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Effect of mono and co-culture fermentation on the nutrient composition of alkaline and biologically pretreated *Indigofera arrecta* seeds.

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Abstract
The effect of mono and co-fermentation on the proximate composition, some mineral elements and antinutritional factors of alkaline pretreated (AlPS) and biologically pretreated (BiPS) *Indigofera arrecta* seeds was studied. Fermentation was carried out using fungal isolates of *Aspergillus niger* and *Trichoderma harzanium* combinations which were found compatible and used for development of co-culture formulations. The proximate composition, some anti-nutrients and the mineral elements of the mono and co-culture formulations pretreated *Indigofera arrecta* seeds were analyzed using standard procedures. These results also show that co-culture formulations of the pretreated samples significantly increased the crude protein content, some minerals and decreased the antinutritional content when compared with monocultures. This study has shown that compatible mixture of *Trichoderma harzanium* and *Aspergillus niger* can further reduce the antinutrients and improve the nutritional content of alkaline and biologically pretreated *Indigofera arrecta* seeds.

Keywords: *Indigofera arrecta*; *Trichoderma harzanium*; *Aspergillus niger*; co-culture fermentation; alkaline pretreated; biologically pretreated

1. Introduction
Legumes (family: Fabaceae) have been recognized to be the 2nd most valuable plant source for human and animal nutrition [1]. They are the most important sources of proteins, carbohydrates and vitamins in the diet of many populations, especially in developing countries [2]. Legume seeds have an average of twice as much protein as cereals and the nutritive value of the proteins are usually high [3]. As a result of the high demand of the common legumes, there is an upsurge in their prices which has compelled the need to look for replacements such as the wild legumes [4]. Legumes have a great potential for delivering quality proteins, but raw legumes have a lower degree of bioavailability and, thereby, lower nutritional value as compared to other foodstuffs [5].
The growth of fungi in natural substrates is usually slow and this limitation must be overcome by suitable mechanical and chemical pretreatment of the raw substrate [6,7]. Pretreatment is an efficient method in enhancing the protein content, mineral composition and in reducing tannins, phytates, alkaloids, saponins, hydrogen cyanide, trypsin inhibitors, lectins and oxalate in the seeds of *Indigofera arrecta* [4]. Fermentation has always been an important part of human lives with exclusive benefits as food. It can produce vital nutrients or eliminate antinutrientss [8]. Mixed culture fermentation is that in which the inoculum consists of two or more organisms [9,10]. The mixed culturing of fungi may lead to better substrate utilization, increased productivity, and it also can strengthen and accelerate the bioconversion process on encouraging compatibility results reported in solid-state bioconversions than for liquid state fermentations [11,12]. Strain compatibility is a critical factor in mixed culturing, and the most important observation was that synergistic interactions between compatible partners may overcome nutritional limitations in poor agricultural residues [9]. The purpose of compatible mixed culturing is to let fungi work together mutually and degrade substrates faster.

Many studies showed that evaluation of fungal possible compatible interactions was first studied in two agar media: potato dextrose agar (PDA) and malt extract agar (MEA) [13, 14]. Mixed culture fermentations are widely used in biotechnology for many processes including the production of antibiotics, enzymes, fermented food, composting, dairy fermentation, bioconversion of apple distillery and domestic wastewater sludge [15]. Studies carried out by Belewu et al. [8] demonstrated that the incubation of substrate with *Trichoderma harzanium* and *Aspergillus niger* markedly reduce the phytate and tannin in Jatropha curcas seeds.

The solid-state fermentation of pretreated *Indigofera arrecta* with *Trichoderma harzanium* and *Aspergillus niger* may be a promising method of enhancing the usage of *Indigofera arrecta* seeds. This study is aimed at evaluating the effect of controlled fermentation using *Aspergillus niger* and *Trichoderma harzanium* in mono and co-culture formulation on the nutrient composition of pretreated *Indigofera arrecta* seeds.

2. Material and methods

2.1 Collection and preparation of samples

Indigofera arrecta fruits were collected from Zaria metropolis with identification done at the Herbarium of the Department of Biological Science, Ahmadu Bello University, Zaria, Nigeria. The voucher number 663 had already been assigned to *Indigofera arrecta.* The fruits were sun-dried for 3-4 days, threshed and winnowed. Dust and other foreign materials were removed to obtain clean seeds which was ground in a bench mill. The ground seeds was then stored in plastic containers for subsequent analysis [4].

2.2 Pretreatments

The following pretreatments were carried out on the ground sample of *Indigofera arrecta*

2.2.1 Alkaline pretreatment

Two hundred grams of the ground sample was mixed with 1L of 0.25M NaOH solutions for 1 hour after which the mixture was neutralized with 0.1M HCl. The residue was washed with distilled water and dried to constant weight at 80^0C in the oven [4, 16].

2.2.2 Biological pretreatment

Forty grams of the sample was moistened with 200ml of distilled water to obtain 70% w/v of moisture in 1L Erlenmeyer conical flask. The mixture was autoclaved for 30min at 121^0C. After cooling, it was inoculated with spore suspension of *Ustilago maydis* and incubated at 28^0C for 21 days [4].

2.3 Microbial Culture

2.3.1 Isolation of *Aspergillus niger*

Isolation of *Aspergillus niger* from soil samples was performed by the soil dilution method [17]. Ten grams of soil sample was added to 90 ml distilled water on a shaker for one hour. Then, 10 mL aliquot from this suspension was transferred aseptically to 90 ml distilled water. This dilution process was repeated to get dilutions up to 10^{-5}. From the dilution suspension, one milliliter of aliquot from soil sample was spread on Petri dishes containing Potato Dextrose Agar (PDA). The identification of *Aspergillus niger* was made by the simple microscopic method of Ellis [18]. Pure isolates of *Aspergillus niger* were sub cultured on slants and stored at 4^0C.

2.3.2 Isolation of *Trichoderma harzanium*

Rice stems and leaves were cut into 5 mm pieces and added to 150 mL distilled water on a shaker for 30 minutes. Then, 10 mL aliquot from this suspension was transferred aseptically to 90 mL distilled water. This dilution process was repeated to get dilutions up to $10^{-5.}$ From the dilution suspension, one milliliter of aliquot from phyllosphere sample was spread on RB-S-F selective medium [19] in Petri dishes. The dishes were incubated at 26±1°C in the dark for 4 days. Individual isolates were identified at the species level using morphological keys and fungal species descriptions [20,21,22]. Pure isolates of *Trichoderma harzanium* were subcultured on slants and stored at 4^0C.

2.4 Fermentation Process

2.4.1 Preparation of inoculum

The *Aspergillus niger* and *Trichoderma harzanium* spore inoculum were separately prepared by adding 10 ml of sterile distilled water containing 0.2% Tween 80 to a fully sporulated slant culture. The spores were dislodged by vigorous shaking and spore number estimated by direct microscopic enumeration using cell-counting hemocytometer. (Neubauer chamber; Merck, S.A., Madrid, Spain). The volume of spores suspension were adjusted to 2.75 x 10^7 spores/ml and the harvested *Aspergillus niger* and *Trichoderma harzanium* spores were used as inoculums in the fermentation of the pretreated *Indigofera arrecta* seeds.

2.4.2 Fermentation of Sample

Each flask containing 10g of pretreated *Indigofera arrecta* seeds was mixed with mineral salt solution (g/l; KH_2PO_4, 2.5; KNO_3, 5.0; $MgSO_4$. $7H_2O$, 1.0; Na_2SO_4, 1.0; $FeCl_2$, 0.02; $ZnSO_4$. $7H_2O$, 0.0015; $CuSO_4$. $5H_2O$, 0.003 and $MnSO_4$, 0.001) in distilled water in the ratio 1:2 autoclaved and inoculated with 2.75x 10^7 per ml spores suspension from each microbe separately and the volume halved in co-culture combination. The samples was then incubated and allowed to ferment for 7 days.

2.5 Biochemical and mineral analysis

The pretreated samples after drying were used for biochemical and mineral analysis. Ash, crude lipid, crude fibre, and crude protein were determined following the methods of A.O.A.C [23]. The anti-nutritional factors in both samples and control were examined as follows: Hydrogen cyanide content was determined according to the method of AOAC [24], tannin content was estimated spectrophotometrically by Folin-Denis method [25], phytic acid was determined using the procedure described by Lucas and Markakas [26]. Saponins was determined by the gravimetric method of AOAC [24], trypsin inhibitor was analysed by using the spectrophotometric method, described by Amtfield *et al*. [27]; lectin content was determined using the spectrophotometric method of Onwuka [28], alkaloid content was determined by the gravimetric method of Harbone [29] and oxalate was determined by using the method of Oke [30].The following minerals: magnesium, calcium, zinc, iron, potassium, phosphorus and sodium were determined using atomic

absorption spectrophotometry as described by AOAC [23]. The Amino Acid profile in the known sample was determined using methods described by Benitez [31].

3.0 Statistical Analysis

Means and standard deviation (SD) of factors examined were calculated. The effects of solid-state fermentation of alkaline and biologically pretreated *Indigofera arrecta* seeds with mono and co-culture combinations of *Aspergillus niger* and *Trichoderma harzanium* on the nutritional, antinutritional and mineral content of *I. arrecta* seeds was resolved by Analysis of variance (ANOVA) using SPSS version 20. Duncan's Multiple Range Test (DMRT) was applied to separate and show means that differed significantly. Significance was accepted at $p \leq 0.05$.

4.0 Results and discussion

4.1 Results

The hyphal interactions and growth of *Aspergillus niger* and *Trichoderma harzanium* as shown in Plates 1C and the reverse in 1D were also studied to confirm the actual outcomes of the interactions. As indicated in Table 1 the proximate composition of the treatment combinations on the seeds of *Indigofera arrecta.* The BiPS - *Tri h* treatment combination had significantly ($P<0.05$) lower percentage crude protein than each of the other five treatment combinations while co-cultures of both AlPS and BiPS treatment combination with values 27.750 ± 0.13 and 28.157 ± 0.02 reduced the carbohydrate content of the seeds. The antinutritional content of AlPS and BiPS treatment combination shown in Table 2 revealed significant decrease in the alkaloid, saponin, trypsin, lectin and hydrogen cyanide content of the BiPS-*Asp.n* and *Tri.h* treatment combination while the AlPS-*Asp.n* and *Tri.h* significantly decreased the tannin and phytate content. The result on Table 3 shows that BiPS– *Asp.n* and *Tri.h* treatment combination had the highest significant reduction in phosphorus and sodium content while AlPS– *Asp.n* and *Tri.h* had the highest increase in sodium content when compared with the other treatment combination.

4.2 Discussion

Invitro interaction of the two different fungi grown adjacently showed that the hyphal growth of *Aspergillus niger* and *Trichoderma harzanium* interacted mutually at the touching point but did not precede long distance into each other as much as mutual compatible cultures should (Fig 1). Mycelium growth and synergetic or antagonistic characteristics of *Aspergillus niger* and *Trichoderma harzanium* shown on Plate 1 (A and B) were categorized based on literature review [32,13,12,11]. The interaction of *Aspergillus niger* with *Trichoderma harzanium* was identified as partial intermingling.

Fermentation of pretreated *Indigofera arrecta* seed flour as shown in Fig 1 increased the crude protein content when compared with that of the pretreated seeds without fermentation [4]. This could be attributed to the addition of mycoprotein (single cell protein) which may be due to the addition of non-protein nitrogen from amide and nucleic acid synthesis by fungal cells [33]. This increase could also be as attributed to the ability of the micro-organism to secrete some extracellular enzymes (proteins) which degrade the materials during fermentation as reported by Oseni and Ekperigin [34]. The AlPS-*Asp.n* and *Tri.h* treatment combination had the highest percentage crude protein content with value of 42.450 ± 0.70. This could be as a result of the synergistic action of the micro-organism.

The lower moisture content of all fermented samples in comparison with the raw sample analyzed as earlier reported by Ahmadu *et al*. [4] revealed that they have better keeping quality. Moisture content in excess of 14% in flours has greater danger of bacteria action and mould growth which produce undesirable changes [35,36]. It was observed that the BiPS – *Asp.n* and *Tri.h* significantly reduced the lipid content of the seeds. The significant ($p \leq 0.05$) decrease in lipid content for BiPS-*Asp.n* and *Tri.h* treatment combination when compared with the other treatment combinations may be an indication that the fungus utilized the lipid as source of carbon and energy for growth and perhaps for the synthesis of protein during pretreatment. In comparison to the raw sample analysed as earlier reported by Ahmadu *et al*. [4], fermentation greatly reduced the carbohydrate content of both treatment combinations. This decrease could be attributed to the ability of fermenting micro floral to hydrolyze and metabolize carbohydrate as carbon source in order to synthesize cell biomass [37]. The synergistic activity of co-cultures of both AlPS and BiPS treatment combination with values 27.750 ± 0.13 and 28.157 ± 0.02 could be responsible for the significant reduction of carbohydrate content when compared with other treatment combinations. There was increase in ash content of all treatment combination when compared to the pretreated samples as earlier reported by Ahmadu *et al*. [4]. This could be due to the breakdown of some organic molecules consequently the release of the mineral elements from the organic phase into the inorganic phase [38]. This could also be due to contribution by fermenting micro-organism [39]. Contrary to expectation, there was an increase in crude fibre content (Table 1) of the BiPS - *Asp.n* and *Tri.h* treatment combination. This may be due to the formation of resistant starch together with condensed tannin-protein complex which could contribute to the increase [40]. It could also be due to the production of some dietary fibre-rich substances such as component parts of mycoprotein deposition in the fermented sample.

The reduction of these complex and toxic molecules as illustrated in Table 2 was attributed to degradation by microorganisms [41]. The significant decrease in the tannin, phytate, alkaloid, saponin, trypsin, lectin and hydrogen cyanide content on Table 2 of the AlPS and BiPS co-culture treatment combination as compared to the mono culture treatment combination could be attributed to the fact that the synergistic interactions between the compatible microorganisms in co-culture fermentation might have resulted in degradation of the antinutrients.

The result on Table 3 shows the mineral composition of the AlPS and BiPS treatment combination. There was significant increase in most minerals when compared with the ground and pretreated sample as earlier reported by Ahmadu *et al*. [4]. This increase could probably mean that fermentation with the fungus caused the breakdown of the antinutrients or the complexes they form with these ions led to the significant increases in their concentrations. The significant reduction in magnesium, iron and zinc content of all AlPS treatment combination could be as a result of leaching or effect of the fermentation inhibitors on the micro-organisms. The reduction in the mineral content

during fermentation could also be attributed to increase in biomass. Minerals are also used by microbes as nutrients source during growth.

5.0 Figures and tables

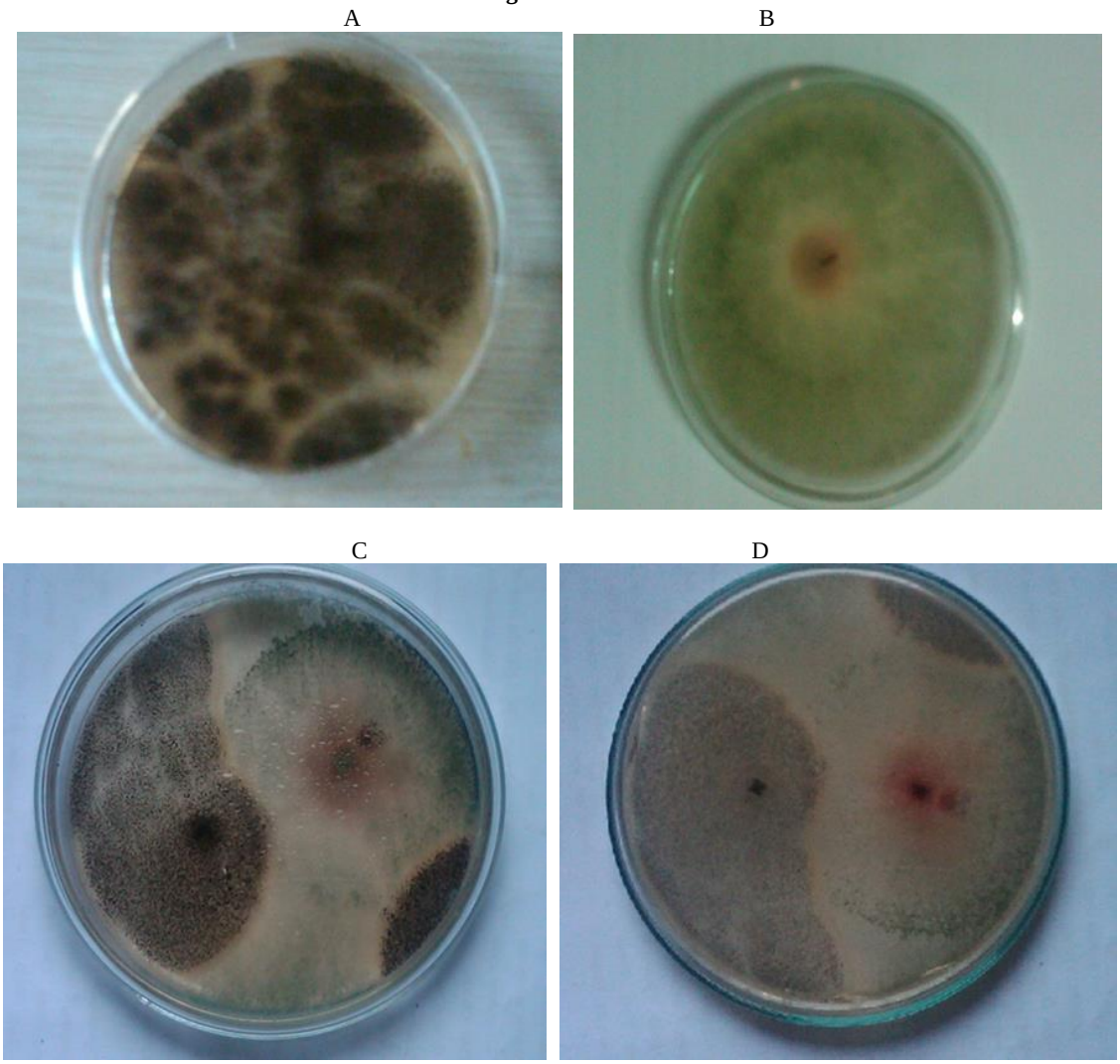

Fig 1. Five- day old colony of A) mono-culture of *Aspergillus niger*, B) mono-culture of *Trichoderma harzanium*, C) Partial compatible mixed culture of *A. niger* and *T. harzanium* and D) reverse of mixed culture of both micro- organism on to PDA medium.

Table 1: Effect of Mono and Co-Culture Fermentation on the Proximate Composition of Alkaline and biologically Pretreated *Indigofera arrecta* Seeds

Treatment Combination	Moisture	Crude Protein	Crude fat	Ash	Crude fibre	Total carbohydrate
AlPS–*Asp.n*	1.990 ± 0.03^{c}	38.937 ± 0.90^{b}	9.363 ± 0.06^{a}	3.820 ± 0.05^{f}	15.086 ± 0.10^{a}	30.320 ± 0.49^{c}
AlPS–*Tri.h*	2.207 ± 0.02^{b}	35.983 ± 0.98^{c}	8.330 ± 0.07^{c}	6.010 ± 0.03^{e}	15.180 ± 0.02^{a}	32.577 ± 0.46^{b}
AlPS-*Asp.n* and *Tri.h*	4.187 ± 0.02^{a}	42.450 ± 0.70^{a}	8.787 ± 0.02^{b}	6.563 ± 0.06^{d}	10.393 ± 0.02^{c}	27.750 ± 0.23^{e}
BiPS-*Asp.n*	2.233 ± 0.01^{b}	42.277 ± 0.42^{a}	8.287 ± 0.25^{c}	8.300 ± 0.01^{b}	9.847 ± 0.04^{d}	28.993 ± 0.02^{d}
BiPS–*Tri.h*	1.133 ± 0.03^{e}	34.620 ± 0.27^{d}	8.080 ± 0.03^{d}	9.903 ± 0.03^{a}	9.847 ± 0.04^{d}	36.070 ± 0.06^{a}
BiPS–*Asp.n* and *Tri.h*	1.983 ± 0.03^{d}	38.067 ± 0.22^{b}	7.990 ± 0.01^{e}	8.193 ± 0.02^{c}	14.203 ± 0.21^{b}	28.157 ± 0.30^{e}

Values are mean ± SD of triplicate determinations. Mean values with different superscripts down the column are significantly different ($p \leq 0.05$). Where; AlPS = Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n = Aspergillus niger, Tri.h =Trichoderma harzanium*

Table 2: Effect of Mono and Co-Culture Fermentation on the Antinutritional Content of Alkaline and biologically Pretreated *Indigofera arrecta* Seeds

Treatment Combination	Tannin %	Phytate %	Alkaloid %	Saponin %	Oxalate %	Trypsin (TIA/g)	Lectin %	Hydrogen cyanide (mg/100g)
AlPS–*Asp.n*	0.157± 0.01^{b}	0.120± 0.02^{ab}	2.113 ± 0.11^{a}	1.927 ± 0.14^{b}	0.027 ± 0.01^{cd}	0.027± 0.01^{a}	0.030 ± 0.01^{a}	0.047 ± 0.01^{a}
AlPS–*Tri.h*	0.287± 0.01^{d}	0.093± 0.02^{bc}	2.077 ± 0.08^{a}	1.920 ± 0.12^{b}	0.019 ± 0.01^{d}	0.019 ± 0.00^{ab}	0.019 ± 0.00^{b}	0.037 ± 0.00^{ab}
AlPS-*Asp.n* and *Tri.h*	0.028± 0.00^{d}	0.073± 0.03^{c}	1.990 ± 0.01^{a}	1.403 ± 0.33 ^{c}	0.017 ± 0.01^{d}	0.016 ± 0.00^{b}	0.017 ± 0.00^{b}	0.029 ± 0.00^{bc}
BiPS-*Aspn*	0.197± 0.02^{a}	0.150± 0.04^{a}	1.343 ± 0.32^{b}	2.870 ± 0.10^{a}	0.110 ± 0.02^{a}	0.013 ± 0.00^{b}	0.006 ± 0.00^{c}	0.020 ± 0.01^{cd}
BiPS–*Tri.h*	0.060± 0.01^{c}	0.107± 0.01^{bc}	0.867 ± 0.11^{c}	1.147± 0.20^{c}	0.077 ± 0.25^{ab}	0.013 ± 0.00^{b}	0.005 ± 0.00^{c}	0.017 ± 0.01^{cd}
BiPS–*Asp.n* and *Tri.h*	0.048± 0.01^{cd}	0.080± 0.01^{bc}	0.610 ± 0.09^{c}	0.543± 0.20^{d}	0.060 ± 0.04^{bc}	0.012 ± 0.00^{b}	0.004 ± 0.00^{c}	0.012 ± 0.00^{d}

Where; Values are means ± SD of triplicate readings down the column are significantly different ($p \leq 0.05$). Mean values with different superscripts. AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n= Aspergillus niger, Tri.h =Trichoderma harzanium.*

Table 3: Effect of Mono and Co-Culture Fermentation on the Mineral Content of Alkaline and Biologically Pretreated *Indigofera arrecta* Seeds

Treatment Combination	Ca	Mg	Fe	Zn	K	P	Na
AlPS–*Asp.n*	2.344± 0.00^{b}	1.616± 0.01^{e}	0.846± 0.00^{d}	0.968± 0.00^{c}	0.603± 00.00^{c}	0.430 ±0.02^{b}	0.623 ± 0.01^{b}
AlPS–*Tri.h*	1.460± 0.00^{c}	1.577± 0.00^{f}	0.525± 0.00^{f}	0.301± 0.01^{d}	1.260± 0.01^{b}	0.180 ±0.01^{d}	0.550 ± 0.01^{c}
AlPS-*Asp.n* and *Tri.h*	1.119± 0.00^{d}	1.985± 0.01^{d}	0.558± 0.00^{e}	0.222± 0.00^{e}	1.550 ± 0.01^{a}	0.583 ±0.01^{a}	0.753 ± 0.04^{a}
BiPS-*Aspn*	2.444± 0.00^{a}	2.399± 0.00^{a}	1.276± 0.00^{c}	4.397± 0.00^{b}	1.287 ± 0.02^{b}	0.583 ±0.01^{a}	0.443 ± 0.02^{d}
BiPS–*Tri.h*	1.013± 0.00^{e}	2.032± 0.00^{c}	1.476± 0.00^{b}	4.589± 0.06^{a}	1.553 ± 0.01^{a}	0.566 ±0.03^{a}	0.270 ± 0.01^{e}
BiPS–*Asp.n* and *Tri.h*	0.863± 0.00^{f}	2.043± 0.00^{b}	1.992± 0.00^{a}	4.566± 0.00^{a}	1.330 ±0.01^{ab}	0.257 ±0.01^{c}	0.227 ± 0.02^{f}

Where; Values are means ± SD of triplicate readings down the column are significantly different ($p \leq 0.05$). Mean values with different superscripts. AlPS= Alkaline pretreated sample, BiPS= Biologically pretreated sample, *Asp.n= Aspergillus niger, Tri.h =Trichoderma harzanium*

6.0 Conclusion

The current investigation has shown that combination of pretreatment and controlled co-culture fermentation of *Indigofera arrecta* are efficient techniques in improving its nutrient, minerals and amino acid composition while reducing its antinutrient. There is still need for further studies on the effect of other types of fermentation methods and pretreatment techniques not studied. Co-culture formulations with cocktail of different microorganisms needs to be employed.

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