# INCREASING PHYTO-CHEMICAL EXPRESSIONS WITH ENZYME INFUSION

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***Abstract***

An investigation was carried out to determine effect of enzyme infusion on concentration of phytochemicals for enhanced antimicrobial expression. To achieve the aim: i. microbial alpha amylase and cellulase were synthesized using solid state fermentation technique, ii. powdery sample of Combretodendrum microcarpon was processed and infused with a consortium of synthases, and iii. infused extract was concentrated and analysed for phyto-inhibitory expressions. The results obtained showed 10% diluted enzymes recorded optimal activities of 3.62 U/ml and 3.23 U/ml at 50ºC and pH 7.0 for alpha amylase while cellulase had activities of 3.54 U/ml and 2.98 U/ml at 60ºC and pH 5.0 respectively. Effect of enzyme infusion on powdery sample recorded a 45% increase in concentration of identified bioactive compounds (alkaloids, flavonoids, steroids, terpenoids, anthraquinone) which was observed in the phyto-inhibition expressions for Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli and Enterobacter aerogenes respectively against controls. The investigation has furthermore proven the importance and relevance of enzyme application in discovery of new compounds for drug discovery.

***Keywords***: **Biocatalyst, Hydrolysis, Plant Microbial Toxicity, Pharmacognosis.**

## INTRODUCTION

Chemically infused methods have been used for the extraction of bioactive compounds from concentrated plant parts and the application of variant solvents have suggested that, greater diffusivity and solubility of an active substance is proportionate to the extraction efficiency. Several factors such as: i. structure, quantity and type of solvents, ii. physiological variables, iii. equipment and iii. time have influenced extraction at different levels of investigation. Also, extraction methods including: maceration, reflux extraction, and percolation have confirmed less effectiveness and relatively long extraction time involving a larger amount of solvent. Currently according to (19), new methods of abstraction including: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), and enzyme-assisted extraction (EAE), have proven to be effective relating to time, reduced solvent consumption, and higher percolation of bioactive compounds. Regardless of these advanced methods,

there is often some natural interference within the plant cellular structure distorting the high yield extraction of useful plant biochemical compounds. Research investigations have recommended that some highly potent metabolites are present in the cytoplasm and vacuoles which makes them not always available for conventional solvents (5, 7). Most of these phyto-compounds have been extracted and used in drug discovery but unfortunately, the limitations in concentration even with the advanced extraction techniques have led to the development of synthetic drugs which usually have risk factors and often expensive. To overcome the challenge in extraction of important plant derived compounds, the application of enzymes in singular or consortia form are currently being considered as a favourable alternative. Enzyme infusion technique allows resistance reduction of natural matters by isomerizing the cleavage of covalent bonds in the presence of water. This process disintegrates cell structures and increases the permeability of the material (26). Another importance is the ability of enzyme to increase water-solubility of derivatives (21). The principle of bioactive compounds involves binding to specific receptors in stem cells; as a result several compounds are present in cytosolic cell spaces and plant cell walls which are difficult to recover (14). This mechanism makes extraction methods difficult to obtain high yield and discover new compounds. The application of enzyme infusion is one of the current methods that is non-toxic to sensitive compounds and its incorporation has been recorded to provide adequate results. The aim of this investigation was to analyse and confirm the effect of enzyme infusion on increasing the concentration and inhibitory expressions of phytochemical compounds present in *Combretodendrum microcarpon* extract.

## MATERIALS AND METHODS

### i. Amylase synthase

Starch hydrolytic ability of *Aspergillus niger* was carried out following a modified method of (3) while using a 1% starch fortified medium. The selected strain was inoculated onto the starch agar medium for 3 days and after incubation at of 25ºC surface of the culture medium was flooded with iodine. The solution was drained and plate was incubated for 10 min. Hydrolytic zone around the colony was observed as a positive reaction. Further synthesis of alpha amylase was carried out using a soluble starch medium composition (%): bacteriological peptone (0.6g), MgSO4.7H2O (0.05g), KCl (0.5g), soluble starch (1.0 g) mixed with 25 ml distilled water to form a solid fermentation medium. 1.0 ml of microbial suspension ($1.2 \times 10^6$) containing extracted *Aspergillus niger* suspended spores was inoculated unto the culture, mixed thoroughly and incubated at 30ºC for 5 days.

### ii. Alpha amylase assay

A method of (35) was used to determine alpha amylase assay using crude extract recovered from the above. Further confirmatory analysis was also carried out according to (27); 250µl of enzyme sample was incubated with 250µl of 1% soluble starch solution in 20mM phosphate buffer pH 7.0 at 37°C for 30 min. The reducing sugar from each sample was measured by adding 250µl of 3,5-dinitro salicylic acid reagent to stop the reaction mixture. The tubes were boiled at 100ºC for 5 min, cooled and measured for optical density (O.D) at 540nm in the UV spectrophotometer (Spinco Biotech. PVT LTD)

### iii. Alpha amylase optimal activities

A modification of (16, 36) as described was followed: the reaction mixtures prepared were incubated at various temperatures of 30ºC - 80ºC. To 1ml of the incubated samples, 1ml of iodine was added and the resulting colour (blue black) was determined for its absorbance at 540nm. Similar reactions with pH values of 3.0 - 9.0, using 6N HCl and 5N NaOH solutions intermittently were used to determine optimal ion concentration. 1 ml of each sample was mixed with 1ml of iodine added to it and incubated for 40 minutes at 30ºC. The resulting colour (blue black) was determined for its absorbance at 540nm. The concentration of reducing sugar released was then calculated for glucose.

### iv. Cellulase synthase

The same *Aspergillus niger* was used on hydrolysing cellulose for synthesis of cellulase and the method of (20) was adopted. A final, 1N NaOH was used to stain the screening which was later incubated for 10 minutes. A clear yellowish zone formation around the isolate indicated a positive reaction for cellulose hydrolysis. A medium composition containing the following (%): L-Glutamic acid, 0.03g; NH4NO2, 1.4; K2HPO4, 0.2g; CaCl2, 0.2g; MgSO4, 0.03g; FeSO4, 0.5g; MnSO4, 0.16g; ZnSO4, 0.14g; dried wheat straw, 3g was mixed with 30 ml of distilled water to form a solid basal fermentation medium which was inoculated with 1ml of the microbial suspended spores. The medium was incubated at 30ºC for 5 days.

**v. Cellulase Assay**

The method of (28) was used to analyse the synthase extract. For filter paper activity Whatman no. 1, filter paper strip of dimension 1.0× 6 cm (50 mg) was placed into each assay tube. The filter paper strip was saturated with 1.0 ml of Na-citrate buffer (0.05 M, pH 4.8) and was heated for 10 min at 50°C, 1ml of enzyme was added to the tube and incubated at 50 °C for 60 min, relevant appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 ml of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for colour development. The reaction mixture was diluted appropriately and measured against a reagent blank at 540 nm in a UVVIS spectrophotometer (Thermo Scientifc Spectramax M3, USA). The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1.0M of glucose per milliliter per minute.

**vi. Cellulase optimal activities**

The method of (23) was used to determine the optimal activities at various temperatures 30 – 80$^{o}$C and pH 3- 9 respectively for cellulase.

**vii. Collection of Sample and processing**

A total of 35 branches of *Combretodendrum macrocarpum* with varying length between 15 – 20 cm were collected from Benin City metropolitan area (6°20′00″N 5°37′20″E) in Nigeria (9.0820° N, 8.6753° E). The samples were wrapped in an airtight bag and taken to the laboratory for further analysis. These samples were removed from the airtight bags and allowed to air-dry on a table in a sterilized room for 7 days. Furthermore, samples were then washed with distilled water to remove dirt and debris and a repeated wash using sodium carbonate to reduce any microbial load present. Sample was later dried in a hot air oven at 25$^{o}$C for 5 days. Fine powder was obtained after grounding and removal of large particle using a 400 micron size sieve mesh.

**viii. Sample Extract Enzyme Infusion Technique**

10.0 g of the fine power sample was weighed into 250ml conical flask (A). Into the same flask, a cocktail of 5.0 ml alpha amylase and 5.0 ml cellulase was added and the mixture was kept in the incubated for 1 hour at 50$^{o}$C for the purpose of hydrolysis. This was used to set up the enzyme infusion technique. Into separate conical flaks (B) and (C), 10.0 g of fine powder was weighed into each, 20 ml of hot water was added to (B) and 20 ml of diethyl ether was added into (C). Extracts from (A), (B) and (C) were collected by filtration using Whatmann Filter No 1. They were concentrated using water bath and furthermore used for phytochemical screen and antimicrobial studies.

**ix. Phytochemical screening**

Phytochemical screening involving chemical tests to determine the presence of alkaloids, saponins, tannins, anthroquinones and flavonoid were carried out on extracts using the methods described by (25).

**x. Antimicrobial studies**

The microorganisms used for this study were *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia and Enterobacter aerogenes.* The method of (30) was used to determine antimicrobial expressions of the extracts. Using the infusion technique, well holes of 0.9 mm diameter were measured and two-fold serial dilutions of the extracts were prepared by first reconstituting in 20% dimethylsulphoxide (DMSO). They were diluted in sterile distilled water to achieve a decreasing concentration range of 50 mg/ml to 8.021 mg/ml. A 50 µl volume of each dilution was introduced in duplicate wells into nutrient agar (NA) plates already seeded with the standardized inoculum ($5 \times 10^5$) of the test bacteria cells. The test plates were incubated at 37$^{o}$C for 24 h. The least concentration of each extract showing a clear zone of inhibition was taken as the MIC.

**RESULT AND DISCUSSION**

Useful Industrial hydrolytic enzymes are being synthesized by fermentation techniques and based on higher yield from microbial source and favourable environmental factors such as temperature (°C) and pH, their relevance to industrial bioprocessing has become overwhelming. Enzymes actively proceed in a singular mode or consortia effectively at a desirable temperature (°C) and pH because they are sensitive and in a relatively short time enabling synthesis of active molecules from selective substrate. This mode of action is relative to pre-treatment of substrates eventually leading to extraction of valuable compounds with industrial, social and economic benefits (29, 32). The application of enzyme in phytochemical extraction from plant parts has been recorded in several investigations (2, 9, 38) and the method has shown effective results regarding increasing concentration of bioactive compounds. Several investigations have shown the presence of active chemical compounds such as tannins, steroids, flavonoids, Phlobatannin, quinones, phenols, caumarins, alkaloids, terpenoids, saponins, Glycosides, cardiac glycoside, anthraquinones, anthocyanin and many more in various plant parts and these phytochemicals have been useful in drug development against several diseases (15, 31, 34, 35). The major challenge till date is not the technology involved in extraction of these compounds rather the yield obtained from samples which has led to production of synthetic drugs. These synthetic drugs are expensive to produce and more so, too expensive to buy, short life span along with slow functionality and sometimes results to further health complications rather than solving a problem. These factors have increased the search for techniques towards finding a lasting solution on increasing the yield of potent plant extracts. Currently one method that has been found to be suitable is the use of enzyme simply because of their functionality in isomerizing cell walls that will promote high secretion of compounds. The main goal of enzyme application is due to the mild expression reaction which is more favourable to plant compounds as compared to other solvents for extraction.

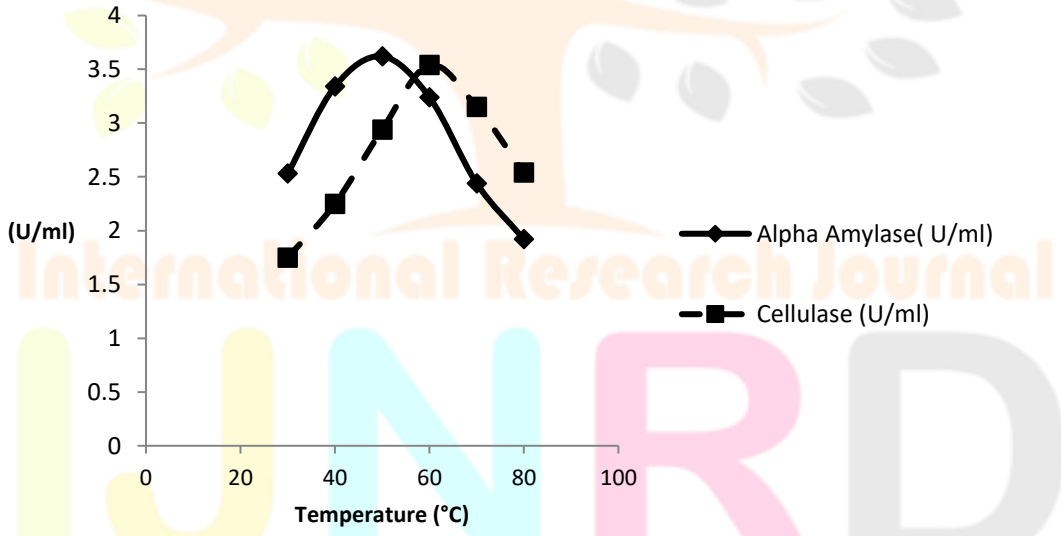


**Figure 1: Enzyme synthase optimal activity measured against temperature (°C)**

Comparable activity curves for alpha amylase and cellulase measured against optimal temperature (oC). Figure 1 showed optimal activities at 50oC and 60oC for alpha amylase and cellulase respectively. In general, when the enzymes are exposed to a high temperature denaturation leading to derangement in the native structure of the protein and active site are observed. Majority of the enzymes become inactive at higher temperature above 70°C. From the results, it was observed that cellulase activity at 60oC was supported by the medium composition and microbial stability

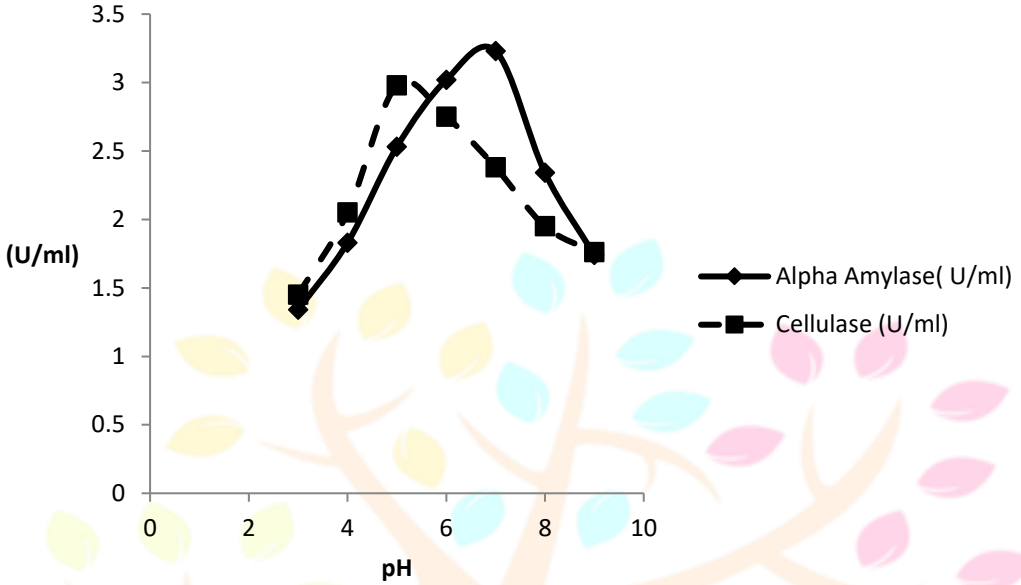


**Figure 2: Enzymes synthase optimal activity measured against pH**

Comparable activity curves for alpha amylase and cellulase measured against optimal temperature (oC). Increase in the hydrogen ion concentration (pH) considerably influences the enzyme activity and a bell-shaped curve is normally obtained as observed in the Figure above. The results showed optimal activities at pH 7.0 and pH 5.0 for alpha amylase and cellulase respectively. Alpha amylase activity was recorded at neutral pH while cellulase was recorded at acidic pH, which will very much support binding with the active sites considering the sensitive of starchy composition and lignocellulose structure present in the plant cell wall.

**Table 1: Differential bioactive compound profile detected in *Combretodendron macrocarpum* branch extract**

| Phyto-compounds | Hot Water | Diethyl Ether | Enzyme Infused Extract |
|---|---|---|---|
| **Alkaloids** | ++ | + | +++ |
| **Flavonoids** | + | + | ++ |
| **Steroids** | + | + | ++ |
| **Terpenoids** | + | + | ++ |
| **Anthraquinone** | - | - | + |

**Heavily present, + + +; Slightly present, ++; Present, +; Absent, –.**

Screening analysis for phytochemical revealed the presence of alkaloids, flavonoids, steroids, terpenoids and anthraquinones shown in the Table 1 above. The results showed that, enzyme infused extract exhibited presence of all phytochemicals with higher increase in alkaloids against hot water and diethyl ether extracts respectively. Also, slight increase in flavoniods, steroids, terpenoids against hot water and diethyl ether extracts. The Table also recorded presence of anthraquinone which was absent in hot water and diethyl ether extracts respectively. Several reports have recorded different benefits of these phytochemicals; alkaloids acting as antioxidants, flavonoids as strong lipid peroxidation inhibitor, steroids as electron donors, terpenoids used against oxidative stress-induced diseases, anthraquinones as anti-inflammatory and anticancer (1, 6, 25)

**Table 2: Table 2: Bioactive compound expression against pathogenic test strains in (mm)**

| Test Strains | Hot Water Extract (mm) | Diethyl Ether Extract (mm) | Enzyme Infused Extract (mm) |
|---|---|---|---|
| ***Pseudomonas aeruginosa*** | **19.8** | **21.9** | **31.8** |
| ***Staphylococcus aureus*** | **21.0** | **20.6** | **30.5** |
| ***Klebsiella pneumoniae*** | **21.0** | **21.8** | **31.6** |
| ***Escherichia coli*** | **22.2** | **0.0** | **32.2** |
| ***Enterobacter aerogenes*** | **19.0** | **0.0** | **27.6** |

The phytochemical expressions of the extracts against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter aerogenes* and the inhibitory activities were recorded ion Table 2 above. From the result, inhibitory effect of enzyme infused extract was highly positive against all the test strains as compared to hot water and diethyl ether extracts respectively. The record showed highest inhibitory activity against *Escherichia coli* (32.2) and lowest against *Enterobacter aerogenes* (27.6 mm) against hot water extract (19.0 mm) and no response from diethyl ether extract for the same strain.

In this experiment, the first step involved production of alpha amylase and cellulase by solid state fermentation using *Aspergillus niger*. The activities of 3.62 U/ml and 3.23 U/ml at 50$^{o}$C and pH 7.0 were recorded for alpha amylase while cellulase expressed activities of 3.54 U/ml and 2.98 U/ml at 60$^{o}$C and pH 5.0 respectively. These records were similar to reports by (4, 8, 10, 18) for alpha amylase, and (13, 24, 33) for cellulase. Also, for the purpose of the investigation a cocktail of alpha amylase and cellulase were used for infusing powdery sample of *Combretodendron macrocarpum* at 50$^{o}$C and pH7.0 for 1 hrs. This allowed the synthases to isomerize the starchy and cellulose contents respectively in the sample and thus promoted an increasing secretion of bioactive compounds. The application of enzyme consortia was also reported report by (38) where a combination of cellulase, pectinase, and tannase were used to extract phenolic compounds at different proportions. Extract was decanted and concentrated using water bath. The concentrate was analysed for phyto compounds and used for antimicrobial expression against common pathogenic strains; *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli* and *Enterobacter aerogene*. Hot water and diethyl ether extracts of *Combretodendron macrocarpum* were also analysed for bioactive compounds and compared to enzyme infused extract as controls. The results obtained from the enzyme infused extract showed inhibitory expressions of 31.8 mm, 30.5 mm, 3.6 mm, 32.2 mm and 27.6 mm for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pnuemoniae*, *Escherichia coli* and *Enterobacter aerogenes* respectively. This was a 45 % concentration increase compared to inhibitory activities of hot water extract with values of 19.8 mm, 21.0 mm, 21.0 mm, 22.2 mm, 19.0 mm for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pnuemoniae*, *Escherichia coli* and *Enterobacter aerogenes* while diethyl ether extract recorded values of 21.9 mm, 20.6 mm, 21.8 mm respectively for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pnuemoniae*. No value was recorded for *Escherichia coli* and *Enterobacter aerogenes* from diethyl ether extract. The results obtained were aligned with the findings of (39) involving the extraction of lycopene from tomato waste using enzyme-treated matrices with respect to untreated controls. In another experiment, enzyme assisted extraction recorded an increase in phenolic content released from grape waste by more than 25% - 30%, with

respect to the control (32). In another report, (9) recovered increase in polyphenols, chlorogenic acid and catechins by the enzymatic pre-treatment procedure. Increasing the efficiency of phytochemical compounds by cellulase, beta-glucosidase, pectinase, protease, tannase and carbohydrase has been reported in different studies according to (11, 12, 17, 22, 37). Common reference from each report stated that there was increase in yield and inhibitory properties of bioactive compounds from different plants compared to control which was in agreement with this investigation.

## CONCLUSION

The use of enzymes in extracting value added biological compounds is an up-and-coming area from laboratory optimization studies to industrial applications. It implies processing plant functional properties for optimal expression of characteristics. However, success in this area requires interdisciplinary research from various life sciences disciplines. An important area of research is investigating the stability of enzymes and their interaction with other food and plant ingredients during processing and storage. However, enzyme-assisted processes are reaching for more sustainable development of innovations in a broad spectrum of industries. In conclusion, this study has demonstrated that enzyme application is a sustainable extraction procedure to be more effective in the recovery of valuable bioactive compounds. Also, the use of a consortium will have a significant increasing effect on the extraction yield of value compounds in the food and pharmaceutical industries.

## DECLARATION OF CONFLICT

The authors declare No Conflict of Interest

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