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ORIGINAL ARTICLE

Protein enrichment, cellulase production and *in vitro* digestion improvement of pangolagrass with solid state fermentation

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Background and Purpose: Pangolagrass, *Digitaria decumbens* Stent, is a major grass for cow feeding, and may be a good substrate for protein enrichment. To improve the quality of pangolagrass for animal feeding, cellulolytic microbes were isolated from various sources and cultivated with solid state fermentation to enhance the protein content, cellulase production and *in vitro* digestion. The microbes, culture conditions and culture media were studied.
Methods: Cellulolytic microbes were isolated from pangolagrass and its extracts, and composts. Pangolagrass supplemented with nitrogen and minerals was used to cultivate the cellulolytic microbes with solid state fermentation. The optimal conditions for protein enrichment and cellulase activity were pangolagrass substrate at initial moisture 65–70%, initial pH 6.0–8.0, supplementation with 2.5% $(NH_4)_2SO_4$, 2.5% KH_2PO_4 and K_2HPO_4 mixture (2:1, w/w) and 0.3% $MgSO_4 7H_2O$ and cultivated at 30°C for 6 days.
Results: The protein content of fermented pangolagrass increased from 5.97–6.28% to 7.09–16.96% and the *in vitro* digestion improved from 4.11–4.38% to 6.08–19.89% with the inoculation of cellulolytic microbes by solid state fermentation. Each 1 g of dried substrate yielded Avicelase 0.93–3.76 U, carboxymethylcellulase 1.39–4.98 U and β-glucosidase 1.20–6.01 U. The isolate *Myceliophthora lutea* CL3 was the strain found to be the best at improving the quality of pangolagrass for animal feeding with solid state fermentation.
Conclusion: Solid state fermentation of pangolagrass inoculated with appropriate microbes is a feasible process to enrich protein content, increase *in vitro* digestibility and improve the quality for animal feeding.

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Introduction

The protein demands for direct human consumption and animal feeding will inevitably increase for the improvement of human living standards.[1] In 2010, Taiwan imported 2.41×10^6 tons of soy beans and 4.52×10^6 tons of corn from other countries, which cost 2.21×10^9 US dollars.[2] Consequently, it is urgent to develop local protein resources with renewable raw materials for animal feed. The substrates traditionally used in solid state fermentation for the production of foodstuffs are rice, wheat, millet, barley, maize and soybean,[3–5] and for the production of feedstuffs are agricultural wastes and crop residues.[5–10] Pangolagrass, *Digitaria decumbens* Stent, is a major grass for cow feeding, and may be a good substrate for protein enrichment. The yield of pangolagrass, *Digitaria decumbens* Stent, was between 50.48 and 80.80 ton/ha, and the cultivation area ranged from 3,113 to 4,991 ha during 1999 to 2009 in Taiwan.[2] The annual production was $2.13–3.86 \times 10^5$ tons.

In order to be economically competitive, the bioconversion of pangolagrass into protein and cellulase production should be performed at the rural level. Solid state fermentation can achieve this purpose by reducing the cost of growing microorganisms, improving the *in vitro* digestibility, and increasing the protein and fat contents of cellulosic materials.[3,11–20] In previous studies, the protein content of sweet potato residue has been enriched from an initial value of 6% protein to 32% protein within 3 days by amylolytic yeasts, and co-cultures of amylolytic fungi,[7,12] and protein content of sugar beet pulp and corncob has been enriched from 2.4% to 21.6% over 4 days by cellulolytic fungi.[6,11] In this article we report our studies on the possibilities to enrich protein content, produce cellulase and improve *in vitro* digestion of pangolagrass by solid state fermentation process with cellulolytic microbes.

Materials and methods

Pangolagrass

Pangolagrass, *Digitaria decumbens* Stent, is a major forage grass in Taiwan. The fresh pangolagrass contained moisture 70.87–78.10%, crude fiber 33.41–35.40%, ether extract 2.69–3.39%, crude protein 5.29–6.90% and ash 5.93–6.92%. After harvest, the pangolagrass was sun dried and stored at room temperature for further study.

Test organisms

Test organisms included 17 isolates and *Myceliophthora lutea* CL3 from pangolagrass and its extracts, 88 isolates from composts and 62 isolates from our laboratory stock cultures. *Streptomyces thermonitrificans* NTU-88 isolated from compost[17,21] and *Trichoderma reesei* QM9414 purchased from Bioresources Collection and Research Center of Food Research and Development Institute, Hsin-Chu, Taiwan, were used as reference strains.

Culture media and conditions

Cellulolytic microbes were isolated on Mandels-Reese medium with carboxymethylcellulose (CMC) as the sole carbon source.[22] Submerged cultivation medium contained (per liter): Avicel, 10.0 g; peptone, 1.0 g; yeast extract, 2.0 g; KH_2PO_4, 1.5 g; K_2HPO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; Tween 80, 2 mL; $FeSO_4 7H_2O$, 0.005 g; $ZnSO_4 7H_2O$, 0.014 g; $MnSO_4 4H_2O$, 0.016 g; and $CoCl_2 6H_2O$, 0.002 g at pH 6.0±0.3, and used for cellulolytic enzyme production and optimization of cultivation conditions.

Solid state fermentation

The basal solid medium comprised pangolagrass, 100 g; nitrogen source [$(NH_4)_2SO_4$, 6 g; urea, 0.4 g; peptone, 0.8 g]; K_2HPO_4, 1.2 g; and KH_2PO_4, 2.5 g at pH 6.8 ± 0.3, and used for protein enrichment, cellulolytic enzyme production and *in vitro* digestion determination. The medium was mixed thoroughly with spores or cells (10^7 spores or cells/mL) that were washed with 5 mL of 0.05% Tween-80 in sterilized water, and incubated in a static flask (the thickness of the medium was about 2 cm) at 30°C for 5–29 days, stirring once a day.[23]

Cellulase activity

The carboxymethylcellulase (CMCase) activity of the culture broth, or culture extract of solid sample with 5× volume of distilled water and shaken for 30 minutes, was determined by the dinitrosalicyclic (DNS) method. The CMC (dissolved in 0.1 M pH 7 phosphate buffer) 1 mL and enzyme extract 1 mL were reacted at 60°C for 60 minutes, and 2 mL of DNS reagent (dinitrosalicyclic acid 1 g and tartarate 30 g were dissolved in 20 mL of 2 N NaOH and then diluted with distilled water to 100 mL) was added and the mixture was boiled for 5 minutes. After cooling, the reaction mixture was diluted with 16 mL of distilled water, and the absorbance at 550 nm was measured. One unit of CMCase activity was defined as the amount of enzyme causing the release of 1 μmol of glucose in 1 minute under the assay conditions.[24] For exoglucanase (Avicelase) and β-glucosidase activities, the enzyme assay substrate was replaced by 2% of Avicel, and 2% of Salicin, respectively.

In vitro digestibility

Sample powder 1 g was suspended in 14 mL of 0.05 M sodium acetate buffer (pH 5.5) with xylanase and cellulase, then added with 54.5 mg of pepsin in 2.6 mL of sodium acetate buffer, 1.4 mL of 1 M HCl at pH 3.0, and 48.1 mg of pancreatin in 1 M $NaHCO_3$ at pH 6.5. After digestion, the mixture was centrifuged at 16,000 rpm with a Sigma 3K20 rotor No. 9137 for 10 minutes. The weight loss during the treatment represents the *in vitro* digestibility.[25]

Chemical analysis

The pH value was measured directly or in 5× volume of distilled water with a pH meter (Good digital pH meter,

model 2002, Taiwan). Moisture content was determined by drying a sample at 105°C for 24 hours to a constant mass. Ash content was measured with an air-dried sample by heating at 550—600°C for 24 hours. Total organic carbon was determined as following: sample powder 0.3 g, 1 N $K_2Cr_2O_7$ 10 mL, and concentrated H_2SO_4 20 mL were mixed thoroughly and stood still for 30 minutes. Distilled water 200 mL and 85% H_3PO_4 10 mL were added. After cooling, diphenylamine 1 mL was added as indicator and the reaction mixture was titrated with 0.5 N of ferrous (II) ammonium sulfate.[26] Soluble nitrogen was extracted with 5× volume of distilled water and shaken for 20 minutes. Soluble and total nitrogen was determined by the modified Kjeldahl method,[27] and protein content was calculated by 6.25× the difference between total nitrogen and soluble nitrogen contents of sample.

Results

Isolation of cellulolytic microbes

Cellulolytic activity of 168 isolates (including 88 phosphate-solubilizing microbes, 18 cellulolytic microbes, 62 laboratory stock cultures) were assayed on Mandels-Reese medium with CMC as the sole carbon source and *Trichoderma reesei* QM9414 was used as a reference strain. It showed that 18 isolates (including 12 bacteria, one actinomycete, and five fungi) had a halo ratio (clear zone/colony size) of 2.7—7.0, 24 isolates (nine bacteria, eight actinomycetes and seven fungi) had a halo ratio of 1.8—2.6, 27 isolates (four bacteria, six actinomycetes and 17 fungi) had a halo ratio of 1.3—1.7, 27 isolates (three bacteria, eight actinomycetes and 16 fungi) had a halo ratio of 1.0-1.2, and 32 isolates could not grow on Mandels-Reese medium. Twenty-six isolates with a high halo ratio and high growth rate were chosen for further studies.

The effect of pH on the growth of 26 tested isolates in Mandels-Reese medium with pangolagrass powder as the sole carbon source was not significant, except with *T. reesei* QM9414 which decreased growth with increasing pH. Isolate SC 57 had the largest colony size (6.48—7.18 cm for 4 days' incubation), then followed by isolates SC 2 (3.10—3.43 cm), SC 73 (3.18—3.33 cm), SC 27 (1.88—2.60 cm), *Myceliophthora lutea* CL 3 (1.95—2.30 cm) and *M. lutea* CL 3-2 (1.78—2.35 cm). While the colony sizes of *S. thermonitrificans* NTU-88 and *T. reesei* QM9414 were only in the ranges of 0.98—1.10 and 0.45-0.80 cm, respectively. All the tested isolates had colony sizes higher than that of *T. reesei* QM9414. The optimal pH of pangolagrass solid substrate medium for microbial growth was between pH 6.3 and 7.8. Therefore, it was not necessary to adjust the pH of pangolagrass solid substrate medium for fermentation.

Effect of sterilization on protein enrichment of pangolagrass

Pangolagrass solid medium was supplemented with $(NH_4)_2SO_4$ to achieve a carbon/nitrogen (C/N) ratio of 20, adjusted initial moisture content 60—63% and incubated at 30°C for 29 days. Substrate pHs increased during cultivation, and nonsterilization medium had a higher pH than sterilization medium (Fig. 1). Moisture contents decreased slightly during the cultivation of sterilization medium, while they increased in nonsterilization medium. Total organic carbon contents and C/N ratios decreased in sterilization medium were larger than those in non-sterilization one. However, ash contents increasing in sterilization medium were higher than those in non-sterilization treatment. Sterilization treatment improved the degradation of solid substrate and stimulated the bioconversion of pangolagrass. Protein enrichment was the highest with the inoculation of *M. lutea* CL 3 (18.89%), followed by isolate SC 2 (16.85%) and *S. thermonitrificans* NTU-88 (11.20%). Blank without inoculation gave the least protein (6.35%). Isolates *M. lutea* CL 3 and SC 2 had high organic carbon conversion, ash and protein contents, and they had low C/N ratios.

After 29 days' incubation, sterilization of pangolagrass gave a lower pH, moisture content, total organic carbon and C/N ratio than did nonsterilization medium, while protein and ash contents were reversed. Sterilization enhanced the growth of the tested organisms and increased the protein content of fermented pangolagrass.

Effect of microbial inoculation on protein enrichment, cellulase activity and *in vitro* digestion

The initial pangolagrass solid substrate had protein 6.48 ± 0.11%, Avicelase 0.54 ± 0.14 U/g, CMCase 0.59 ± 0.23 U/g, β-glucosidase 0.64 ± 0.13 U/g, and *in vitro* digestion 4.02 ± 0.41%. However, sterilization destroyed the enzyme activity. The blank without inoculation had Avicelase, CMCase and β-glucosidase 0.01 U/g only after 18 days of cultivation. Sterilized pangolagrass medium with the inoculation of tested organisms had higher protein content, Avicelase, CMCase and β-glucosidase activities, and *in vitro* digestion than those of nonsterilized pangolagrass medium except the blank without inoculation (Table 1). After 18 days' incubation, the sterilized pangolagrass substrate with inoculation of tested organisms had protein content 9.02—15.81%, Avicelase 1.72—2.16 U/g, CMCase 1.66—3.03 U/g, β-glucosidase 1.33—2.33 U/g, and *in vitro* digestion 16.31—24.81%. While in the nonsterilized pangolagrass substrate with inoculation had only protein content 8.02—11.63%, Avicelase 0.95—1.46 U/g, CMCase 1.02—1.52 U/g, β-glucosidase 0.98—1.24 U/g, and *in vitro* digestion 15.55—21.05%. Isolate *M. lutea* CL3, isolate SC 2 and *S. thermonitrificans* NTU-88 had higher protein content, cellulase activity and *in vitro* digestion than those of *Aspergillus niger*, *Mucor hiemalis* and isolate SC 99.

Protein enrichment, cellulase activity and *in vitro* digestion of pangolagrass with solid state fermentation

Protein enrichment, cellulase activity and *in vitro* digestion of pangolagrass with solid state fermentation by different tested organisms are demonstrated in Table 2. When the pangolagrass was supplemented with 2.5% $(NH_4)_2SO_4$ as a nitrogen source and 2.5% of the mixture of KH_2PO_4 and K_2HPO_4 (2:1, w/w) as phosphate source at initial moisture content 68—72% and initial pH 6.8—7.0, the substrate pH

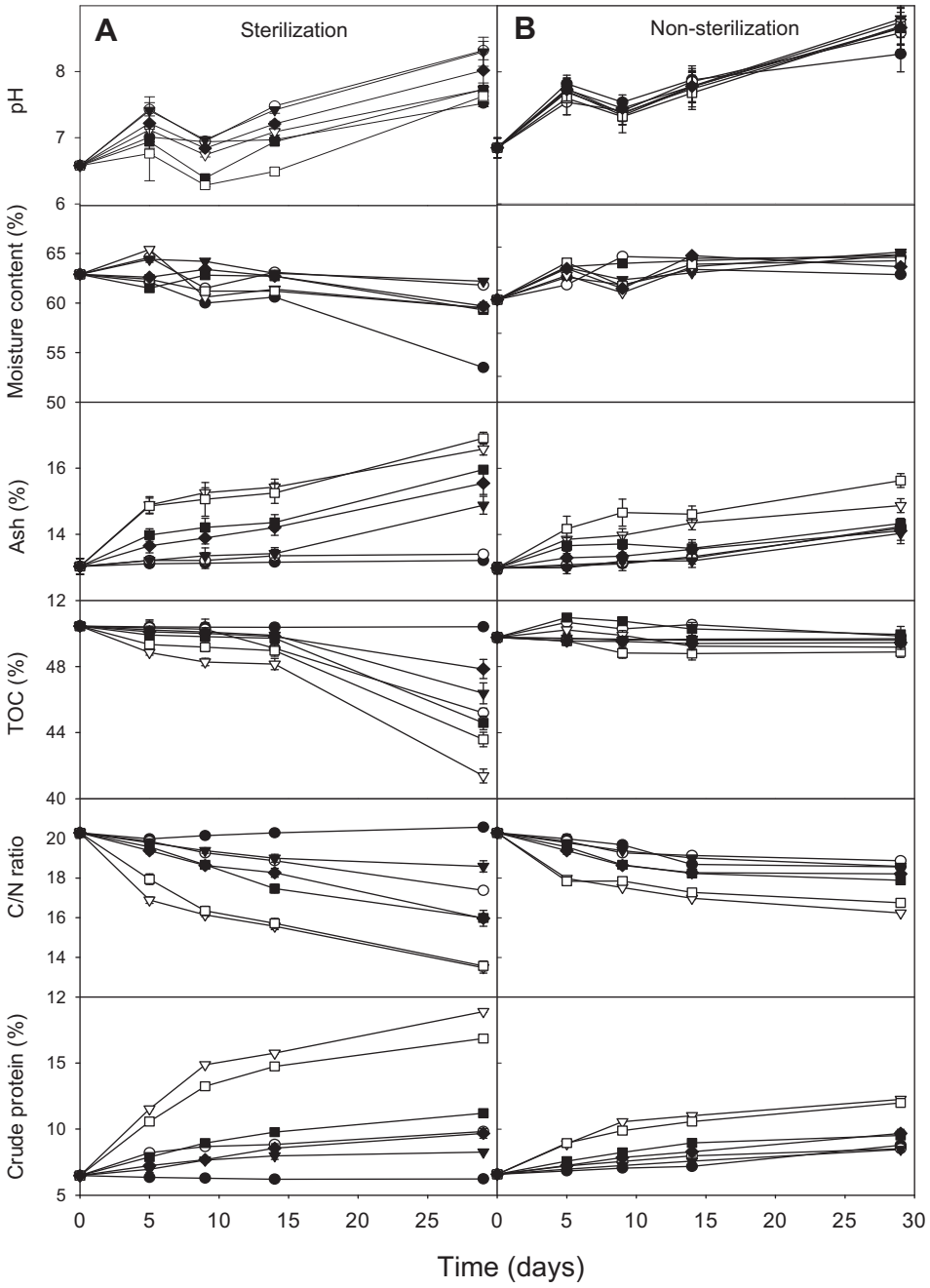

Figure 1. Effect of sterilization on physical and chemical properties of pangolagrass solid substrate. (A) Sterilization; (B) Nonsterilization. ●: Blank, ○: *Aspergillus niger*, ▼: *Mucor hiemalis* IFO 2216, ▽: *Myceliophthora lutea* CL3, ■: *Streptomyces thermonitrificans* NTU-88, □: Isolate SC2, ◆: Isolate SC99. C/N = carbon/nitrogen; TOC = total organic carbon.

values were between 6.05 and 7.65, and moisture contents increased from 69.75% to 71.08—74.67% after 6 days of fermentation at 30°C. Bacterial isolates showed the highest rise in moisture and ash contents, followed by fungal isolates, and the actinomycete isolates had the lowest. Total organic carbon decreased slightly from 45.72% to 42.49—45.54%, and C/N ratio decreased from 14.97 to 13.01—14.80; while ash content increased from 13.50% to 14.15—16.01%. The decrease in total organic carbon and the increase in ash content during the cultivation are as a result of carbon metabolism and the release of carbon dioxide; while the increase in moisture content is because of metabolic water production of tested microbes.

Each 1 g of pangolagrass substrate produced Avicelase 0.93—3.76 U, CMCase 1.39—4.98 U, and β-glucosidase 1.20—6.01 U. *M. lutea* CL 3, isolates CL 5, CH 23, SC 27, SC 2 and SC 73 had high Avicelase activities; *S. thermonitrificans* NTU-88, isolates SC 73, *M. lutea* CL 3, SC 27, SC 57 and CL 5 had high CMCase activities; and isolates SC 50, SC 27, SC 2, SC 73, *M. lutea* CL 3, and M6-2 had high β-glucosidase activities. Protein content increased from 5.88% to 7.09—16.96%, and *in vitro* digestion enhanced from 4.12% to 6.08—19.89%.

Table 1 Effect of sterilization on protein enrichment, cellulase activity and *in vitro* digestion of pangolagrass

Tested organisms	Crude protein (%)	Avicelase (U/g)	CMCase (U/g)	β-glucosidase (U/g)	*In vitro* digestion (%)
(a) Sterilization					
Blank	6.50 ± 0.09	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	4.02 ± 0.35
Aspergillus niger	9.33 ± 0.08	1.72 ± 0.05	2.54 ± 0.23	1.36 ± 0.12	19.65 ± 0.14
Mucor hiemalis	9.02 ± 0.24	2.02 ± 0.01	3.03 ± 0.11	1.45 ± 0.10	16.31 ± 0.41
Streptomyces thermonitrificans					
NTU-88	10.49 ± 0.19	1.74 ± 0.01	2.83 ± 0.12	2.32 ± 0.12	24.81 ± 0.42
Myceliophthora lutea					
Isolate CL 3	13.63 ± 0.02	2.03 ± 0.02	2.69 ± 0.03	1.77 ± 0.03	21.40 ± 0.20
Isolate SC 2	15.81 ± 0.25	2.16 ± 0.01	2.69 ± 0.24	2.33 ± 0.24	20.91 ± 0.67
Isolate SC 99	9.12 ± 0.05	1.91 ± 0.13	1.66 ± 0.29	1.33 ± 0.13	20.20 ± 0.73
(b) Non-sterilization					
Blank	7.99 ± 0.07	1.43 ± 0.24	1.50 ± 0.13	1.16 ± 0.01	7.13 ± 0.23
A. niger	8.25 ± 0.08	1.35 ± 0.01	1.40 ± 0.24	1.03 ± 0.01	16.30 ± 0.32
Mucor hiemalis	8.02 ± 0.16	0.95 ± 0.01	1.35 ± 0.12	1.15 ± 0.12	15.55 ± 0.51
S. thermonitrificans					
NTU-88	9.24 ± 0.15	1.46 ± 0.01	1.02 ± 0.11	0.98 ± 0.10	21.05 ± 0.52
Myceliophthora lutea					
Isolate CL 3	11.63 ± 0.02	1.40 ± 0.03	1.42 ± 0.03	1.11 ± 0.03	18.85 ± 0.29
Isolate SC 2	11.29 ± 0.19	1.35 ± 0.01	1.34 ± 0.13	1.02 ± 0.22	15.82 ± 0.65
Isolate SC 99	8.99 ± 0.13	1.45 ± 0.13	1.52 ± 0.01	1.24 ± 0.13	18.80 ± 0.68

The solid substrate was pangolagrass at initial moisture content 60—63%, initial pH 6.5—6.6 and cultivated at 30°C for 18 days. Means ± SD (n = 3).
CMCase = carboxymethylcellulase.

Isolates *M. lutea* CL 3, SC 27, SC 2, SC 73, JP 3 and SC 62 had high protein enrichment. Protein enrichment was high in fungal isolates (3.79—10.84%), followed by actinomycete isolates (3.50—5.10%), and bacterial isolates was the least (1.01—1.54%). *S. thermonitrificans* NTU-88, isolates *M. lutea* CL 3, CL 5, SC 73, SC 27 and SC 2 had high *in vitro* digestion. From the protein enrichment, cellulase activity and *in vitro* digestion of pangolagrass with the inoculation of tested isolates, isolate *M. lutea* CL3 had the highest protein content (16.96%) and Avicelase activity (3.76 U/g), the second highest *in vitro* digestion (17.82%), the third highest CMCase activity (4.40 U/g), and appropriate β-glucosidase activity (4.13 U/g). Therefore, isolate *M. lutea* CL3 could be used for protein enrichment and *in vitro* digestion improvement of fermented pangolagrass for animal feed.

Discussion

Solid state fermentations are distinguished from submerged cultures by the fact that microbial growth and product formation occur at or near the surfaces of solid materials with low moisture content.[1,4,6,7] Because of the low moisture content of the substrate in solid state fermentation, the microbes used in solid state fermentation were limited to a large number of filamentous fungi, some yeasts, some actinomycetes and few bacteria.[7] Protein enrichment and *in vitro* digestion improvement of fermented pangolagrass in this study was also high with the inoculation of fungal isolates, followed by actinomycete isolates, and bacterial isolates was the least.

Substrate moisture contents decreased in the blank without inoculation because of evaporation, while the moisture contents with the inoculation of tested microbes increased with the production of metabolic water. The same phenomena were observed in protein enrichments, enzymes, antibiotics and polyunsaturated fatty acid productions by solid state fermentation of starchy and cellulosic materials.[6—8,11,12,23,28—37] During cultivation, total organic carbon and C/N ratio decreased, while protein and ash contents increased as a result of the degradation of substrate carbon, release of carbon dioxide and bioconversion of pangolagrass to the biomass. Similar results were found in the protein enrichments of sweet potato residue and corncob, the preparations of biofertilizer and compost with the inoculation of appropriate microbes.[7,8,11,14,18,21,38—44]

Sterilization treatment may cleave the lignocellulose structure, improve the degradation of solid substrate, stimulate the microbial growth, enhance the bioconversion of pangolagrass, and increase the protein content of pangolagrass. Sterilization also increased the cellulolytic enzyme activities and *in vitro* digestion of fermented pangolagrass. The same phenomena were reported by Yang and Cheng in protein enrichment of corncob[11] and by Singh and Hayashi in cellulase production.[45] Wen et al[46] also showed that homogenized treatment reduced the length of fiber size and increased the cellulase production.

Pangolagrass has crude protein 5.29—6.90%, crude fiber 33.41—35.40%, and *in vitro* digestion 4.02—4.12% and is not a good source of protein for animal feeding. However, a solid state fermentation process with cellulolytic

Table 2 Protein enrichment, cellulase activity and *in vitro* digestion of pangolagrass with solid state fermentation

Tested	pH	Moisture content (%)	Total organic carbon (%)	Ash content (%)	C/N ratio	Protein content (%)	Avicelase (U/g)	CMCase (U/g)	β-glucosidase (U/g) (%)	*in vitro* digestion
Blank	6.83 ± 0.17	69.75 ± 0.12	45.72 ± 0.21	13.50 ± 0.29	14.97 ± 0.08	5.88 ± 0.05	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	4.12 ± 0.14
M. elongta [CL11]	7.36 ± 0.13	72.23 ± 0.36	44.08 ± 0.28	15.03 ± 0.15	14.56 ± 0.37	9.89 ± 0.19	0.93 ± 0.19	1.39 ± 0.20	1.20 ± 0.01	7.26 ± 0.14
Streptomyces thermonitrifican NTU-88	7.30 ± 0.07	71.76 ± 0.51	43.09 ± 0.28	15.84 ± 0.24	14.27 ± 0.39	11.09 ± 0.23	2.60 ± 0.05	4.98 ± 0.24	2.61 ± 0.21	19.89 ± 0.51
Streptomyces vendargensis	6.45 ± 0.18	71.74 ± 0.23	43.98 ± 0.28	14.96 ± 0.28	14.39 ± 0.24	9.78 ± 0.06	1.91 ± 0.11	2.00 ± 0.08	1.37 ± 0.14	10.08 ± 0.13
Isolate CH 23	7.15 ± 0.12	73.12 ± 0.19	44.06 ± 0.28	15.06 ± 0.24	14.65 ± 0.12	10.03 ± 0.11	2.97 ± 0.11	3.32 ± 0.01	2.63 ± 0.09	14.52 ± 0.04
Isolate CL 3	6.74 ± 0.21	74.67 ± 0.14	43.12 ± 0.30	15.13 ± 0.26	13.01 ± 0.15	16.96 ± 0.21	3.76 ± 0.14	4.40 ± 0.19	4.13 ± 0.13	17.82 ± 0.08
Isolate CL 5	6.59 ± 0.11	72.46 ± 0.14	43.59 ± 0.28	14.89 ± 0.14	14.42 ± 0.43	10.29 ± 0.08	2.98 ± 0.23	3.06 ± 0.21	1.95 ± 0.14	16.82 ± 0.12
Isolate JP 3	6.98 ± 0.17	73.26 ± 0.21	43.19 ± 0.32	15.35 ± 0.28	14.04 ± 0.42	12.85 ± 0.07	1.33 ± 0.21	1.86 ± 0.20	2.59 ± 0.11	8.79 ± 0.19
Isolate M 6-2	6.88 ± 0.18	73.13 ± 0.14	43.52 ± 0.33	15.47 ± 0.23	14.32 ± 0.38	10.48 ± 0.11	1.38 ± 0.09	2.11 ± 0.21	2.91 ± 0.13	9.38 ± 0.14
Isolate SC 2	6.32 ± 0.21	74.16 ± 0.16	43.89 ± 0.24	15.01 ± 0.23	13.64 ± 0.18	14.86 ± 0.15	2.77 ± 0.14	2.94 ± 0.12	5.18 ± 0.12	15.02 ± 0.12
Isolate SC 14	6.45 ± 0.10	72.49 ± 0.08	44.55 ± 0.21	14.68 ± 0.24	14.79 ± 0.19	7.52 ± 0.12	1.84 ± 0.16	2.02 ± 0.13	2.85 ± 0.06	6.79 ± 0.14
Isolate SC 18	7.32 ± 0.08	73.15 ± 0.19	42.49 ± 0.28	16.01 ± 0.28	13.84 ± 0.18	11.96 ± 0.14	1.24 ± 0.16	1.99 ± 0.17	2.75 ± 0.09	6.08 ± 0.13
Isolate SC 27	6.35 ± 0.14	73.83 ± 0.21	43.88 ± 0.24	14.75 ± 0.32	13.73 ± 0.20	14.96 ± 0.11	2.93 ± 0.21	3.83 ± 0.12	5.55 ± 0.06	15.42 ± 0.14
Isolate SC 50	6.99 ± 0.16	72.79 ± 0.14	43.64 ± 0.35	15.51 ± 0.33	14.56 ± 0.22	9.89 ± 0.12	1.84 ± 0.25	2.13 ± 0.08	6.01 ± 0.13	10.19 ± 0.13
Isolate SC 57	6.88 ± 0.14	71.51 ± 0.26	44.39 ± 0.21	14.49 ± 0.28	14.77 ± 0.27	8.82 ± 0.01	1.91 ± 0.19	3.47 ± 0.12	1.40 ± 0.17	9.19 ± 0.24
Isolate SC 62	7.06 ± 0.28	74.04 ± 0.13	43.27 ± 0.10	15.56 ± 0.22	14.01 ± 0.24	12.58 ± 0.09	0.98 ± 0.32	1.78 ± 0.12	1.45 ± 0.23	8.07 ± 0.18
Isolate SC 65	6.88 ± 0.18	71.34 ± 0.16	44.38 ± 0.28	14.80 ± 0.16	14.67 ± 0.03	8.96 ± 0.03	1.50 ± 0.09	1.80 ± 0.19	1.73 ± 0.08	7.96 ± 0.13
Isolate SC 73	6.05 ± 0.19	72.84 ± 0.14	43.45 ± 0.31	14.83 ± 0.24	13.71 ± 0.07	14.29 ± 0.05	2.76 ± 0.20	4.57 ± 0.19	4.59 ± 0.31	15.63 ± 0.12
Isolate SC 75	6.92 ± 0.24	71.25 ± 0.21	44.58 ± 0.28	14.61 ± 0.27	14.69 ± 0.08	8.83 ± 0.08	1.40 ± 0.21	2.15 ± 0.19	2.28 ± 0.20	8.49 ± 0.21
Isolate SC 76	6.88 ± 0.14	72.58 ± 0.14	45.54 ± 0.32	14.15 ± 0.14	14.73 ± 0.20	7.15 ± 0.04	1.83 ± 0.17	3.11 ± 0.12	2.36 ± 0.08	7.96 ± 0.14
Isolate SC 99	7.65 ± 0.16	71.08 ± 0.65	45.21 ± 0.33	14.86 ± 0.28	14.80 ± 0.22	7.78 ± 0.01	1.02 ± 0.13	1.50 ± 0.12	1.50 ± 0.08	7.53 ± 0.36
Isolate SC 142	7.06 ± 0.14	71.49 ± 0.28	45.48 ± 0.28	14.48 ± 0.23	14.80 ± 0.27	7.09 ± 0.02	1.01 ± 0.12	1.92 ± 0.12	2.23 ± 0.05	7.28 ± 0.51

Blank: without inoculation. Sterilized pangolagrass 25 g (dry wt. basis) was at initial moisture content 68 − 72% initial pH 6.8 − 7.0 and incubated at 30°C for 6 days.
Means ± SD (n = 3).
C/N = carbon/introgen.

microbes could enrich the protein content and improve the digestibility. Theoretically a 2.5% nitrogen supplement is necessary to enrich the protein content from 5—6 to 16—20%. The protein content was 16.95% and the *in vitro* digestion was 17.82% with a 2.5% supplement of $(NH_4)_2SO_4$ in fermented pangolagrass. The fractional supplement of nitrogen could result in higher protein enrichment than supplement added only at zero time for the maintenance of substrate pH, lowering the nitrogen source inhibition and increasing the nitrogen efficiency.[7]

Although 29 days of pangolagrass fermentation gave the highest protein content and *in vitro* digestion, the fermentation rate increased only slowly after 6 days. Therefore, 6 days of solid state fermentation of pangolagrass was recommended for protein enrichment and *in vitro* digestion improvement in animal feed preparation. Protein enrichment of sweet potato residue or corncob increased with increasing inoculation density of tested microbes.[7,11] Increasing inoculation density also reduced the maturity period during biofertilizer or compost preparation.[18,21] The inoculation density of each 1 g of fermented pangolagrass was 5×10^5 spores or cells, so the fermentation period was slightly longer than those in protein enrichment of sweet potato residue or corncob.[7,11]

In conculsion, pangolagrass solid substrate supplemented with 2.5% $(NH_4)_2SO_4$, 2.5% the mixture of KH_2PO_4 and K_2HPO_4 (2:1, w/w) and 0.3% $MgSO_4 7H_2O$, incubated with cellulolytic *M. lutea* CL3 by solid state fermentation at 30°C for 6 days could be used to enrich the protein content and improve the *in vitro* digestion of cellulosic materials for animal feed. The protein content of fermented pangolagrass increased to 16.96% and the *in vitro* digestion improved to 17.82%. Therefore, solid state fermentation of pangolagrass with appropriate microbes is a feasible process to enrich the protein content, enhance the *in vitro* digestion and improve the quality for animal feeding.

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