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Full Length Research Paper

Protein enrichment of cassava peel by submerged fermentation with *Trichoderma viride* (ATCC 36316)

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Cassava (*Manihot esculenta Crantz*) peel is one of the solid wastes produced as a consequence of cassava processing. It is low in protein but contains a large amount of carbohydrate, causing an environmental problem with disposal. In order to add-value to this major cassava processing waste and also reduce its resultant environmental pollution, this study investigated the effect of submerged fermentation using *Trichoderma viride* ATCC 36316 on the protein content and amino acid profile of enzyme and non-enzyme pre-treated cassava peel. Compositional analysis of the product obtained with *T. viride* in the fermentor revealed that dry biomass increased in crude protein, true protein, crude fat, crude fibre, ash and total dietary fibre. The crude protein increased from 4.21 to 37.63 and 36.52% for enzyme and non-enzyme pre-treated fermented samples respectively with 31.6% as true protein for the former and 29.03% as true protein for the latter while the starch contents reduced considerably in both samples. Starch reduction was from 51.93 to 24.34 and 26.07% for enzyme and non-enzyme pre-treated fermented samples, respectively. The fermented products contained all the essential amino acids; however the chemical score of essential amino acids indicated methionine as the limiting amino acid.

Key words: Cassava peel, *Trichoderma viride,* enzyme, submerged fermentation, protein, amino acids.

INTRODUCTION

Protein-energy malnutrition remains a major public health problem in many developing countries and there is the need to increase daily intake of protein, especially animal protein, using cheap and non-conventional sources such as agricultural wastes and by-products of food processing. The food processing industry generates a large amount of wastes annually including crop residues (peels, husks, cobs, shells, etc), sugarcane baggase and cheese and whey permeates that pose serious disposal challenges (Gomez et al., 2005). Such wastes are usually rich in sugars and are easily assimilated by microorganisms. This makes them very suitable as raw materials for growth of microorganisms.

Cassava (*Manihot esculenta Crantz*) is a staple food in Tropical Africa and Central and South America. Nigeria with an annual production of 34 - 40 million tonnes is the largest producer of the crop (CBN, 2003). Cassava is fermented to produce several different or similar products in different parts of Africa and Latin America. Such products include farina in Brazil, Costa Rica and Bolivia, garri, fufu, lafun, chiwangue and myondo in West, East and South Africa (Nestel, 1973; Subrahmanyan, 1990; Akinrele, 1967 and Giraud et al., 1991). Chief among the wastes obtained from cassava processing is the cassava peel that accounts for 10 - 20% of the root. It is estimated that about 4 million tonnes of cassava peel are generated from cassava processing in Nigeria annually (Hahn and Keyser, 1985). It is of low protein content which limit its use as animal feed. As grain production remains insufficient to meet human and animal feeding, the alternative is to employ feed ingredients which do not have direct human value. It is therefore important to enhance the nutritive value of cassava peel with respect to its protein

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content in order to provide a valuable ingredient for animal feed formulation in Nigeria and other cassava producing developing countries. It will also help in releasing some other materials exclusively as food for human consumption. Technology exists for recovery of food materials in waste by chemical and microbiological means either directly for human food or indirectly by conversion to animal feed. Protein production by fermentation using renewable resources including organic waste has received world wide attention (Oliveira, 2001) as a solution to environmental and industrial waste (Duru and Uma, 2003). Okpako et. al., (2008) fermented cassava peel with a mixture of *Aspergillus niger* and *Lactobacillus rhamnosus* to increase the crude protein content from 5.50 to 24.40%. *Trichoderma viride* has been previously used in the fermentation of several food processing wastes; De Gregorio et al. (2002) fermented lemon pulp with *T. viride and A. niger* by slurry-state fermentation, the protein content obtained in the residue was higher with *T. viride* than with *A. niger*. Palm-tree leaflets and midribs both of very low protein content, when chemically hydrolysed and used for the growth of *T. viride* were enriched with protein (Abou-Zeid, 1991).

The objectives of this study are to evaluate the performance of *T. viride* in protein enrichment of cassava peels by submerged fermentation and also investigate the effect of enzyme pretreatment prior to the fermentation process on the enriched product. The study reports changes that occur during the fermentation process.

MATERIALS AND METHODS

The peel from fresh cassava tubers, variety TME I, obtained from a farm at Ajibade village in Akinyele local government area, Ibadan was used for this study.

Micro-organism

The filamentous fungi *T. viride* ATCC 36316 was obtained from American Type Culture Collection.

Maintenance medium

T. viride ATCC 36316 was maintained on malt extract agar slants and stored at 4°C. The organism was sub-cultured once every 3 months.

Vegetative medium for *T. viride*

The vegetative medium contained the following ingredients g/l: Glucose, 10.0; $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 1.9; MgSO4.7H2O, 0.5; $MnSO_4.7H_2O$, 0.05; FeSO4.7H2O, 0.005 (Abou-Zeid, 1991). The medium was portioned into 250 ml flask, each containing 50 ml of the medium and sterilised at 121°C for 15 min. After cooling to room temperature, it was then inoculated with maintenance medium slants and incubated on a rotary shaker (150 rpm) at 30°C for 48 h.

Mineral medium for *T. viride*

The mineral medium contained the following in g/l: $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01 and NaCl, 0.01 (Qureshi and Blaschek, 1999). This was prepared as 100x strength and cold sterilized.

Growth or fermentation medium for *T. viride*

The fermentation medium (modification of Abou-Zeid, 1991) contained the following ingredients (g/litre): cassava peels, 50.0; $(NH_4)_2SO_4$, 10.0; KH_2PO_4, 2.0; KCl, 0.5.

The pH of the fermentation medium was adjusted to 5.00 ± 0.1 and the medium was autoclaved at 121°C for 20 min. 10 ml of mineral solution was added followed by inoculation with 5% volume of the vegetative medium.

Submerged fermentation conditions

Submerged fermentation was carried out at an agitation speed of 150 rpm and an aeration rate of 500 ml/l at incubation temperature of 28 ± 1°C.

Compositional analysis of cassava peel

Moisture content of the cassava peel was determined by drying at 105°C to constant weight (AOAC., 1995). The crude protein was by Kjeldahl method (total nitrogen x 6.25), crude fibre, fat, ash carbohydrates (estimated by difference), total dietary fibre, nitrogen free extract and gross metabolizable energy were quantified as described by AOAC methods (AOAC., 1990). True protein content was determined by the method of Lowry (1951). Total carbohydrate was estimated using phenol sulphuric acid method as described by Dubois et al. (1956). Total cyanide of dried samples was determined by phosphoric acid extraction, hydrolysis of cyanogenic glucosides with linamarase from cassava, followed by colorimetric determination of cyanide (Cooke, 1978).

Amino acid analysis and chemical score

The amino acid analysis of the biomass was carried out using an amino acid analyser (Beckman 6300) after hydrolysis of the sample protein in 6 N HCl for 24 h (Uysal et al., 2002). Chemical score was calculated following method of AOAC (1984).

Enzyme treatment of cassava peel

Termamyl 120 L (Novo Industry A/S, Bagsvaerd, Denmark) was used for liquefaction at a concentration of 0.06% on starch dry weight (equivalent to 0.036% (w/w) on dry peel weight) according to the manufacturer's dosage recommendation. Saccharification was accomplished with a fungal amyloglucosidase, Novo AMG 300L at a concentration of 0.15% (on starch dry weight) was used.

Procedure for liquefaction and saccharification

Dried and milled cassava peel (0.3 - 0.6 mm) suspended in distilled water (about 5% peel on dry weight and 8% moisture content) was adjusted to pH 6.5 with 3 M KOH and heated to 92.5°C in a boiling water bath. 8 mg per litre of Ca^{++} (0.029 g/l of $CaCl.2H_2O$) was added to the mixture prior to heating. The enzyme (termamyl 120 L) was added to the slurry when the temperature approached 60°C. After liquefaction, the medium was cooled to 60°C and the pH adjusted to 4.5 with 3 N H_2SO_4 prior to the addition of AMG. Hydrolysis was allowed to proceed for 9 h and stirring was maintained throughout.

Preliminary submerged fermentation of cassava peel using *T. viride*

Prior to fermentation, mineral solutions ($(NH_4)_2SO_4$ (10.0 g/l), KH_2PO_4 (2.0 g/l), KCl (0.5 g/l)) were added to the 5% gelatinised cassava peel before pH adjustment (5.00 ± 0.1) and autoclaving at 121°C for 20 min. The autoclaved medium was allowed to cool to 28°C and 10 ml of mineral solution added prior to inoculation. The following preliminary fermentations were carried out:

i) Batch fermentation with the pH uncontrolled throughout the fermentation period except at the initial setup
ii) Batch fermentation with the pH constantly being adjusted to 5.00 ± 0.1 throughout the fermentation period
iii) Fed-batch fermentation with the pH constantly being adjusted to 5.00 ± 0.1 throughout the fermentation period and supplementation of the fermentation medium with inorganic nutrients and mineral medium during the course of fermentation.

Procedure for submerged batch fermentation of cassava peel using *T. viride*

The observations from preliminary submerged fermentations (results not included) suggested subsequent submerged fermentations using batch technique with the pH being constantly adjusted to 5.00 ± 0.1.

Gelatinized cassava peel (5% w/w) was placed into a 2 L benchtop fermentor (BIOSTAT® B, B.Braun Biotech International, Germany) and sterilised at 121°C for 20 min. The sterile medium was cooled to 28°C, enriched with mineral medium and inoculated with 5% (v/v) spore suspension to obtain a 1 L working volume. Filtered air was introduced through air sparger at the bottom of the fermentor at a flow rate of 500 ml/min. The temperature of incubation was maintained at 28 ± 1°C with agitation at 150 rpm. Fermentation proceeded for 7 days and samples were collected daily for soluble protein, soluble solids and starch analysis.

The procedure was repeated for enzyme pre-treated cassava peel slurry. Enzyme hydrolysis of the substrate was by commercial external enzymes (α-amylase and gluco-amylase) prior to batch fermentation with the pH constantly being adjusted to 5.00 ± 0.1 throughout the fermentation period. Unfermented or sample without *T. viride* served as the control.

The fermentations were terminated by freeze drying and the dried product subsequently subjected to chemical analysis.

Analysis of supernatants and protein enriched cassava peels

Supernatants of samples withdrawn during the fermentation processes were analysed for soluble protein content by the method of Lowry (1951) and total sugars were by estimated by phenol-sulphuric acid method (Dubois et al., 1956). Starch concentrations of the samples were determined by acid hydrolysis and the samples assayed immediately for reducing sugar using the 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959).

Starch concentration was calculated as: 0.9 x reducing sugar (glucose equivalent)

Glucose concentration was determined using a hexo-kinase- and glucose phosphate dehydrogenase (Sigma Chemicals)-coupled enzymatic assay. The fermentation medium was centrifuged (microfuge centrifuge) at 10,000 g for 3 min at 4°C. A portion of the supernatant (10 µl) was mixed with glucose (HK) 20 reagent (1.0 ml) and incubated at room temperature for 5 min. Standard solutions of anhydrous D-glucose containing 1 - 5 g of glucose L^{-1} of distilled water were prepared. 10 microliters of each of the standard solution was mixed with glucose (HK) 20 reagent (1.0 ml) and incubated at room temperature for 5 min. A blank (de-ionized water) (10 µl) was incubated with the reagent and was used for zero adjustment of the spectrophotometer. After 5 min, the absorbance was measured at 340 nm using a Beckman DU 640 spectrophotometer and the glucose content in the sample was computed by least squares linear regression using a standard curve.

The resulting protein enriched cassava peel from submerged fermentation using *T. viride* with and without enzyme pre-treatment was analysed for proximate composition, nitrogen free extract, gross metabolizable energy and amino acid profile as previously described.

RESULTS AND DISCUSSION

Submerged fermentation of cassava peels using *T. viride*

The highest soluble protein for fermentation with enzyme pre-treatment was recorded on the 3rd day of fermentation while for fermentation without enzyme pre-treatment the highest was recorded on the 4th day (Figure 1). Fermentation with external enzyme prior to inoculation produced a higher percentage increase in soluble protein content presumably because glucose was readily available glucose for the organism to utilise for bioconversion to soluble protein from the initial enzyme hydrolysis. The enzyme protein added prior to fermentation may also have contributed to the higher soluble protein content. The initial increase and further decrease of the glucose and total sugars concentration in the fermentation broth culture might be due to simultaneous hydrolysis of the starch to simple sugars and subsequent utilisation of the sugars by the organism (Figures 2 and 3). The reduction in the percentage starch content for both enzyme and non-enzyme pre-treated fermentation (Figure 4) indicated a substantial utilisation of the starch in the cassava peel by *T. viride.* It should be noted that soluble protein, glucose and total sugars increased gradually at time zero while starch content reduced for enzyme pretreated sample, this was as a result of inclusion of enzyme for liquefaction and saccharification of the carbohydrates for enzyme pre-treatment prior to fermentation.

Glucose accumulation and increase in protein content in relation to hydrolysis of cassava peel starch during the early growth period of the fungus in unpretreated cassava peel is a fundamental innovative argument for improvement of the nutritional and industrial value of cassava peel meal. A similar observation was made during the solid state fermentation of cassava pulp with *Rhizopous* sp. (Soccol et al., 1994).

Agitation of the fermentation broth could not go beyond the 4th day for non-enzyme pre-treated fermentations due to low liquid volume, while agitation was still possible until the 6th day for enzyme pre-treated fermentation broth. This may be due to high liquefaction of the fermentation medium through the initial activity of the external enzymes. After stopping the agitation, pH adjustment was discontinued. It was however noted that henceforth the pH of the fermentation broth without enzyme pre-treatment

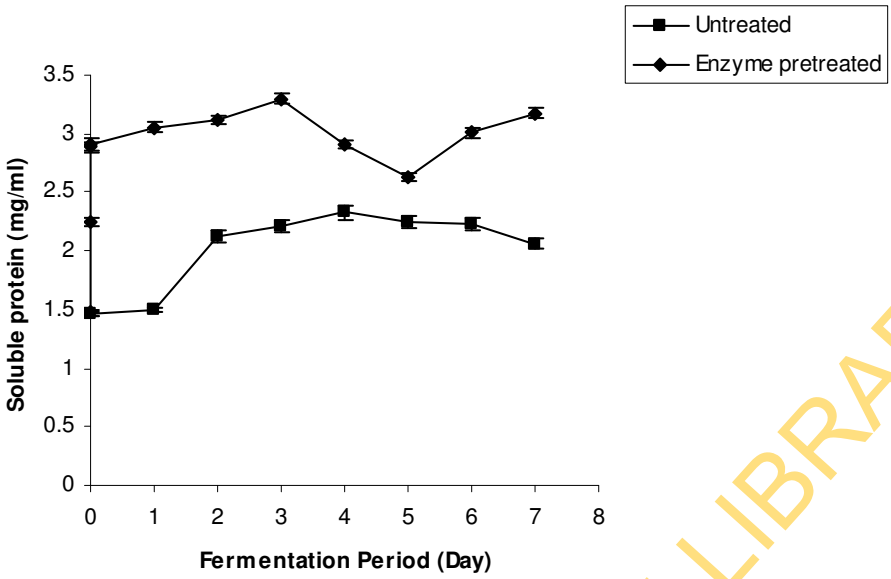

Figure 1. Changes in soluble protein content in batch submerged fermentation of cassava peel with *T. viride.*

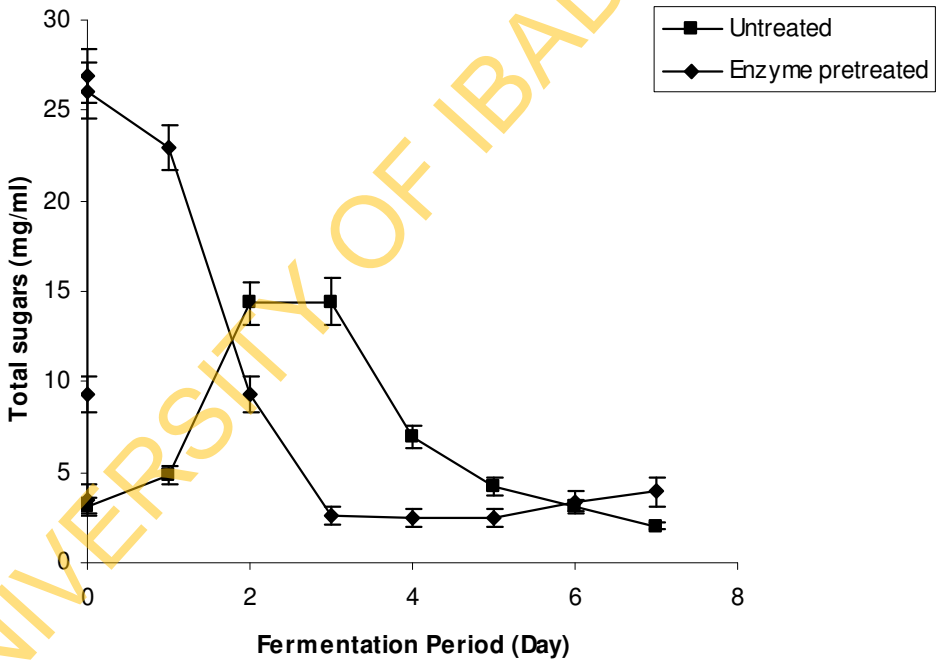

Figure 2. Changes in total sugars during batch submerged fermentation of cassava peel with *T. viride.*

tended towards acidic range while the pH of enzyme pre-treated broth tended towards neutrality. Consequently, the non-enzyme pre-treated fermentation could be terminated on the fourth day while the enzyme pre-treated fermentation could be terminated earlier on the third day. The resulting fermented products were freeze dried and analysed for proximate composition and amino acid profile.

The compositional analysis of the product obtained with *T. viride* in the fermentor (Table 1) revealed that dry biomass increased in crude protein, true protein, crude fat, crude fibre, ash and total dietary fibre. There were significant differences in all the measured parameters but hydrocyanic acid for fermented and unfermented

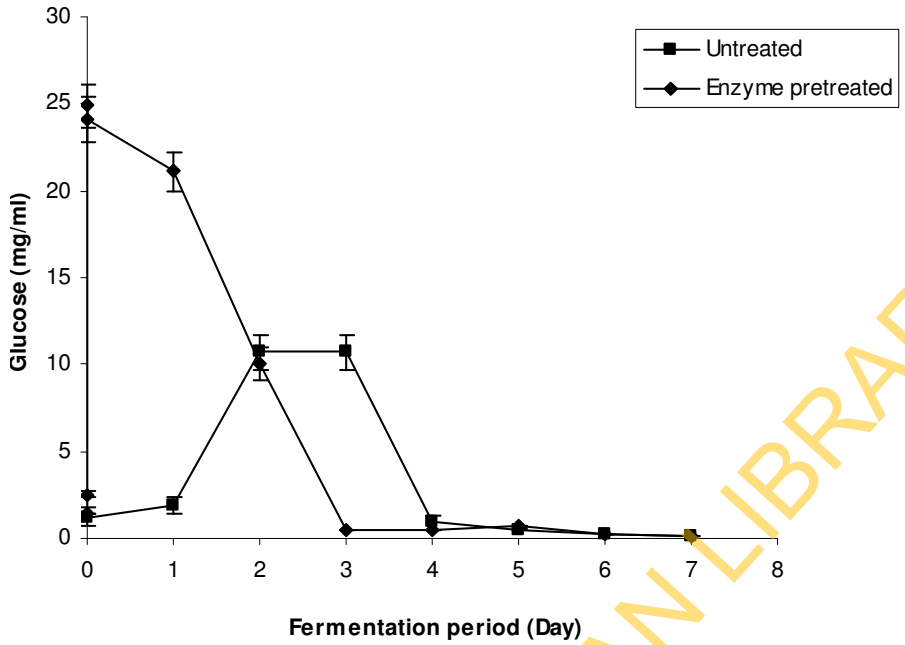

Figure 3. Changes in glucose concentration during batch submerged fermentation of cassava peel with *T. viride.*

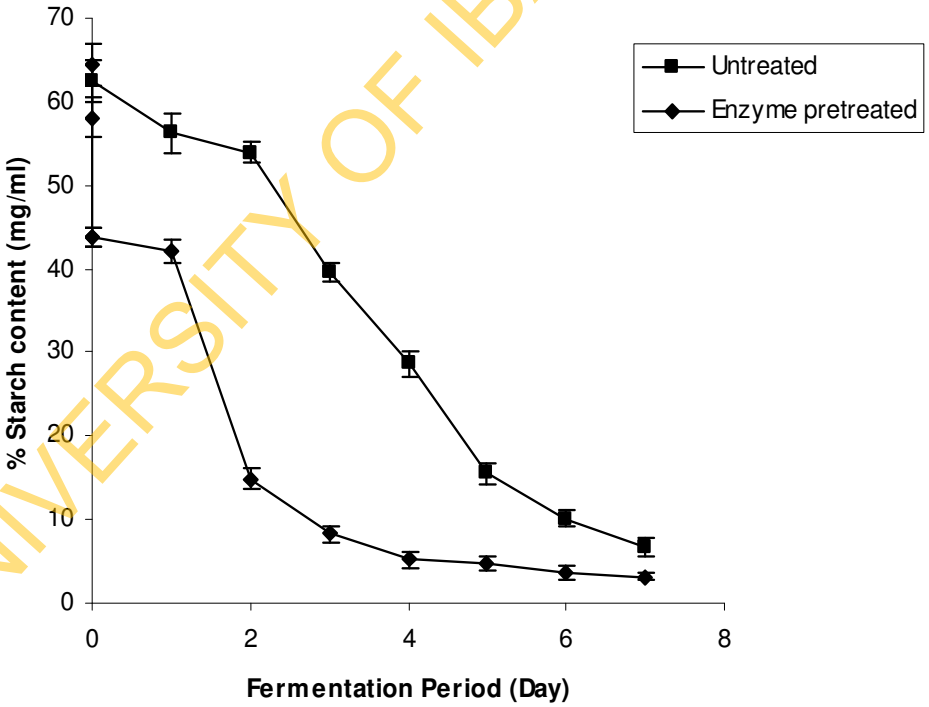

Figure 4. Changes in percentage starch content during batch submerged fermentation of cassava peel with *T. viride.*

samples. With the exception of values for true protein, crude fat, ash, starch and gross calories there were no significant differences in the fermented, enzyme pre-treated and untreated samples. The crude protein

Table 1. Composition of unfermented and fermented cassava peels samples.

Composition (%)	Unfermented cassava peel Sample	Fermented cassava peel without enzyme pre-treatment	Fermented cassava peel with enzyme pre-treatment
Crude protein	4.21[b]	36.52[a]	37.63[a]
True protein	1.36[c]	29.03[b]	31.60[a]
Moisture	8.73[a]	7.66[b]	7.29[b]
Crude fat	1.37[c]	2.23[a]	1.83[b]
Crude fiber	8.46[b]	12.88[a]	11.93[a]
Ash	3.27[c]	15.49[b]	17.80[a]
Carbohydrates by difference	91.15[a]	45.77[b]	42.72[b]
Starch	51.93[a]	26.07[b]	24.34[c]
HCN(mg/100 g)	0.72[a]	0.70[a]	0.70[a]
Gross calories (kcal/100 g)	393.79[a]	349.46[b]	338.53[c]
Total dietary fiber	24.96[b]	27.36[ab]	29.35[a]

Results are expressed on a dry matter basis. Each value is a mean of three independent experiments. Means followed by the same superscript in the same row are not significantly different ($p \leq 0.05$).

increased from 4.21 to 37.63 and 36.52% for enzyme and non-enzyme pre-treated fermented samples respectively with 31.6% as true protein for the former and 29.03% as true protein for the latter while the starch contents reduced considerably in both samples. This indicates good nitrogen and carbon sources utilization by the organism. Single cell protein product reported by Singh et al. (1991) contained 30.4% crude protein and *Kluyveromyces fragilis* biomass grown on deproteinized whey supplemented with 0.8% diammonium hydrogen phosphate contained 37% crude protein (Paul et al., 2002). Similarly yeast cell mass reported by Chanda and Chakrabarti (1996) and Meyer et al. (1993) contained 54.3 and 47% crude protein respectively. The increase in the ash content may have resulted from the addition of mineral medium to the fermentation medium. With regards to cyanide content no significant reduction was noticed. There was a slight reduction in the gross calories for the fermented samples.

Protein quality

The microbial protein sources are of great value for ruminant as well as monogastric animals. Hence the evaluation of the protein quality in terms of *in vitro* rumen digestibility or amino acid composition would indicate the suitability of the microbial protein as a protein feed supplement.

Quantitative estimations of the amino-acid content of submerged fermented cassava peel in comparison with unfermented cassava peel samples are presented in Table 2 while Table 3 showed the chemical score of the fermented samples using FAO/WHO amino acid reference pattern (FAO/WHO, 1957).

The amino acid profile of cassava peel was greatly improved by fermentation with *T. viride*. The improvement in amino acid profile and amino acid score of the fermented cassava peel over the unfermented cassava peel could be attributed to the luxuriant growth of the fungus in the cassava peel substrate. Total amino acid content was increased 4 times in the fermented cassava peel samples. The amino acid scores varied from 11.36 to 27.14% in unpretreated sample and from 10.45 to 26.42% in enzyme pre-treated sample, threonine had the highest amino acid score. Although the amino acid profile fell short of FAO/WHO amino acid reference pattern (FAO/ WHO, 1957), the fermented products contained all the essential amino acids with methionine that had a chemical score of 10.45% for enzyme pre-treated samples and 11.36% (Table 3) for untreated samples as the limiting amino acid.

Conclusion

Trichoderma viride ATCC 36316 grew well on cassava peel especially on un-pretreated cassava peel. Fermentation for 3 to 4 days increased crude protein content of cassava peel 8-fold (4.2% to up to 37.6%) and true protein content 22-fold (1.4% to up to 31.6%). Further study is required on optimising the fermentation medium to improve the amino acid profile of the product.

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Table 2. Amino acid Profile of Unfermented and fermented Cassava Peel Samples

Amino acid	Unfermented cassava peel (mg/100 g)	Fermented cassava peel without enzyme pre-treatment (mg/100 g)	Fermented cassava peel with enzyme pre-treatment (mg/100 g)
Taurine	80 ± 1.1	110 ± 2.0	120 ± 2.3
Aspartic acid	300 ± 2.5	1340 ± 28.7	1290 ± 29.5
Threonine	130 ± 2.0	760 ± 5.6	740 ± 5.4
Serine	120 ±1.3	630 ± 3.4	630 ± 4.3
Glutamic acid	510 ± 4.5	1660 ± 13.3	1750 ± 5.4
Proline	140 ± 2.1	690 ± 0.36	640 ± 4.3
Glycine	160 ± 1.2	750 ± 4.4	720 ± 3.3
Alanine	190 ± 1.4	1100 ± 21.1	1020 ±18.9
Cysteine	60 ± 0.9	260 ± 1.9	230 ± 1.2
Valine	190 ± 1.4	1030 ± 15.6	1010 ± 16.3
Methionine	60 ± 0.9	250 ± 3.0	230 ± 4.1
Isoleucine	150 ± 1.7	680 ± 8.9	650 ± 12
Leucine	240 ± 1.6	1160 ± 25.4	1090 ± 23.0
Tyrosine	70 ± 0.8	370 ± 4.9	370 ± 2.9
Phenylalanine	140 ± 1.1	640 ± 12.3	610 ± 12.2
Ornithine	10 ± 0.4	20 ± 1.1	30 ± 2.4
Lysine	180 ± 2.2	1080 ± 27.2	980 ± 16.7
Histidine	90 ± 1.5	360 ± 2.4	330 ± 2.3
Arginine	190 ± 2.7	840 ± 16.6	810 ± 15.4
Tryptophan	<40	50 ± 2.0	40 ± 1.0
Total amino acid content (mg/100 g)	3180	14240	13640
Total amino acid content (%)	3.2	14.2	13.6

Results are expressed on a dry matter basis. Each value is a mean of 3 independent experiments.
± Stands for standard deviation among replicates.

Table 3. Chemical score of fermented cassava peel.

Amino acid	FAO/WHO amino acid pattern (mg/g)	Available percentage in cassava peel fermented without enzyme pre-treatment (%)	Available percentage in cassava peel fermented with enzyme pre-treatment (%)
Threonine	28	27.14	26.42
Valine	42	24.52	24.04
Methionine	22	11.36	10.45
Isoleucine	42	16.19	15.48
Leucine	48	24.17	22.71
Phenylalanine	28	22.86	21.79
Lysine	42	25.71	23.33
Histidine	-	-	-
Tryptophan	-	-	-

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