	33.5c	56.6n	19.0g	3.8h	20.0c
	80	12.7d	32.5c	46.2c	33.2c	80.0k	33.3e	6.3f	18.9d
	100	9.3f	36.2b	46.6b	34.3b	136.7f	73.3c	14.7e	20.1c
	120	7.4h	39.2a	49.9a	34.6a	108.8h	73.3c	14.7e	20.1c

*DAS: Day after sowing, CP: Crude protein, CF: Crude fiber, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, a, b, c, d, e, f, g, h, i, j, k: Duncan's multiple range tests ($P<0.05$, n=3)

Table 3: Fermentation characteristics of the silage of six Kenaf cultivars with different date of harvesting

Cultivar	Harvest date (DAS*)	pH	Lactic acid (%)	Butyric acid (%)	Acetic acid (%)	Ammonia-N (%)
Jangdae	60	3.89d	2.00k	0.06d	0.11c	10.88g
	80	3.74b	2.54c	0.12b	0.20a	10.62d
	100	3.80b	2.93b	0.04d	0.08d	10.50d
	120	3.72b	2.80b	0.05d	0.12c	10.45c
Jinju	60	3.75b	2.54c	0.06d	0.12c	10.96h
	80	3.72b	2.34e	0.06d	0.12c	10.94h
	100	3.95d	2.29g	0.06d	0.12c	10.61d
	120	3.76b	2.28g	0.06d	0.12c	10.51d
Auxu	60	3.85c	2.57c	0.05d	0.11c	10.87g
	80	3.70b	3.18a	0.09c	0.23a	10.38c
	100	3.88d	3.19a	0.10b	0.22a	10.21b
	120	4.00f	3.20a	0.10b	0.21a	10.24b
Bakma	60	3.79b	2.11j	0.06d	0.11c	10.98i
	80	3.97e	2.67c	0.11b	0.23a	10.66e
	100	3.94d	2.62c	0.10b	0.20a	10.56d
	120	3.93d	2.61c	0.10b	0.19b	10.64e
Jukbong	60	3.80b	2.55c	0.12b	0.10c	10.73f
	80	3.43a	2.24h	0.12b	0.20a	9.95a
	100	3.93d	2.31f	0.16a	0.20a	9.78a
	120	3.67a	2.20i	0.18a	0.20a	10.07b
C14	60	3.87c	2.14j	0.05d	0.10c	10.89g
	80	3.55a	2.65c	0.10b	0.20a	10.73f
	100	3.93	2.52c	0.04d	0.10c	10.67e
	120	3.59a	2.42d	0.05d	0.20a	10.63d

*DAS: Day after sowing, a, b, c, d, e, f, g, h, i, j: Duncan's multiple range tests ($P<0.05$, n=3)

significant interaction between harvest date and cultivar. The pH of silage was not influenced by any interaction between cultivar and harvest date; all pH values were under 4.0 due to high lactic acid levels. Butyric acid concentrations were low in all cultivars at all harvesting times. The ammonia-N content all cultivars were about 9.95–10.98%. The lowest ammonia was recorded in the silage of all cultivars at 100 DAS.

Thus, the optimum harvesting time for silage production, in terms of yield, fermentation characteristics and nutritive value, was 100 DAS.

Table 4: Mineral composition of silage of various Kenaf cultivars at 100 DAS

Composition	Unit	Jangdae	Jinju	Auxu	Bakma	Jeokbong	C14
Ca	%	1.61a	1.41b	1.42b	1.22c	1.45b	1.56a
P	%	0.40a	0.38a	0.37a	0.40a	0.45a	0.39a
K	%	2.50a	2.48a	2.39a	1.98b	1.99b	1.72c
Mg	%	0.42a	0.33b	0.32b	0.29b	0.25c	0.29b
Na	ppm	175.02b	131.82d	185.57b	204.94a	164.54c	180.59b
Zn	ppm	25.74c	25.00c	28.12b	26.94c	29.98b	38.82a
Fe	ppm	207.10b	180.83d	206.50b	203.99c	210.17b	231.74a

[a, b, c] : Duncan's multiple range tests ($P<0.05$, n=3)

Mineral and Amino Acid Content

Silage mineral contents at the optimal harvest date (100 DAS) are shown in Table 4. Calcium and potassium were present at high levels. The Jangdae and C14 cultivars had the highest calcium contents. Silage from early-maturing cultivars contained less potassium than did silage from late-maturing and mid-late maturing cultivars. The phosphorus contents of silage from the six cultivars were similar. Jangdae silage had the highest magnesium content, and the C14 cultivar had the highest zinc and iron contents.

Concentrations of amino acid of hay and ensiled Kenaf were shown in Table 5. The total amino acid content in the hay was higher than silage from Jangdae cultivar at 100 DAS. The present observations show that contents of threonine, valine, phenylalanine, histidine and arginine were reduced in comparison with the hay from Jangdae cultivar at 100 DAS, however contents of aspartic acid, isoleucine, leucine, γ-Amino-n-butyric acid in the silage were induced. Thirty-two amino acids were identified at varying levels, of which seven (lysine, phenylalanine, leucine, isoleucine, methionine, valine, and threonine) were essential amino acid. Especially, the levels of valine, leucine, phenylalanine and lysine differed significantly between hay and silages. Of all essential amino acids, leucine attained the highest levels in silage (Supplement Fig. 1).

Table 5: Amino acid composition of hay and ensiled Kenaf (mg/kg)

Components	Hay	Silage
Phosphoserine	63.84	43.76
Taurine	15.36	9.85
Phospho ethanol amine	169.32	62.75
Urea	379.92	ND
Aspartic acid	91.08	195.58
Threonine	308.04	213.35
Serine	143.16	124.55
Glutamic acid	56.28	28.32
α-aminoadipic acid	10.08	49.9
Hydroxy proline	3.00	14.86
Proline	253.56	265.91
Glycine	43.20	121.24
Alanine	533.4	418.95
Citrulline	3.96	ND
α-amino-n-butyric acid	11.64	15.46
Valine	530.76	394.56
Cystine	27.00	123.13
Methionine	12.12	109.53
Cystathionine	27.00	7.63
Isoleucine	192.36	289.75
Leucine	172.80	659.35
Tyrosine	166.56	47.72
Phenylalanine	900.6	369.91
β-Alanine	33.24	17.45
β-Aminoisobutyric acid	4.44	21.80
γ-Amino-n-butyric acid	358.56	743.06
Ethanol amine	63.72	26.54
Hydroxylysine	0.96	1.46
Ornithine	ND	51.61
Lysine	62.76	126.64
Histidine	79.68	20.32
Arginine	369.48	ND
TAA*	5087.88	4574.94
TEAA**	2565.84	2056.77

*Total amino acid content, **Total essential amino acid content, ND: not detected

Identification of Fungi

We used universal primers to clone the ITS and 26S rDNA regions of microbes associated with Kenaf (Table 6 and Table 7). Yeasts were isolated from silages prepared from the six cultivars and identified to the species level; they were >97% similar in sequence to reference strains described by Barnett *et al.* (2000). Only one species of yeast (*Pichia fermentans*) was isolated. The length variations were ITS, 383–422 bp (Fig. 2), and 26S rDNA, 914–960 bp (Fig. 3). A BLAST search of the NCBI database revealed that the sequences were nearly identical (98–100%) to those of *P. fermentans* (KR089901 and GQ458040). Thus, *P. fermentans* was the dominant yeast in silage prepared from Kenaf harvested at the 100 DAS.

Discussion

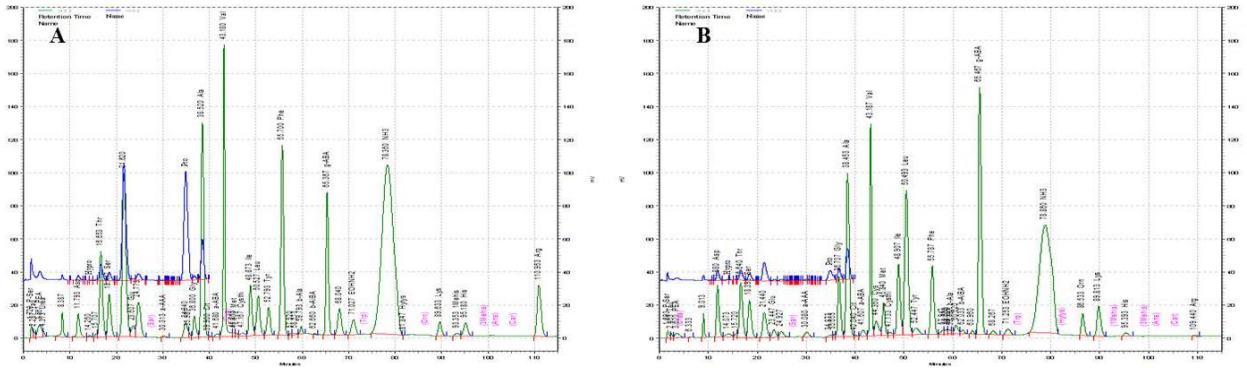
The DM yields of all six cultivars increased with growth duration. Jinju allowed the highest DM yields at 100 DAS. The yields were significantly lower than those recorded by Najid and Ismawaty (2001), who found that introduced Kenaf cultivars (Chingpi, Evergrades-41, and Tainung-2) DM yielded 21–28 ton·ha^{-1} when grown in Korea. Kang *et al.* (2004) found it impossible to harvest seed from late-maturing cultivars grown in Korea, such as Chingpi, Evergrades-41, and Tainung-2. The Jinju cultivar matures late, increasing the risk of seek shattering and reducing seed quality. Jangdae, which afforded both a high biomass and a high seed yield, has been tested by the Korea Seed and Verity Service (KSVS) for 3 years under Korea environment (Grant No. 4560).

Table 6: The length of ITS region and GenBank accession numbers of the sequences from Kenaf silage and its related species

Cultivar name	Cloned sequence		Blast analysis			
	Contig length (bp)	GenBank number	GenBank number	Coverage	Identity (%)	Description
Jangdae	383	KU820958	KR089901	100	99	*Pichia fermentans*
Jinju	411	KU820960	KR089901	100	100	strain ATCC 10651
Auxu	422	KU820955	KR089901	100	99	
Baekma	411	KU820966	KR089901	100	100	
Jeokbong	409	KU820959	KR089901	100	100	
C14	414	KU820957	KR089901	100	100	

Table 7: The length of 26S rDNA region and GenBank accession numbers of the sequences from Kenaf silage and its related species

Cultivar name	Cloned sequence		Blast analysis			
	Contig length (bp)	GenBank number	GenBank number	Coverage	Identity (%)	Description
Jangdae	935	KU820952	GQ458040	100	99	*Pichia fermentans*
Jinju	925	KU820954	GQ458040	100	99	strain YH003
Auxu	944	KU820951	GQ458040	100	99	
Baekma	914	KU820949	GQ458040	100	99	
Jeokbong	928	KU820953	GQ458040	99	98	
C14	926	KU820950	GQ458040	100	99	



Supplement Fig. 1: Amino acids chromatogram of hay and ensiled Kenaf from Jangdae cultivar. A: Hay, B: silgae

The rate of DM observed in this study was significantly lower than the result from corn silage reported by Do *et al.* (2012). Despite the low rate of DM of the Kenaf, the low concentrations of butyric acid in Kenaf silage seem to be resulted from the low pH of Kenaf silage.

The chemical compositions of Kenaf silage and alfalfa hay are similar in terms of CP, ADF and NDF (National Research Council, 2001). Chantiratikul *et al.* (2009) found that the CP content (14.34–6.58%) of Kenaf decreased significantly ($p < 0.05$) as harvesting was delayed, but the NDF (41.99–48.74%) and ADF (27.20–30.57%) levels increased ($p < 0.05$). The CP contents observed in Kenaf silage were much higher than the good quality of corn silage. However, higher forage protein can become more soluble and can be catabolized to non-protein nitrogen (NPN) which is resulted in higher ammonia-N content (Muck, 1987). Normally, upon maturation, the lower leaves succumb to senescence, decreasing the proportion of leaves and elevating that of stems (Webber, 1993). Plant maturity is the most important mediator of forage quality, which is associated with NDF and ADF levels. Usually, forage plants mature so rapidly that significant declines in forage quality must be carefully avoided. However, in the present study, neither the NDF nor ADF level of any Kenaf cultivar increased significantly upon maturation. Similarly, Kang *et al.* (2004) grew introduced Kenaf cultivars (Tainung, Everglade, and Dowling) in Korea and found that neither the NDF nor ADF content increased significantly with maturity. Several studies have found that the initial pH of Kenaf silage is low (Xiccato *et al.*, 1998). Silage of high moisture content often produces a high level of butyric acid. Gaspari (1990) reported that Kenaf of high moisture content (DM <200 g kg^{-1}) harvested at early growth stages was not suitable for ensiling. The Kenaf silage showing high concentrations of ammonia-N were observed in all cultivars at each harvest stage. Ammonia-N content of alfalfa silages reported by Tremblay *et al.* (2001) was similar to our result. The protein degradation can be evaluated by level of ammonia-N concentration in silage (Muck, 1988). Protein degradation resulted in large induction of ammonia-N

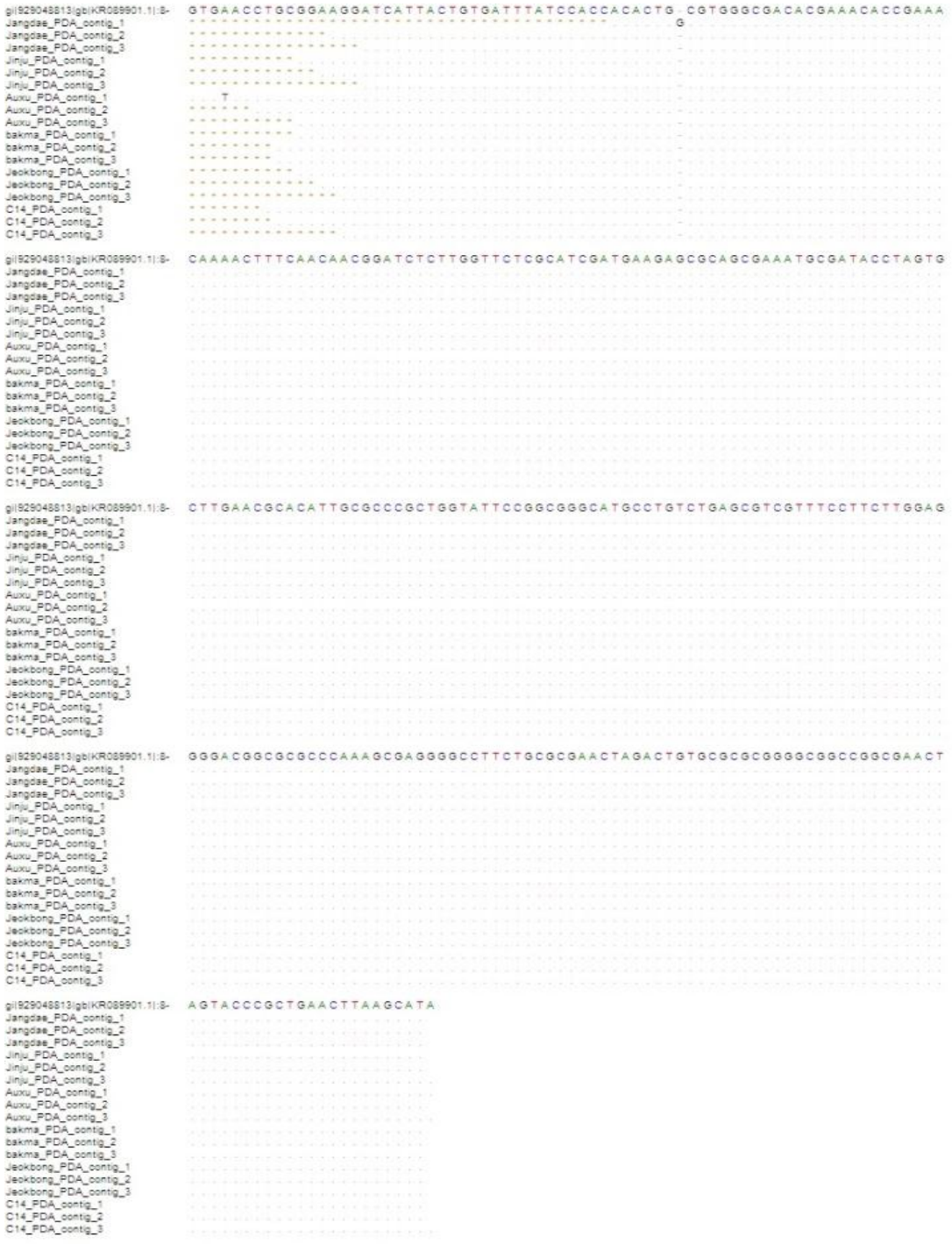


Fig. 2: Multiple alignment of the ITS sequences of the Kenaf silage fungi produced from PDA medium

concentration in the silage. Proteolysis is a major factor in reducing silage protein utilization and efforts to reduce proteolysis should enhance silage protein utilization. Xiccato *et al.* (1998) reported that the addition of beet pulp onto fermentation process slightly decreased ammonia–N concentration in Kenaf silage.

Kenaf silage contained high levels of calcium and potassium. The calcium contents in Kenaf silage were much higher than the alfalfa silage, and the potassium contents of alfalfa silages were similar to values reported by Kume *et al.* (2001). Alexopoulou *et al.* (2013) found that addition of Kenaf leaves to other feed improved the overall calcium content. Calcium plays a key role in supporting cell walls and is therefore found principally in fibrous fractions.

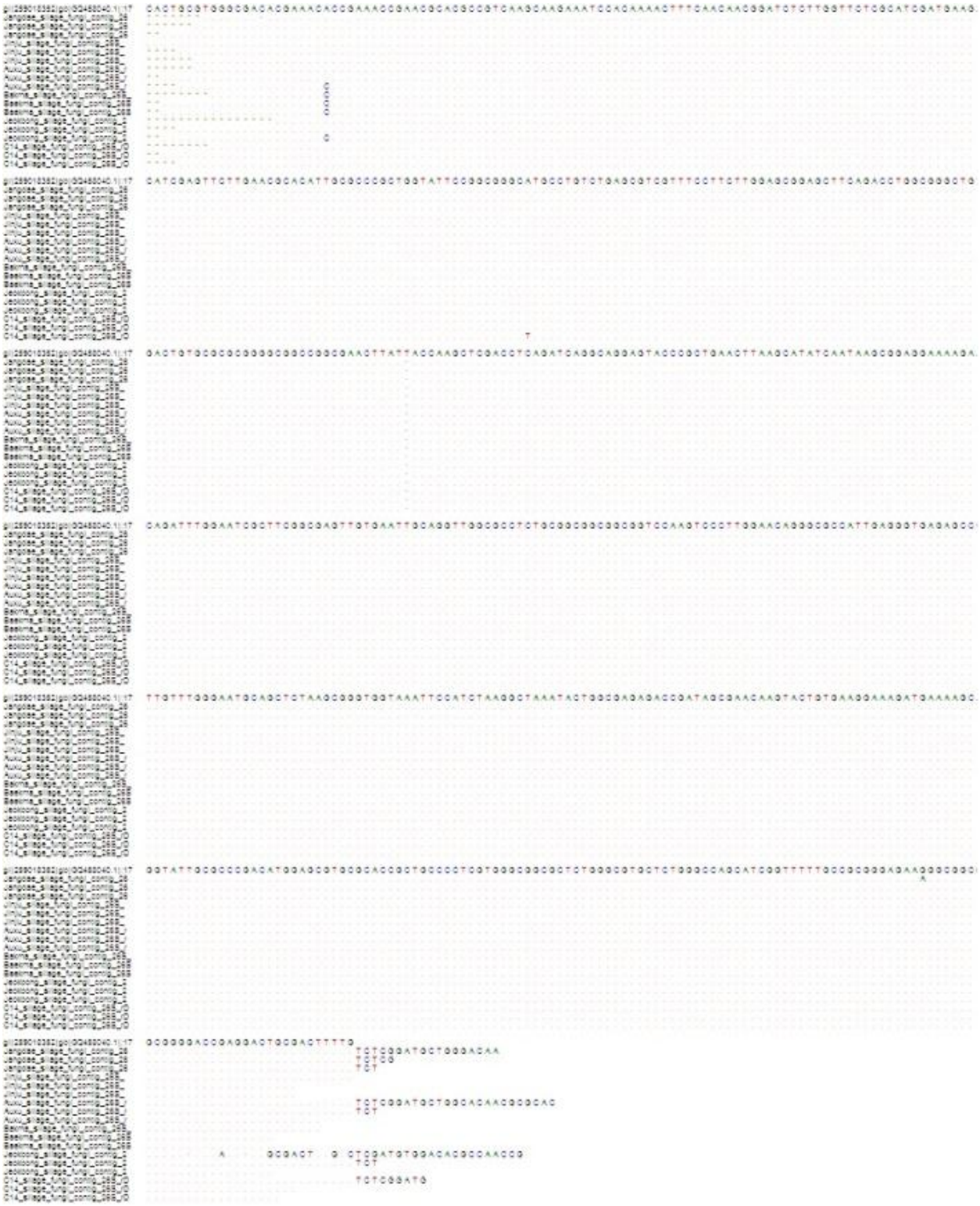


Fig. 3: Multiple alignment of the 26S rDNA sequences of the Kenaf silage fungi produced from EC medium

This study measured the amino acid compositions of hay and silages prepared from Kenaf harvested at 100 DAS. Results show that concentrations of amino acid in the silage were lower than that in the hay. The similar findings that amino acid breakdown of alfalfa silages have been reported by Guo *et al.* (2008) and Wang *et al.* (2009). Purwin *et al.*

(2014) found that the total amino acid content/100 g protein was affected by both the ensiling process and the plant species used (both p-values <0.05). The quality of red clover protein was affected by ensiling, as the levels of all essential amino acids except Cys and Met were reduced. Arrigo (2006) showed that the levels of amino acids in roughage fell during preservation and storage, and herbage conservation reduced these levels (Coblentz *et al.*, 1998). The amino acid profile attained during ensiling depends on the proteolytic potential of the ensiled plant (as reflected by protease and polyphenol oxidase activities), the tannin content, and the rates of wilting and acidification during ensiling. Do *et al.* (2012) found that the total amino acid content of corn silage prepared in Korea ranged from 3,831.01 to 4,480.77 mg/100 g protein, and leucine was the principal free amino acid.

P. fermentans was the dominant yeast in silage from all tested Kenaf cultivars; this is a novel observation. The nucleotide sequences of the twelve ITS and 26S rDNA (Accession number KU820949 to KU820960) were registered in the GenBank database (Table 6, Table 7). *P. fermentans* occurred on bales harvested from forage of a lower DM content later in the summer, stored on their ends (O'Brien *et al.*, 2007). In addition, *P. fermentans* numerically increased in colony numbers from November to February and produces mainly yeast-like cells on media containing millimolar concentrations of urea and diammonium phosphate (O'Brien *et al.*, 2007; Sanna *et al.*, 2012). Similarly, silages prepared from sugarcane, maize, alfalfa, and white clover in various regions contained the lactate-assimilating *P. fermentans* (Rossi and Dellaglio, 2007; Avila *et al.*, 2010). Such low-level yeast biodiversity suggests that the above plants and Kenaf undergo similar fermentation reactions upon ensiling and that the yeasts assimilate related sources of carbon and nitrogen (Avila *et al*., 2010). Yeasts are undesirable during ensiling, and their biochemical and physiological characterisation will be important to identify microbial metabolites affecting silage quality.

Conclusion

The obtained results establish the optimum harvest date depending on the yield and nutritive value for silage from novel Kenaf cultivars, and their amino acid composition and identification of microorganisms at optimal harvest date. It was presumed that an optimal harvest time for Kenaf silage production should be at 100 DAS in Korean. The CP contents observed in Kenaf silage were much higher than other crop silage. However, the Kenaf silage showed high concentrations of ammonia-N and low DM were observed in all cultivars at each harvest stage. These results suggest that Kenaf silage may require development of additive agents which can enhance fermentation efficiency. Less knowledge has been gained regarding the amino acid composition of Kenaf silage. Our study demonstrated abundant amino acid (leucine, isoleucine, valine, and phenylalanine) in the Kenaf silage at optimal harvest date. This study is the first to identify the yeast species *P. fermentans* as a dominant species on silage of Kenaf.

Acknowledgement

This work was supported by a grant from the Korea Atomic Energy Research Institute (KAERI), Republic of Korea.

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(Received 09 April 2016; Accepted 08 August 2016)