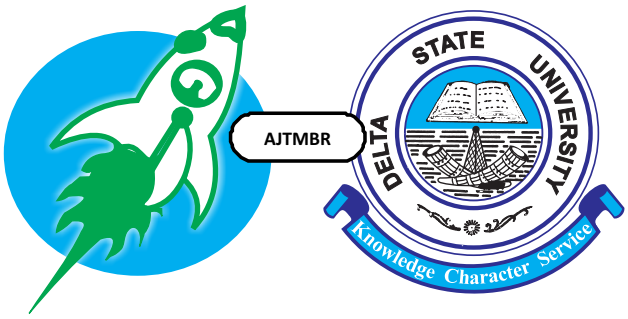
African Journal of Tropical Medicine and Biomedical Research (AJTMBR)



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Focus and Scope

The African Journal of Tropical Medicine and Biomedical Research is a multidisciplinary and international journal published by the College of Health Sciences, Delta State University of Abraka, Nigeria. It provides a forum for Authors working in Africa to share their research findings on all aspects of Tropical Medicine and Biomedical Sciences and to disseminate innovative, relevant and useful information on tropical medicine and biomedical sciences throughout the continent. The journal will publish original research articles, reviews, editorials, commentaries, short reports, case reports and letters to the editor. Articles are welcome in all branches of medicine and dentistry including basic sciences (Anatomy, Biochemistry, Physiology, Pharmacology, Psychology, Nursing etc) and clinical sciences (Internal Medicine, Surgery, Obstetrics and Gynaecology, Dental surgery, Child Health, Laboratory Sciences, Radiology, Community Medicine, etc). Articles are also welcome from social science researchers that document the intermediating and background social factors influencing health in countries of Africa. Priority will be given to publication of articles that describe the application of the principles of primary health care in the prevention and treatment of diseases.

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Production of L-lysine under submerged fermentation by *Corynebacterium glutamicum* using different agricultural plants leaves

Theresa Ezedom[1], Egoamaka Oliseneku Egbune[2], Solomon Adanoritsewo Atseponu[2], Mary Ogochukwu Charles[2], Blessed Achugbue Benson[2], Diana Ebbah[2], Promise Chika Amechi[2], Oghenetega Benjamin[2], Akpevweoghene Rejoice Egbodje[2], Lucky Ebinum[2], Blessing Ifechi Chukwudozie[2], Stephen Eboe[2], Ifeanyi Benedict Alexander[2], Sophia Fejiro Edijana[2] and Nyerhovwo Tonukari[2]*

ABSTRACT

Introduction: This study investigated the production of L-lysine using *Corynebacterium glutamicum* and various leaf extracts (cassava, palm tree, maize, cowpea, cocoyam, and plantain). The study also explored the activities of amylases and proteases, as well as the levels of total soluble proteins, reducing sugars, glucose, flavonoid and phenolic contents, and pH changes.

Materials and Methods: Different treatments (extract, boiled extract, extract + *C. glutamicum*, boiled extract + *C. glutamicum*) were examined for their effects on L-lysine concentration. Additionally, the activities of amylases and proteases, as well as levels of total soluble proteins, reducing sugars, glucose, flavonoid and phenolic contents, and pH changes, were analyzed.

Results: Maize leaf extract + *C. glutamicum* exhibited the highest L-lysine concentration (1.771a±0.1 mg/g), while boiled cassava leaf extract showed the lowest concentration (0.023b±0.1 mg/g). Palm tree leaf extract had significantly higher reducing sugar levels compared to other extracts. Boiled plantain leaf extract fermented by *C. glutamicum* had the highest total soluble protein level (9.5±0.2 mg/g), while cassava leaf extract had the lowest (2.1±1.2 mg/g).

Conclusion: Submerged fermentation of leaf extracts using *C. glutamicum* can be utilized for L-lysine production. The study highlights the influence of different leaf extracts and treatments on L-lysine production, as well as on amylase and protease activities, total soluble protein levels, reducing sugars, glucose, flavonoid and phenolic contents, and pH values. These findings provide valuable insights into the potential application of this approach for lysine production.

Key words: Agricultural leaves, submerged fermentation, *Corynebacterium glutamicum,* extract, boiling

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Introduction

Agricultural products are currently being used as low-cost carbohydrate source for the production of high value-added products such as amino acids[1-3].Amino acids are very important macromolecules required for both human and animal to function properly. L-Lysine is one of the nine essential amino acids required for human and animal nutrition. Studies have shown that the demand for L-lysine has been steadily increasing[4], with several hundred thousand tonnes produced annually[1,5]. Being an essential amino acid, its addition to foods and feeds improves protein quality resulting to better growth and tissue synthesis[5]. Different methods utilized in the production of lysine include chemical synthesis, fermentation, enzymatic method, and protein hydrolyzate extraction[6]. Of

these methods, fermentation remains the most practical and economical approach for the production of lysine as it requires low-cost carbon sources, low pressure and even moderate temperatures[7].

Corynebacterium glutamicum is majorly used for the production of amino acids such as L-glutamate and L-lysine industrially[8]. *C. glutamicum* is capable of utilizing different carbohydrates, organic acids and alcohols as sources of carbon and energy required for growth and production of amino acids[9-10]. Various organic and inorganic salts and compounds such as urea, ammonium salts and yeast extract can be utilized as nitrogen sources[11].

The use of agricultural plants or products as substrates for fermentative production of amino acids might provide cheap alternative for the manufacturing of lysine. Large-scale production of lysine using leaf extracts has not been reported in literature. The present study investigates the application of leaf extracts of tropical agricultural plants (cassava leaves, palm tree leaves, maize leaves, cowpea leaves, plantain leaves and cocoyam leaves) as alternative substrates for the production of L-lysine by *C. glutamicum*.

MATERIALS AND METHODS

Collection of materials

Leaves of cassava, palm tree, maize, cowpea, plantain, and cocoyam were harvested, identified, and authenticated by Plant Taxonomy and Molecular Systematics, Department of Botany, Delta State University, Abraka (Voucher numbers DELSUH 047, 132, 117, 068, 064, respectively). They were blended with water (1:10) using a commercial grinding machine and stored at room temperature.

Microorganism and preparation of inoculum for submerged fermentation

Corynebacterium glutamicum was obtained from Tonukari Biotechnology Laboratory in Delta State, Nigeria, and stored on glycerol at 4°C. For inoculation, a loop full of microorganisms was transferred to an inoculum media containing glucose, yeast extract, tryptone, NaCl, agar, and distilled water at pH 7. Homogenization was performed using 10 g of the pulverized sample. A 2 ml culture was used to inoculate a 100 ml Erlenmeyer flask with 20 ml fermentation broth. After a 72-hour incubation period at 30°C and 160 rpm on a rotary shaker, growth and lysine accumulation were assessed. Control flasks were not inoculated. Each extract underwent four different treatments: extract, boiled extract, extract + *C. glutamicum*, and boiled extract + *C. glutamicum*, with boiling lasting for 30 minutes. The reported values represent the average of at least two congruent samples.

Estimation of L-lysine and other biochemical parameters

L-lysine concentration was estimated using the acid ninhydrin method of Chinard[12]. Total soluble proteins were determined following the procedure by Gornall *et al.*[13] with bovine serum albumin as the standard. Reducing sugars were estimated using the 3,5-dinitrosalicylic acid (DNS) colorimetric technique[14]. Glucose content was determined using the Randox glucose kit as per the manufacturer's instructions. α-amylase activities were determined based on the procedure of Nouadri *et al.*[15], while protease (caseinolytic) activity was assayed using the method of Kunitz[16]. Total flavonoid and phenolic contents were determined using colorimetry methods described by Jia *et al.*[17] and Singleton and Rossi[18], respectively. pH measurements were taken using a Mettler Toledo pH meter.

Statistical analysis

All data were subjected to statistical analysis. Values were reported as mean ± standard deviation and the experimental results were analyzed using analysis of variance (ANOVA). The results were considered significant at p-values of less than 0.05 (95% confidence level; $p<0.05$).

Results

The results of the study on the determination of L-lysine concentration in submerged fermented agricultural by-products using *C. glutamicum* are summarized in Figure 1. Maize leaf extract + *C. glutamicum* showed the highest lysine production (1.771±0.1 mg/g), while boiled cassava leaves exhibited the lowest concentration (0.023±0.1 mg/g).

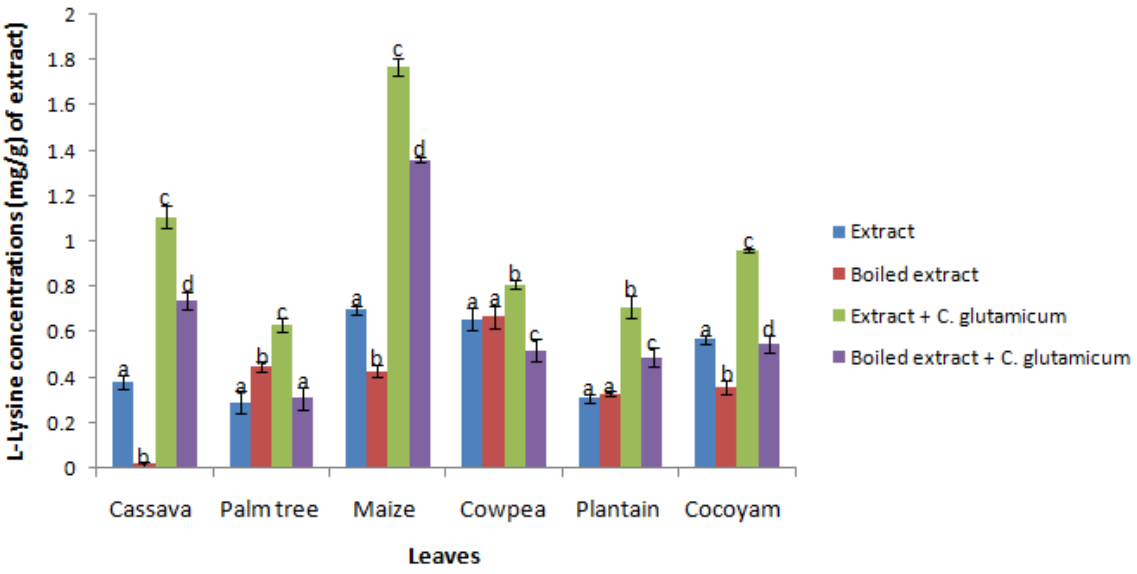

Figure 1. L-Lysine concentrations in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

Figures 2, 3, and 4 present the levels of total soluble proteins, reducing sugars, and glucose, respectively. Palm tree leaves showed a significant increase in reducing sugar content, while plantain leaves exhibited a significant decrease in all test groups compared to the extract. Palm tree leaves boiled before introducing C. glutamicum had the highest level of total soluble proteins (9.5±0.2 mg/g), whereas cassava leaf extract had the lowest (2.1±1.2 mg/g). Glucose levels significantly increased in all test groups compared to the control.

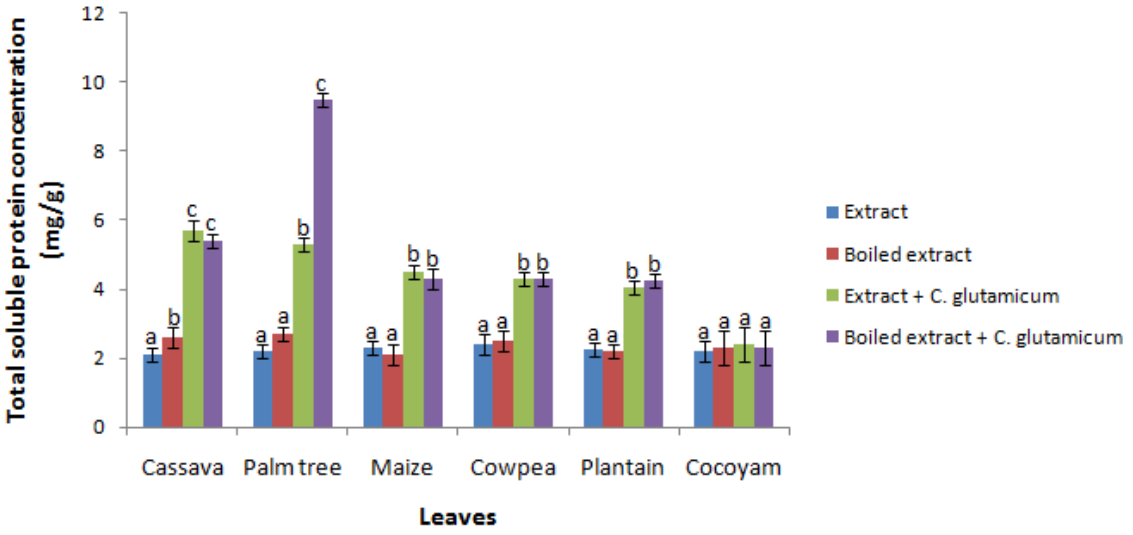

Figure 2. Total soluble protein concentrations in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

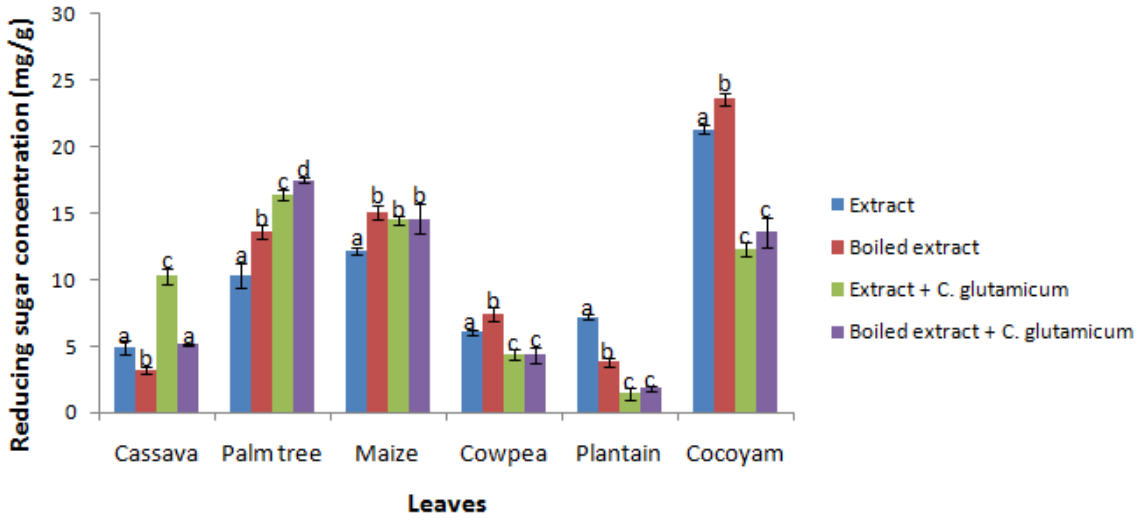

Figure 3. Reducing sugar concentrations in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

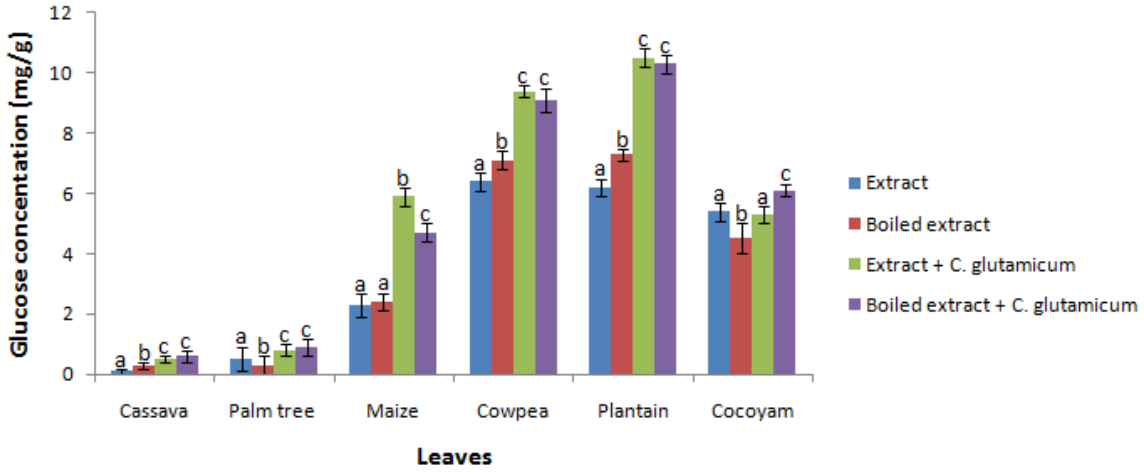

Figure 4. Glucose concentrations (mg/g) in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

Figures 5 and 6 illustrate the effect of submerged fermentation using C. glutamicum on amylase and protease activities, respectively. Cassava leaves boiled in the presence of C. glutamicum showed the highest amylase activity, while the combination of extract and C. glutamicum exhibited the highest protease activity. Beans extract and boiled plantain leaves had the lowest amylase and protease activities, respectively.

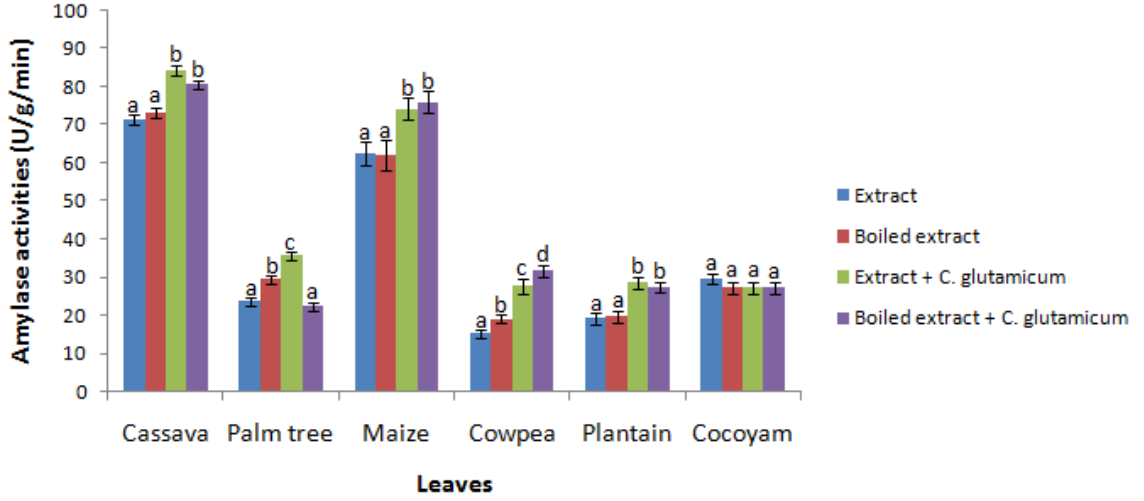

Figure 5. Amylase activities (U/g/min) in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

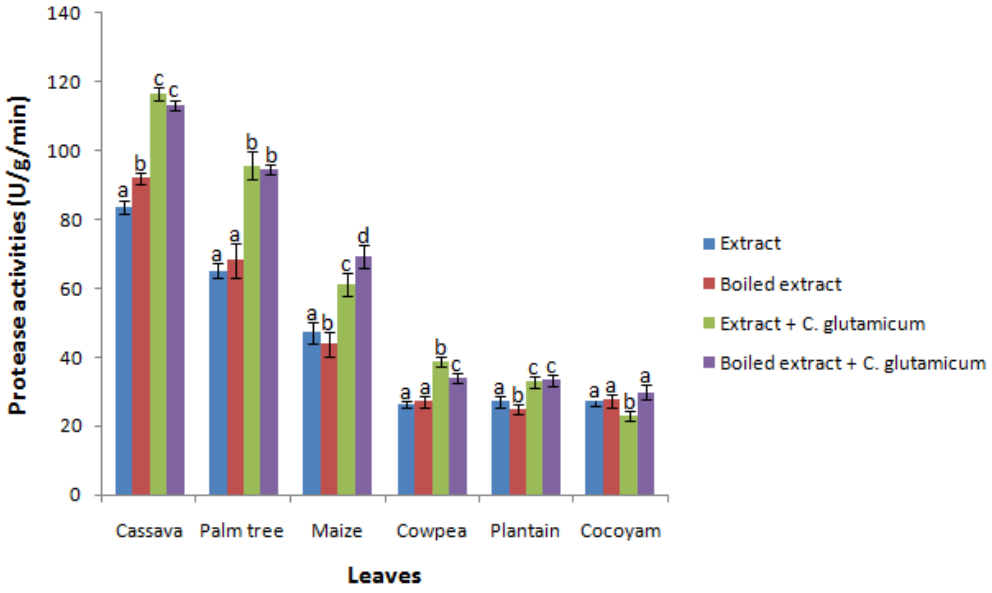

Figure 6. Protease activities (U/g/min) in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

The effects of the experimental treatment on total flavonoid and phenolic contents are shown in Figures 7 and 8, respectively. All test groups for the agricultural products displayed a significant increase in total flavonoid content.

The total phenolic content in palm tree leaves varied among the treatment groups (extract, boiled extract, extract + C. glutamicum, boiled extract + C. glutamicum) with values of 17.3±2.1, 19.5±2.1, 34.3±1.9, and 37.5±1.5 µg/ml, respectively (Figure 8).

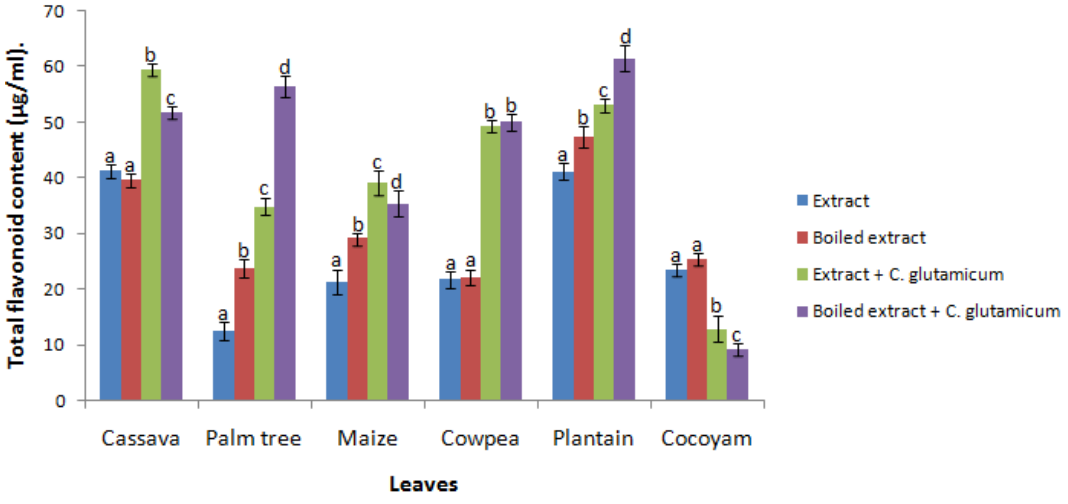

Figure 7. Total flavonoid content (TFC) (µg/ml) in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

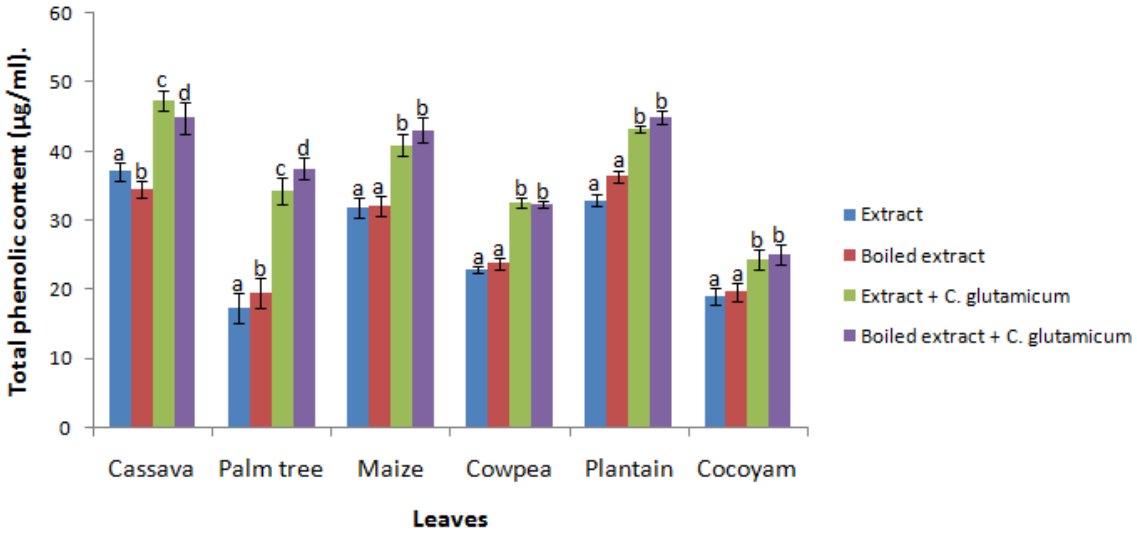

Figure 8. Total phenolic content (TFC) (µg/ml) in leaves extracts fermented by *C. glutamicum*. Bars with different superscript differs significantly at ($p<0.05$).

Figure 9 demonstrates that the treatment had no significant effect on pH, except for maize, plantain, and cocoyam leaf extracts + C. glutamicum, and maize leaf boiled extract + C. glutamicum.

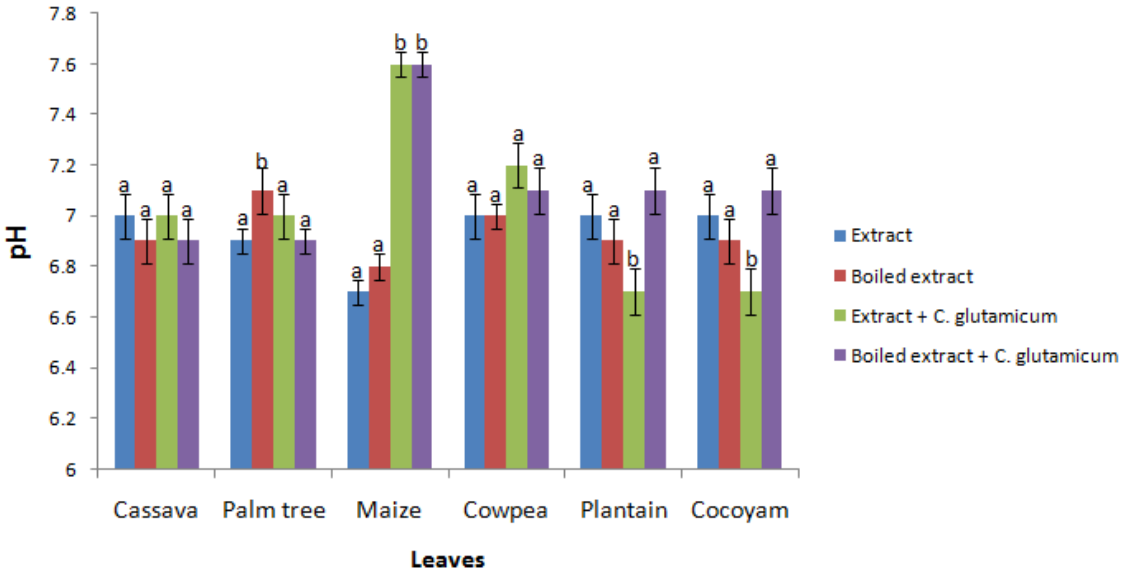

Figure 9. pH values in leaves extracts fermented by *C. glutamicum*. The leaf extracts were made by grinding 1g of leaf in 10 ml water. Bars with different superscript differs significantly at ($p<0.05$).

Discussion

The study revealed variations in lysine concentration among different treatments. Maize extract combined with *C. glutamicum* exhibited the highest lysine concentration, consistent with previous reports by Teniola [19] and Cui *et al.* [20], highlighting the potential of fermentation to enhance lysine levels. Ezemba *et al.* [21] also observed a significant increase in lysine yield through submerged fermentation. *Corynebacterium*, *Arthrobacter*, *Bacillus*, and *Brevi bacterium* are among the yielding strains used for fermentation, as reported by Ekwealor and Obeta [7]. Boiling agricultural products, with or without *C. glutamicum*, significantly reduced lysine concentration, as shown in the current study. Amaechi and Oluagha [22] and Hurrell and Finot [23] emphasized the susceptibility of lysine to damage during processing, and boiling has been shown to reduce its levels. Lysine, an essential amino acid, inhibits viral growth, aids in calcium absorption, reduces serum triglyceride levels, and contributes to the production of hormones, antibodies, and enzymes [24-25].

The study revealed variations in the levels of total soluble protein among different agricultural leaves and treatments. The highest level of total soluble protein was observed in palm tree leaves boiled in the presence of *C. glutamicum*, consistent with the findings of Zhu *et al.* [26], who reported increased proteinase activity during fermentation. Similar increases in protein levels have been reported by Ezekiel *et al.* [27] for cassava peels fermented with Trichoderma viride and by Egbune *et al.* [28] for fermented Pearl millet using *Rhizopus oligosporus*. Amaechi and Oluagha [22] highlighted the protein-increasing effect of boiling, which may contribute to the observed results. Specific *C. glutamicum* strains can secrete hydrolytic enzymes and utilize lipids and starch to produce proteins, as reported by Ezedom *et al.* [29], Vijayaraghavan *et al.* [30], and Ray *et al.* [31].

In this study, the levels of reducing sugar varied among the different agricultural by-products and treatments. Cocoyam leaf, beans, and maize exhibited the highest levels of reducing sugar after boiling. Wei *et al.* [32] demonstrated that cooking can increase total sugar content, particularly reducing sugars, with variations depending on cultivars and cooking methods. The study also observed a significant reduction in reducing sugar levels in plantain leaves, palm tree leaves, and cassava leaves compared to the control. This reduction could be attributed to the loss of components in hot moist conditions and other factors such as cooking temperature, time, and species differences, as reported by Lai *et al.* [33] and Bian and Liu [34]. Lu *et al.* [35] attributed the decrease in reducing sugars in sweet potatoes to the loss of amylase activity.

The levels of glucose were significantly increased in all agricultural leaves treated with extract + *C. glutamicum* and boiled extract + *C. glutamicum*. This increase can be attributed to the fermentative ability of *C. glutamicum*. Nkhata *et al.* [36] and Osman [37] demonstrated that fermentation activates starch-hydrolyzing enzymes like α-amylase and maltase, leading to the degradation of starch into malto-dextrins and simple sugars. El-Hag *et al.* [38] also reported an increase in glucose levels during the early stages of fermentation. Anigboro *et al.* [39] observed an increase in glucose levels during the solid-state fermentation of maize (Zea mays) offal. Conversely, Wei *et al.* [32] reported a decrease in glucose levels in agricultural by-products subjected to boiling.

Amylases play a crucial role in various biotechnological applications across industries such as food, fermentation, detergent, pharmaceutical, brewing, textile, and paper [40-41]. In the present study, a significant increase in amylase activities was observed in the groups treated with extract + *C. glutamicum* and boiled extract + *C.*

glutamicum, particularly in cassava leaves and maize leaves. Similar increases in amylase activities due to fermentation have been reported by Dou *et al.* [42] and Divakaran *et al.* [43]. The heat stability of amylase, retaining about 80% enzyme activity at high temperatures, makes it valuable for industrial applications [44]. The enzyme exhibits activity under acidic to neutral conditions (pH 6-7), enabling its use in various food industry processes such as dough preparation, juice and fruit processing, baking, and brewing [1,45].

Submerged fermentation of agricultural by-products using *C. glutamicum* resulted in the highest protease activities in cassava leaves, palm tree leaves, and maize compared to the control group. Punniyakotti *et al.* [46] demonstrated enhanced protease production using agricultural waste materials in submerged fermentation with *Bacillus subtilis* B22. Mathias *et al.* [47] also reported increased proteolytic activity values in residual brewer's waste utilizing Lactobacillus delbrueckii. Proteases find wide biotechnological applications in the food, detergent, textile, pharmaceutical, peptide synthesis, leather, and paper industries [48-49]. They play a crucial role in hydrolyzing protein molecules, breaking down bonds to produce peptides or amino acid units.

The total flavonoid content of the agricultural by-products was determined and showed significant increases in the fermented groups (extract + *C. glutamicum* and boiled extract + *C. glutamicum*) compared to the unfermented by-products, except for cocoyam leaf. Similar findings were reported by Adetuyi and Ibrahim [50], Yao *et al.* [51], Moktan *et al.* [52], and Ademiluyi and Oboh [53]. The increase in flavonoid content could be attributed to the increase in acidity during fermentation, which liberates bound flavonoid components and enhances their bioavailability. Microbial enzymes generated during fermentation have been reported to play a significant role in breaking down the plant matrix and extracting flavonoids [54-56]. However, β-glucosidase of microbial origin can hydrolyze flavonoids, resulting in either an increase or decrease in flavonoid levels, as observed in cocoyam leaves [59]. Flavonoids are polyphenolic compounds known for their antioxidant and free radical scavenging abilities [60].

The fermented agricultural by-products exhibited significantly higher total phenolic contents compared to the non-fermented samples, consistent with previous studies [61-62]. Phenolic compounds in their natural form are often bound to sugars, reducing their bioavailability. However, during fermentation, microbial proteolytic enzymes hydrolyze these complexes, releasing soluble free phenols [63].

The pH values of the fermented and non-fermented agricultural leaves showed non-significant changes compared to the control, except for maize, which exhibited a significant increase in pH. Generally, during fermentation, the pH value tends to decrease initially due to the production of organic acids by microorganisms. However, as fermentation progresses and nutrients are depleted, microorganisms start utilizing the organic acids as a nutrient source, leading to a reduction in their levels and subsequent increase in pH [64,65].

In conclusion, the present study demonstrates the advantage of submerged fermentation using *C. glutamicum* in increasing lysine yield compared to non-fermented methods. The study also highlights the effects of various treatments on the nutritional value, pH, and activities of amylase and proteases in agricultural leaves. The observed variations can be further optimized for specific lysine production by employing suitable methods

and conditions. Given the increasing demand for L-lysine in the food, pharmaceutical, and animal feed industries, these methods offer potential for improving production technology, reducing costs, and utilizing unconventional resources.

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