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Fermentation of Seeds of Teff (*Eragrostis teff*), Grass-pea (*Lathyrus sativus*), and Their Mixtures: Aspects of Nutrition and Food Safety

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Fermentation of pure teff (*Eragrostis teff*), pure grass-pea (*Lathyrus sativus*), and their mixtures, 9:1 and 8:2 (teff/grass-pea) has been done at two temperatures (room temperature and 35 °C) in duplicate using the strains of *Lactobacillus plantarum*, for bacterial fermentation, and *Aspergillus oryzae* and *Rhizopus oligosporus* in succession for solid-state fungal fermentation as inocula. In addition, the natural or spontaneous and back-slopping methods of bacterial fermentation have been done on the above four substrate groups. The pH and essential amino acid profiles of the different fermentation processes were compared. The back-slopping in teff at a temperature of 35 °C gave the sharpest pH drop. All fermentations done at 35 °C showed a steeper slope in their pH versus time plot compared to their room temperature counterpart. Fungal fermentation gave an improved amino acid profile for the essential ones in all of the substrate groups, except in pure grass-pea. Fermented teff/grass-pea (8:2) in this fungal fermentation has been found to be quite comparable in essential amino acid profile to an ideal reference protein recommended for children of 2−5 years of age. None of the bacterial fermentations produced a net change in their essential amino acid profile in any of the substrate groups investigated. Solid state fungal fermentation on pure grass-pea using the fungal strains *R. oligosporous* and *A. oryzae* in succession has shown that the neurotoxin β-*N*-oxalyl-α,β-diaminopropionic acid (β-ODAP) in grass-pea has been removed by 80% on average for the high-toxin variety and by up to 97% for the low-toxin variety as determined by an improved chromatographic method with bioelectrochemical detection coupled on-line with refractive index detection.

KEYWORDS: Fermentation; grass-pea; chromatography; biosensor; β-ODAP

INTRODUCTION

Fermentation is one of the oldest methods of food processing and food preservation used by mankind. In the present time specific cultures are prepared for different fermentation processes, and these are carried out under defined conditions of time, temperature, pH, water content, etc. Consequently, the finished product is anticipated to have good quality, desirable characteristics, and safety (*1*). Fermentation is claimed to have a number of beneficial effects. For example, it may increase the protein content and improve its quality and digestibility, it may increase vitamin levels and mineral bioavailability, and it may reduce certain toxic substances, including cyanide and hemoglutinins (*1*, *2*). Fermented food products generally have a very good safety record even in developing countries, where they are processed by common people who do not know the underlying principle (*3*).

The safety of fermented foods is generally related to several principles. The first principle is that the fermenting food already overgrown with desirable, edible microorganisms becomes resistant to invasion by spoilage, toxic, or food-poisoning microorganisms (*3*). In tempeh fermentation (a solid state fungal, *Rhizopus oligosporous*, fermentation), antibacterial activity observed by Wang and Hesseltone (*4*) could explain the safety factor involved in tempeh fermentation. Moreover, the optimum temperature for this mold, which is 35−42 °C (*5*), is already too high for many bacteria and molds to grow. Still another safety factor comes from the carbon dioxide produced by the mold, which further inhibits some potential spoilage organisms. Generally the combination of relatively low pH, no free water, and a high temperature in tempeh fermentation enables *R. oligosporus* to overgrow soybeans in 18 h (*3*).

Low pH and anaerobic conditions could also account for the principles behind the safety of fermented foods. The antimicrobial effect of lactic acid and other metabolites that are formed during fermentation gives rise to a good safety record

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of the second major class of fermented foods—lactic acid fermented foods (*5*). Dairy products, vegetable foods, and vegetable/fish/shrimp mixtures are preserved around the world by lactic acid fermentation (*6*−*9*).

The high-alkaline condition in some fermentations is another safety principle. The highly proteolytic desirable microorganisms such as *Bacillus subtilis* and related Bacilli hydrolyze proteins and amino acids to release ammonia (*3*). The pH under these conditions rises and reaches as high as 8 or even higher. The combination of high pH and free ammonia along with very rapid growth of the essential microorganisms at relatively high temperatures, >40 °C, makes it very difficult for spoilage microorganisms to grow.

A valid concern in food safety is toxins such as mycotoxins and others that are present in many cereal grains and legume substrates. Van Veen (*10*) reported that the ontjom mold *Neurospora* and the tempeh mold *R. oligosporus* could decrease the aflatoxin content of peanut press-cake by 50 and 70%, respectively, during fermentation. Bau et al. (*11*) showed that a 24 h fermentation of rapeseed meal using *R. oligosporus* eliminated 56.5% of alkenyl glucosinolates and 93% of indol glucosinolates. Recently Lambein et al. (*12*) reported that fermentation of grass-pea seed with *A. oryzae* NRRL 1998 and *R. oligosporus* sp T-3 in succession for 48 h each reduced the neurotoxin, *β*-*N*-oxalyl-α,*β*-diaminopropionic acid (*β*-ODAP), to <10% of the original level.

In the present work fermentation of the cereal teff and the legume grass-pea either in pure form or in mixtures (9:1 and 8:2, teff/grass pea) was carried out with different inocula for the purpose of comparison of the essential amino acid and pH profiles of the fermentation product. Mixing teff (a cereal) with grass-pea (a legume) is a way of improving the amino acid profile of the final fermented product, as they are complementary in their amino acid compositions (*13*). Particularly, the relatively high content of lysine in grass-pea (>3 times higher than in teff) is attractive as admixture in the development of nourishing cereal-based foods.

The major objective of the double solid state fermentation of grass-pea using *A. oryzae* and *R. oligosporus* in succession is to investigate its potential in bringing detoxification and hence safe consumption of the final product. The presence of the neurotoxin, *β*-ODAP, is both a cause of public health problem and a barrier to the utility of a very beneficial staple crop, grass-pea. In a previous paper (*12*) it was shown that the toxin in grass-pea could be reduced by up to 90% by the same solid state fungal fermentation as used in the present study; however, the sample pretreatments used in that work have been deliberately avoided as it is well-known that soaking and/or cooking of grass-pea has a toxin removal effect (*14*−*17*). Moreover, control samples that have passed through all of the sample pretreatments except inoculation by the microbes have been used to calculate the percent toxin removal by fermentation. An attempt is therefore made hereby to exclusively determine the exact role of the solid state fermentation in toxin removal.

MATERIALS AND METHODS

Materials. Teff seeds (var. DZ-01-96) were generously provided by the Ethiopian Agricultural Research Organization (Addis Ababa). Grass-pea seeds were collected from Fogera and Bichena districts of northwestern Ethiopia. Lyophilized preparations of *Aspergillus oryzae*, *Rhizopus oligosporus*, and *Lactobacillus* (LAB) *plantarum* were purchased from Culture Collection, University of Göteborg (CCUG), Göteborg, Sweden. L-Glutamate oxidase (*Streptomyces* sp. code 7804, lot 01105, EC 1.4.3.11, 50 units) was from Yamasa Corp. (Tokyo, Japan). Horseradish peroxidase (catalog no. P-8375, type VI, EC 1.11.1.7) and α-aminoadipic acid (Sigma catalog no. a-7275) were from Sigma Chemical Co. (St. Louis, MO). Poly(1-vinylimidazole){osmium (4,4′-dimethyl-bpy)$_2$ Cl}$^{3+/2+}$ was generously provided by TheraSense Inc. (Alameda, CA). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences, Inc. (Warrington, PA, catalog no. 08210). Polyethyleneamine (PEI), L-glutamic acid (EC no. 200-293-7), *o*-phthaldehyde (OPA), *β*-ODAP (catalog no. 0-5382), and diaminopropionic acide (DAP) were from Sigma. Tris(hydroxymethyl)-aminomethane and potassium chloride were from Merck (Darmstadt, Germany). Pure water was produced by Milli-Q water equipment (Millipore, Bedford, MA) and was used in all preparations involving water.

Sample Preparation for Fermentation. Teff and grass-pea seeds were manually cleaned, washed with tap water, rinsed with distilled water, and dried immediately in an oven at 50 °C for ∼6 h under a circulating air. Grits and flours were then prepared for fungal and bacterial fermentations, respectively. Clean seeds (whole seed for teff and dehusked seeds for grass-pea) were ground to flour to pass through a 1 μm sieve by a standard mill (Cyclotec 1093 sample mill, Tecator, Höganäs, Sweden). Grits for fungal fermentation were prepared by grinding clean whole seeds of teff and dehusked grass-pea by a mill (Cemotec 1090 sample mill, Tecator) and sieving between sieves of mesh sizes 32 and 62.

Inoculum Preparation. *L. plantarum* was grown on autoclaved MRS broth (Merck) and counted on MRS agar (Merck) by a standard plate counting method. The bacterial stock suspension in the MRS broth was stored under sterile condition in a cold room (4 °C) until activation for immediate inoculation to the fermenting medium. The fungal strains of *A. oryzae* and *R. oligosporus* were grown on autoclaved potato dextrose agar (Merck) until complete spore formation (3 days for *Rhizopus* and 4 days for *Aspergillus*). The spores were then harvested by scratching/washing the surface of the agar medium with peptone (Merck) solution (0.1%). The number of spores per milliliter of peptone was obtained by a standard plate counting method, using potato dextrose agar as growth medium. The spores were then stored in a sterile bottle suspended in peptone (0.1%) solution in a cold room (4 °C) until inoculation into the fermenting sample.

Fermentation. *Bacterial Fermentation.* Four groups of fermentation substrates were identified as pure teff, pure grass-pea, and teff and grass-pea mixtures of 9:1 and 8:2. Exactly 30 g of flour sample from each group (duplicate) was mixed with 75 mL of distilled water in a 250 mL narrow-base (∼7 cm i.d.) beaker. The contents of the beakers were thoroughly mixed after addition of the appropriate inoculum and covered with perforated aluminum foil. Those beakers for room temperature (23 ± 1 °C) fermentation were allowed to stand on a laboratory bench, whereas those for fermentation at 35 °C were immersed in a thermostated water bath at the specified temperature. Spontaneous fermentation was the one without deliberate addition of inoculum, that is, making use of the natural microflora. Back-slopping fermentation was done by transferring about 5% of a previously fermented lot (called *ersho* by the local people) to the fermenting substrate as a starter culture.

The initial pH and its changes throughout the fermentation time were followed by dipping a pH electrode into the beaker slightly above the bottom of the beaker. The fermentation was stopped when the pH had reached a value of ∼4. All fermentations except solid state fermentation of grass-pea were done in duplicate.

Fungal Fermentation. Fungal fermentation was carried out for the above substrate groups at room temperature and at 35 °C, in duplicate, except for pure grass-pea, which was done in triplicate. Exactly 30 g of grit samples was steamed at 121 °C for 10 min, cooled to room temperature, and acidified with dilute lactic acid (50 g L^{-1}) to bring to a pH of 4. The acidified substrate was then inoculated with a suspension of *A. oryzae* CCUG 33812 (10^6 spores/5 g of substrate), mixed thoroughly, and spread on a glass Petri dish. The dishes were covered with perforated aluminum foil, and those for room temperature fermentation were kept in a cabinet, whereas those for fermentation at 35 °C were placed in an incubator at the specified temperature. Following 48 h of fermentation, both groups of samples were steamed for 10 min at 121 °C to destroy *A. oryzae* CCUG 33812. The samples were then cooled to room temperature before the next inoculation with

R. oligosporus sp T3 (10^6 spores/5 g of substrate), and the procedure was repeated as above. The fermented samples were then freeze-dried and ground into powder (particle size ≤ 1 μm) with analytical mill A10 (Janke & Kunkel, IKA Labortechnik, Germany) for subsequent analysis. Control samples were prepared according to the same procedure except inoculation with the fungal strains.

Preparation of Biosensor. A bilayer bienzyme electrode involving a mediator, poly(vinylimidazolyl) osmium (4,4′-dimethylbipyridine)$_2$, $Cl^{2+/3+}$, denoted PVI_{12}-dmeOs, a cross-linking polymer [poly(ethylene glycol) (400) diglycidyl ether] (PEGDGE), and a highly positively charged polymer, polyethyleneimine (PEI), with two enzymes, glutamate oxidase (GlOx) and horseradish peroxidase (HRP), was used as a postcolumn electrochemical detector. The construction of the redox hydrogel biosensor is exactly the same as described in ref *18*.

Liquid Chromatography. The chromatographic system is made up of an isocratic pump (model 2150, LKB, Bromma, Sweden), a Carbopac anion exchange column (PA1, 4 × 250 i.d. model P/N 35359, Dionex, Sunnyvale, CA), and a guard column (PA1 guard, 10-32, P/N 43096, Dionex). The configuration of the instrumental setup and sample extraction procedures used for the chromatographic analysis were exactly the same as the one described in ref *19*.

Spectrophotometry. For the screening of grass-pea collections for their toxin level by spectrophotometry, ~80 mg of dehusked and powdered grass-pea sample was suspended in 8 mL of Milli-Q water and subjected to 1 h of sonication at 40−45 °C to extract ODAP (*20*). Then it was centrifuged at 4000 rpm, and a 0.1 mL aliquot of the supernatant was transferred to a 10 mL test tube and subjected to basic hydrolysis to convert ODAP to L-2,3-diaminopropionic acid (DAP), that is, formed after cleavage of the −COCOOH group using 0.2 mL of 3 M KOH in a boiling water bath for ~30 min. After neutralization of the hydrolysate with 0.2 mL of 3 M HCl, and making the volume up to 1 mL, 2 mL of OPA reagent, composed of 200 μL of mercaptoethanol and 100 mg of *o*-phthalaldehyde dissolved in 100 mL of 5 M potassium tetraborate tetrahydrate (*21*), was added to form a colored adduct. Absorbance of the colored adduct was measured at 476 nm against a reagent blank after 30 min of standing time, after which the color was fully developed.

Dry Substance Determination. About 1 g of powder sample was put into a preweighed crucible and was kept in an oven at 110 °C for ~24 h. Crucibles with dry matter were reweighed after cooling to room temperature in a desiccator. The percent of dry matter in each sample was determined by weight difference.

pH Measurement. *Bacterial Fermentation.* The pH of the fermenting substrate was measured at an interval of 2 h by the use of a pH electrode (Orion) connected to a digital readout unit (Orion, model 8103 Ross, Beverly MA). The electrode was dipped well inside the fermenting slurry, leaving some space above the base of the beaker. The measurement was continued until the value reached ~4 (marking the end of fermentation) for the fermenting sample in the beaker.

Fungal Fermentation. For measurement of the pH change in the fungal solid state fermentation, 0.2 g of the fermenting sample was withdrawn at intervals of 4 h and suspended in 1 mL of deionized water. The electrode tip was then immersed into the suspension for reading the pH.

Amino Acid Analysis. A sample of powdered fermented food product, 50 mg for grass-pea and 200 mg for the rest of the substrates, was weighed and carefully transferred to the bottom of a 25 mL test tube, to which was added 5 mL of 6 M HCl containing 0.1% phenol. The tube was then sealed by melting in a flame. The sealed tube was then kept in an oven at 110 °C for 24 h for complete hydrolysis of the protein. The hydrolysis products were filtered with glass wool in a funnel, out of which 2 mL of the filtrate was transferred into a round-bottom flask. To this was added 100 μL of internal standard (α-aminoadipic acid). The mixture was then evaporated to dryness with a rotavapor (water bath at 40 °C). The dry sample left behind was redissolved with 5 mL of buffer and filtered through a 0.45 μL membrane filter into an Eppendorf tube. Analysis of individual amino acids was done with an amino acid analyzer (model LC 5001, Biotronic, München, Germany).

For quantitative determination of cysteine and methionine, both of them were first converted into their stable oxidized forms by using freshly prepared performic acid. For the preparation of performic acid 45 mL of 88% formic acid was mixed with 5 mL of 30% hydrogen peroxide solution (Merck). After 1 h of standing time at room temperature, the mixture was cooled in an ice bath for 10−15 min. For low-protein samples (teff, teff/grass-pea mixtures of 9:1 and 8:2) 500−600 mg and high-protein samples (grass-pea) 200 mg, respectively, were weighed and poured into a 100 mL round-bottom flask in an ice bath, into which was added 5 mL of the cold performic acid solution. The samples were sonicated only when necessary for dissolution and kept in an ice bath in a cold room for ~16 h. Then half a teaspoon of sodium disulfite and 50 mL of 6 M HCl were added to each flask and subjected to a reflux for 24 h, covered with aluminum foil to prevent exposure to light. After refluxing, the contents of the flask were quantitatively transferred to a 100 mL volumetric flask and diluted to the mark with deionized water. The rest of the procedure was like that of the other amino acids.

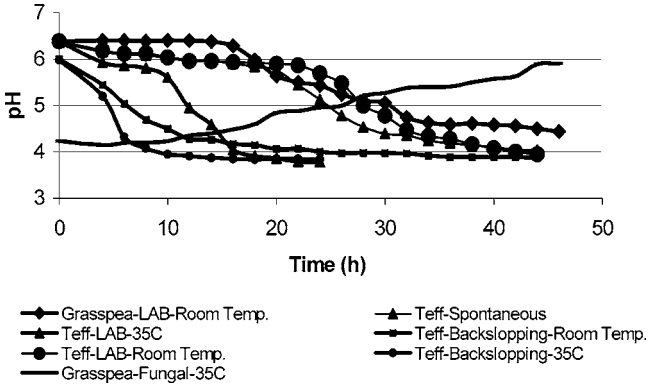

Figure 1. pH profiles of some representative fermented samples.

RESULTS AND DISCUSSION

pH Profile. The pH of all of the fermented samples was measured at an interval of 2 h, and the results for a few representative fermentations are plotted as pH versus time (**Figure 1**). Fermentation by the method of back-slopping at room temperature for pure teff gave a fast pH drop, reaching a value of 4 in <20 h of fermentation. The same fermentation at 35 °C reached a pH of 4 in <10 h. The back-slopping method of fermentation, as used in the household, has been done by using some portion of the previously fermenting lot (ersho) as a starter culture. The indigenous microflora in the fermenting teff by the back-slopping method is believed to be already well adapted and activated enough to the condition of fermentation. Hence, the microbes did not take much time to proliferate and resume maximum growth rate in a short while. The production of lactic acid by the indigenous microflora and the fast pH drop account partially for the inhibitory effect of fermented foods on spoilage and pathogenic microorganisms. In the bacterial fermentations, the organic acids produced as determined in a similar fermentation (*22*, *23*) such as lactic acid ($pK_a = 3.86$) and acetic acid ($pK_a = 4.75$) are predominantly in their protonated forms at such low pH values. The inhibition by organic acids has been attributed to the protonated forms of these acids, which are uncharged and may therefore cross biological membranes. The cytoplasmic conditions in the microorganisms, however, allow dissociation of the acids and give rise to inhibition of growth (**Figure 2**). Inhibition is caused by acidification and/or accumulation of anions inside the cell (*2*, *24*). The quick pH fall in back-slopping at 35 °C, therefore, gives little chance for infection.

Spontaneous fermentation of teff and that with pure LAB as a starter culture at room temperature showed similar pH profiles, the spontaneous fermentation being slightly lower in pH at any

Table 1. Amino Acid Profile of Raw and Fermented Substrate Groups[a]

	fermented, mg/g, dry wt																	raw, mg/g, dry wt			
AA	T-Sp RT	9:1 Sp	8:2 Sp	T BS RT	T Bs 35°C	8:2 Fung 35°C	8:2 Fung RT	9:1, BS RT	8:2, BS RT	Gp, BS RT	T LAB RT	9:1 LAB RT	8:2 LAB RT	T Fung 35 °C	9:1 Fung RT	8:2 Fung RT	Gp Fung RT	T	9:1	8:2	Gp
Thr	3.4	5.0	6.0	3.6	3.4	8.7	7.8	4.5	5.5	9.8	3.6	5.6	6.3	5.4	6.3	7.8	11.6	4.9	5.5	6.6	12.0
Val	4.4	5.0	5.9	4.3	4.4	8.9	8.7	5.3	6.3	12.4	4.5	5.3	6.1	6.1	6.9	8.7	13.8	4.8	5.5	6.1	14.2
Ile	2.7	4.2	4.9	3.1	2.8	7.8	7.6	3.9	5.2	11.4	3.1	3.9	4.9	4.7	5.9	7.6	12.7	3.6	4.2	5.0	12.1
Leu	7.8	9.6	9.9	7.3	7.6	14.0	13.1	9.8	10.9	22.2	8.3	9.9	10.5	10.1	10.7	13.1	21.7	7.4	9.0	10.0	23.3
Phe	4.7	5.6	6.2	4.5	4.5	8.2	7.9	5.8	6.5	12.5	4.7	5.8	6.4	5.9	6.6	7.9	12.9	4.6	5.8	6.2	13.7
Lys	3.1	5.1	6.3	2.9	2.9	9.6	8.7	5.2	6.7	20.0	3.2	5.2	6.8	4.5	6.1	8.7	19.6	3.8	5.7	7.3	22.0
His	2.1	2.9	3.0	1.9	1.9	3.9	3.6	2.8	3.3	7.7	2.1	2.9	3.3	2.2	2.8	3.7	6.9	2.0	2.6	3.2	7.1
Met	1.9	1.0	0.8	1.5	1.7	2.9	3.0.3	0.9	0.8	0.3	2.1	1.5	0.6	2.8	2.9	3.3	0	1.0	1.0	0.8	1.1

[a] T = teff; Sp = spontaneous fermentation; BS = back-slopping fermentation; LAB = *Lactobacillus* fermentation; Fung = fungal fermentation; RT = room temperature; 9:1 = substrate teff/grass-pea (9:1 ratio); 8:2 = substrate teff/grass-pea (8:2 ratio); Gp = grass-pea. Values given are calculated with RSD level ≤5% (n = 3) and dry weight basis.

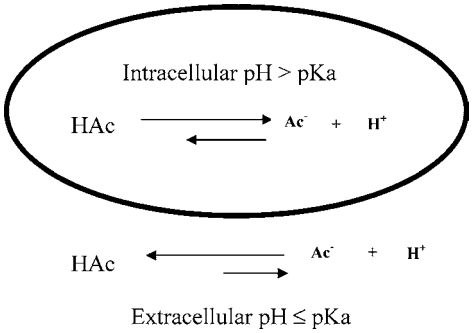

Figure 2. Diffusion of a weak organic acid into a microbial cell and its dissociation yielding protons and potentially toxic anions.

time during the fermentation period. This may be due to the effect of mixed culture in the case of the spontaneous fermentation. Comparison of fermentation of teff and grass-pea at room temperature, using LAB as a starter culture, showed that the pH of teff was significantly lower than that of the grass-pea in most of the time of fermentation. This could be ascribed to the high protein content of grass pea, which renders a buffering capacity and hence a slower change in pH. The reversal in the trend for some short interval in the middle of the plots, however, may be due to experimental error.

In the fungal fermentation experiment, the trend in pH change was a progressive increase to >7 (**Figure 1**). This could be due to the active proteolysis and deamination of amino acids by the mold (*3*). The increase in pH with fermentation time is not an unusual phenomenon. A similar trend was reported in tempeh fermentation (*25*).

Amino Acid Profile. At the end of fermentation, marked by a pH of ∼4, the edible fraction of the fermented product was analyzed for total amino acid content, except tryptophan (tryptophan is completely destroyed by the hydrolysis step). The analysis was done in duplicate (i.e., two separate fermentations). For the purpose of evaluation of the precision of the method, triple fungal fermentations of grass-pea were done, and analysis of total amino acids was shown to differ to a maximum of 5% (i.e., RSD of ≤ 5, n = 3). Therefore, a difference of ≤5% RSD in milligrams of amino acid per 100 g of dry sample is considered to be within the experimental error. The results are discussed only with regard to the essential amino acids as they are interesting from a nutritional point of view.

For teff, the spontaneous fermentation showed decreases in threonine, valine, isoleucine, and lysine by 30, 8, 20, and 17%, respectively (**Table 1**). Amino acids such as leucine, phenylalanine, and histidine showed no significant change, whereas methionine was increased by 106%. The situation is very similar in spontaneous fermentation of a teff/grass-pea mixture (9:1), except that leucine and histidine showed increases of 7 and 11%, respectively (**Table 1**). The substrate teff/grass-pea (8:2) has undergone decreases in threonine and lysine (8 and 13%, respectively) and a nonsignificant change for all other essential amino acids (**Table 1**). The same fermentation done at 35 °C gave essentially the same effect on the essential amino acid profile. This was a common observation in almost all types of fermentation on almost all substrate groups (**Table 1**).

The back-slopping fermentation of teff gave essentially the same pattern of essential amino acids as that of the spontaneous counterpart (**Table 1**). This similarity of the back-slopping and spontaneous fermentation also holds true for the substrates teff/grass-pea (9:1) (**Table 1**) and (8:2) (**Table 1**), respectively. The back-slopping fermentation of grass-pea was, however, notably different from the other substrate groups in that all of the essential amino acids except histidine (which increased by 8%) tended to decrease (**Table 1**).

Fermentation with LAB as inoculum gave different patterns among the four substrate groups. For teff, threonine, isoleucine, lysine, and valine decreased by 28, 14, 17, and 6%, respectively (**Table 1**). There was an increase in the amounts of leucine (12%) and methionine (112%), whereas phenylalanine and histidine remained practically the same. For the mixture 9:1 (teff/grass-pea), isoleucine and lysine were found to undergo marginal decreases, 6 and 9%, respectively. Methionine, histidine, and leucine marginally increased. There was no significant change in the contents of threonine, valine, and phenylalanine (**Table 1**). The other mixture substrate, that is, 8:2, showed a marginal increment for leucine (6%) and the same marginal decreases in threonine and lysine (6 and 7%, respectively) (**Table 1**). The fermentation of grass-pea with this inoculum was different from all other bacterial fermentations in one important aspect—lysine did not change. Lysine consistently decreased in all other cases. This is probably due to the already high lysine content in grass-pea. In general, the bacterial fermentations did not improve the essential amino acid profile of the fermented food product. A similar observation was made by Steinkraus, who studied acid fermentation of some indigenous foods (*26*). It is therefore considered that this has no important consequence, in particular to the status of dietary protein. The decrease in the lysine content of the fermented food product in almost all cases of bacterial fermentation is rather disadvantageous as fermentation counteracts the complementary action of mixing of a cereal and a legume in their amino acid profile.

The fungal fermentation, on the other hand, improved the essential amino acid profiles of teff and teff/grass-pea (9:1 and 8:2) mixtures. In all of these substrate groups almost all amino

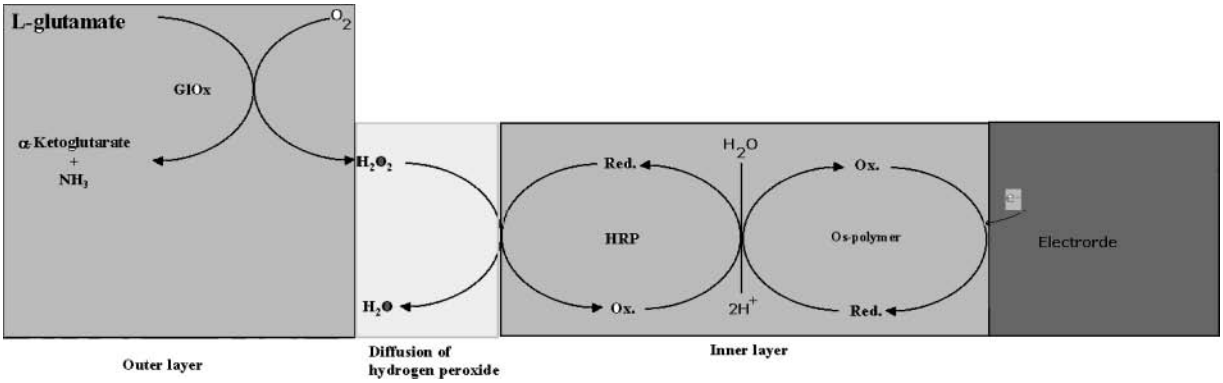

Figure 3. Reaction sequence of the bilayer bienzyme electrode for the detection of L-glutamate and β-ODAP.

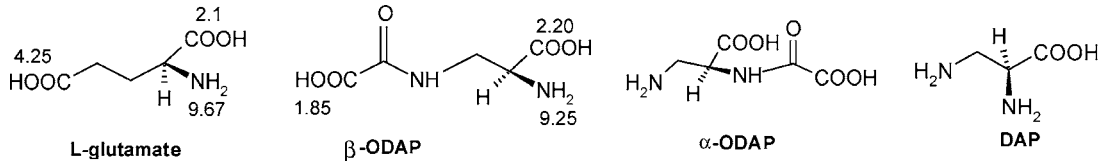

Figure 4. Structures of L-glutamate, β-ODAP, and α-ODAP.

Table 2. Comparison of Fungal Fermented Teff and Mixtures of Teff with Grass-pea (9:1 and 8:2) for Amino Acid Pattern with an Ideal Reference Protein[a]

	amino acid composition, mg/g of protein, dry wt basis					
amino acid	ref	raw T	BS-T-RT	Fung T35	Fung 9:1-RT	Fung 8:2-35
His	26	26	26	30	28	31
Ile	46	45	38	59	57	62
Leu	93	92	103	127	103	111
Lys	66	48	40	56	59	76
Met + Cys	42	17	30	50	36	39
Phe + Tyr	72	103	97	132	113	115
Tre	43	62	45	66	61	69
Trp	17					
Val	55	59	56	76	67	71

[a] Ref = recommended reference protein for ages 2–5 years. BS-T-rt = backslopping fermentation of teff at room temperature. Fung T35 = fungal fermentation of teff at 35 °C. Fung 9:1RT = fungal fermentation of teff/grass-pea (9:1) at room temperature. Fung 8:2-35 = fungal fermentation of teff/grass-pea (8:2) at 35 °C. Results given have RSD of ≤5%, $n = 3$.

acids increased from 15 to 55% (**Table 1**). The effect was even higher for methionine, which increased from 100 to 300%. This improvement in amino acid profile could be due to the metabolizing ability of the fungal strains (*R. oligosporus* and *A. oryzae*) on the nonprotein nitrogenous components of the substrates. The same effect, however, was not observed when pure grass-pea was used as a substrate for the fungal strains. It showed an increase for isoleucine (8%), decreases for leucine and lysine by 7 and 13%, respectively, and nonsignificant changes for threonine, valine, phenylalanine, and histidine (**Table 1**).

The protein qualities of fermented samples of teff, teff/grass-pea mixtures of 9:1 and 8:2 were compared with an ideal protein having the essential amino acid composition of the recommended (*13*) level for 2−5-year-old children (**Table 2**). Teff, fermented by the back-slopping technique, was found to be deficient in lysine (as is the unfermented raw teff), and in sulfur-containing amino acids. Fungal fermentation of teff, however, improved the situation and gave a profile comparable to the reference protein. The highest improvement in the quality of protein is achieved by fungal fermentation of a mixture of teff and grass-pea in an 8:2 ratio. In this substrate group, with the exception of the sulfur-containing amino acids, which are nearly the same as the ideal reference protein, all other essential amino acids have been at a level quite higher than the recommended level for the age group.

***β*-ODAP Levels of Fermented Grass-pea.** Prior to fermentation, collected grass-pea samples were screened for their toxin levels by a spectrophotometric assay (*21*) to select one high-toxin variety (HTV) and another low-toxin variety (LTV) for further processing by fermentation and an accurate estimation of the toxicity level by a β-selective method. The highest toxin level found was 0.83% and the lowest 0.57%, on a dry weight basis. It should be noted that this method is nonselective and measures the total amount of α- and β-ODAP (i.e., nontoxic and toxic isomers of ODAP, respectively). Nonetheless, the content of the nontoxic α-isomer is ∼5% in raw seed and the method is good enough for screening purposes.

With the analytical system employed, fully described in ref *19*, it was possible to determine simultaneously the total concentration of ODAP (i.e., the sum of α-ODAP and β-ODAP), selectively that of the toxic isomer, β-ODAP, and free glutamate in fermented and control *Lathyrus sativus* seed samples. The reaction sequence of the bioelectrochemical detection system is illustrated using L-glutamate as analyte (**Figure 3**). Structures of interest for the present analysis are shown in **Figure 4**. Chromatograms of fermented/control samples are shown in **Figure 5.** Grass-pea was fermented in triplicate, and each fermentation product was measured in triplicate (i.e., three extractions) for the toxin content. The measurements showed that the fungal strains used for the fermentation reduced the toxin level of the HTV on the average by ∼80% and that of the LTV by 96%. In view of the fact that certain processings such as soaking, cooking, and some others (*14*−*17*) have some toxin removal effect, avoiding such sample pretreatment for the fermentation in the present work has showed the exact role played by the microbes in detoxifying the raw material by fermentation. The present study therefore shows (**Table 3**) that the majority of the toxin contained by grass-pea seeds could be removed by simple solid-state fungal fermentation. Validation of the analytical system for β-ODAP determination has been described in ref *19*.

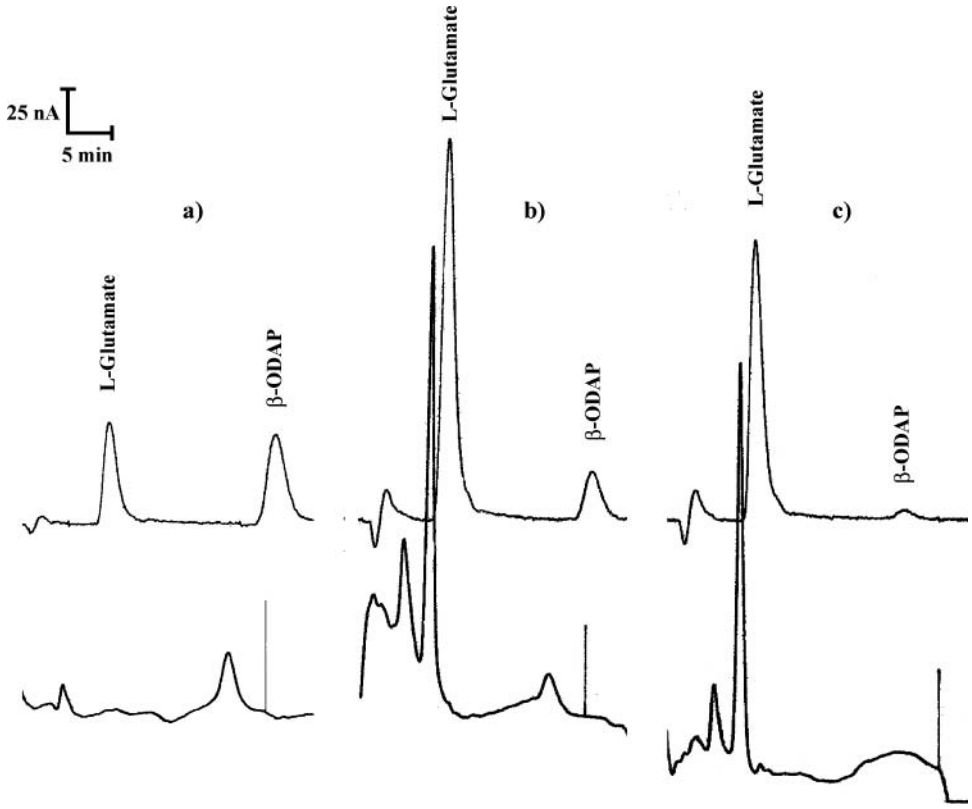

Figure 5. Chromatograms of real sample analysis: bioelectrochemical detection (upper) and refractive index detection (lower): (**a**) control sample; (**b**, **c**) fermented HTV and LTV, respectively. Conditions: carrier buffer, Tris/Cl^- (10:10 mM, pH 7.5); flow rate, 0.8 mL/min; injection volume, 30 μL.

Table 3. Effect of Solid State Fungal Fermentation of Grass-pea on β-ODAP and Glutamate Level

sample code	% β-ODAP, dry wt basis		% β-ODAP decrease	% glutamate, dry wt basis	
	control[a]	fermented[b]		control[a]	fermented[c]
F_{LTV1}-I	0.52	0.016	97	0.16	0.29
F_{LTV1}-II	0.52	0.016	97	0.16	0.28
F_{LTV2}-I	0.52	0.04	92	0.16	0.28
F_{LTV2}-II	0.52	0.03	94	0.16	0.28
F_{LTV3}-I	0.52	0.005	99	0.16	0.41
F_{LTV3}-II	0.52	0.017	97	0.16	0.41
F_{HTV1}-I	0.76	0.21	72	0.11	0.44
F_{HTV1}-II	0.76	0.20	74	0.11	0.45
F_{HTV1}-I	0.76	0.14	82	0.11	0.40
F_{HTV2}-II	0.76	0.15	80	0.11	0.40
F_{HTV3}-I	0.76	0.14	82	0.11	0.32
F_{HTV3}-II	0.76	0.14	82	0.11	0.32

[a] Dry weight basis, mean value of three determinations ± 0.017 (mean SD). [b] Dry weight basis, mean value of three determinations ± 0.014 (mean SD). [c] Dry weight basis, mean value of three determinations ± 0.021 (mean SD).

L-Glutamate Levels of Fermented Grass-pea. With the present analytical system it was also possible to measure the amount of free L-glutamate in raw and fermented grass-pea. In all of the samples the glutamate level increased after fermentation. The results are shown in **Table 3**. A dramatic increase in free glutamate level after fermentation has been reported in a similar work (*12*). L-Glutamate is an important amino acid to determine in foodstuffs. It serves as a flavor enhancer and hence is used to evaluate food quality. Quantification of L-glutamate is also useful in clinical diagnosis of some myocardial and hepatic diseases. The present method therefore offers an accurate and sensitive analytical potential for such purposes.

Conclusion. Grass-pea is a very interesting crop from nutritional and agricultural points of view. It is a cheap source of protein for the low-income part of populations in some countries and withstands adverse agricultural conditions. The presence of the neurotoxin in it has been the major drawback in the safe consumption of the crop. Many attempts have been made in the past to detoxify the crop in the field or postharvest; however, the problem of lathyrism, a crippling disease caused by β-ODAP, continues to occur and even some times in epidemic proportions. This study attempted to address nutritional and safety aspects of food products containing grass-pea.

Mixing of teff and grass-pea in a ratio indicated and the subsequent fermentation have led to a product having an amino acid profile comparable to the one recommended for 2−5-year-old children. The results obtained with the 8:2 teff/grass-pea mixture are very encouraging. The amino acid profile is even better than the recommended level. Ascertaining the safety level of food products containing grass pea is a primary task as no food, however nourishing, is acceptable if it poses a health risk. In this regard the present investigation has followed a systematic approach to evaluate the exact role the microbes have in bringing detoxification to the seeds of grass pea. Moreover, the β-ODAP selectivity of the detection method used was of utmost help to accurately determine the toxin content in the food product. The huge reduction of the toxin contained in grass-pea after the solid state fermentation (up to 96%) is really attractive as the only drawback in the consumption of this otherwise highly beneficial crop is the presence of the neurotoxin, β-ODAP. Coupling fermentation with other food-processing methods such as soaking, steeping, and roasting could result in total toxin removal.

The results in the present investigation have therefore convincingly indicated that fast, simple, cheaper, and safer food processing by fermentation is possible for food products containing grass-pea. The implication is that this cheap source of protein can help to develop an affordable nutrient-dense

weaning food in developing countries. However, before recommendation of the present technique for household and/or large scale food processing, animal experiments for toxicity and a proper sensory evaluation may be necessary.

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