# Gene Modulation Effect on Phyto-Compound Expression

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## ABSTRACT

Biocatalyst hydrolytic function, socio-economic importance and industrial relevance were identified in this investigation and the aim of the research was to determine effect of enzyme infusion on phyto-compounds yield and antimicrobial expression. The following activities were carried out to achieve the objective: i. construct of a hyperactive *Bacillus subtilis* sub specie using a modulation kit, ii. production of amylase, cellulase and pectinase using the successfully modulated strain, iii. Applying a consortium of the enzymes to infuse powdery samples of *Calotropis procera Ait Leaf* under synthase proximate conditions of pH 6 at 60$^{o}$ C for 1 hours, and iv. analysis on extract to ascertain phyto-compund yield and antimicrobial expression. The following results were recorded: i. infused extract with a consortium of catalysts showed high impact of 35% increment in secretion of terpenoids, flavonoids, anthraquinone, alkaloids, steroids, tannins, and saponins that were analysed and identified to be present compared to extracts of dimethyl ether, 70% ethanol, and methonal; ii. results also recorded high impact of boioactive agent expression against test strains with the following phyto-restraint articulations of 33.3mm, 28.6 mm, 30.4 mm, 31.6 mmm, 30.6 mm, and 22.3 mm for *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Candida albicans* respectively against concentrates of dimethyl ether, 70% ethanol, and methanol used as controls. The outcome of this investigation demonstrated the significance of enzyme application in drug discovery toward alleviating challenges from pathogens causing progressive diseases that are influencing healthcare and overall environmental declination.

**Keywords:** Gene Modulation, Medicinal Plants, Pathogen Resistance,

## INTRODUCTION

Medicinal plants are comprised of a diverse group of structurally complex compounds that are found in a variety of biosynthetic pathways. These biosynthetic pathways make it possible for medicinal plants to develop progressive adaptability, which is an essential quality in the face of harsh environmental conditions. Several significant phytocompounds, including carotenoids, polyphenols, isoprenoids, phytosterols, and saponins, have been found to have high expressions against pathogens that cause health conditions [Kumar et al. 2023, Sharma et al., 2018]. Extraction of significant plant synthetic compounds has displayed an unmistakable property in relativity for solvents, temperature, water activity, and plant material bringing about the choice of dissolvable influencing the nature of the recuperated phytochemical and its application [Cooperstone and Schwartz, 2016]. Additionally, various solvents have been found to have varying degrees of compatibility and non-compatibility with phyto-compounds' functional properties. In addition,

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compelling fundamentals like the structural type, molecule size, sample pre-treatment, and solid-solute interaction are necessary for effective extraction [Kumar et al. 2023]. There has been a constant search for high-quality plant bioactive agents and phytochemical compounds toward the development of drugs with fervent potency to alleviate the production of synthetic drugs, thereby reducing the cost of production and meeting end-user demand, in response to the growing observation of drug resistance in pathogens. Despite the fact that relevant reports have demonstrated proportionate effects of a variety of conventional methods, such as maceration, percolation, decoction, reflux extraction, Soxhlet extraction, pressurized liquid extraction, high hydrostatic pressure extraction, microwave-assisted extraction, ultrasound-assisted extraction, pulsed electric field extraction, vibro-cavitation extraction, extraction under vacuum-oscillating boiling conditions, extractions in mills, extractions in rotary-pulsation apparatus, it is basic to choose a more reasonable strategy for phytochemical extraction with more financial, natural, and medical advantages [Gonzale et al. 2021, Pontillo et al., 2021, Zhang et al. 2018, Carreira-Casais et al. 2021, De Silva et al. 2016]. The utilization of microbial chemicals as hydrolytic specialists has shown to be effective for extraction, and alteration of complicated bioactive substances. A compound's ability to hydrolyse cell walls by disrupting underlying intricacy alongside the properties of explicitness and regioselectivity offers the best appropriate technology that can work with the decrease of ecological difficulties. Microbial gene modulation techniques for the secretion of hyperactive enzymes have been developed in response to the difficulty of discovering novel phyto-compounds and these techniques can be used to disrupt the cytoplasm and vacuole of cells that contain potent metabolites [Cooperstone and Schwartz, 2016]. The capacity to isomerize the structural bonds that are present, thereby increasing permeability and the production of water-soluble derivatives is yet another aspect of hyperactive enzyme infusion application that is crucial [Lang and Wai, 2003, Puri et al., 2012 Marathe et al., 2017]. Literature has demonstrated the significance and relevance of genomic modulation in the improvement of extracellular enzyme secretion machinery, fusion capacity, and synthase expression [Throop and LaBaer, 2015]. Due to an increase in affinity and fusion capacity, this suggests that enzymes with a modulated genome can be utilized to overcome the difficulties of recovering beneficial plant materials from the cytoplasm and vacuole.

Although enzymes with hyperactivity secreted from modulated genomes will provide a more distinct impact towards the recovery of novel compounds extracted from plant materials and furthermore increase yield. It will also reduce the identified drug production challenges by balancing the energy level of interaction between molecules in a bioreaction, thus utilization of enzyme infusion has been reported to be a useful technique because it is non-toxic to sensitive plant materials. This study sought to determine how phyto-compound yield and bioactive agent expressions were affected by synthases isolated from modulated microbial genes

## MATERIALS AND METHODS

### Strain Modulation

A novel highly efficient *Bacillus subtilis* sub specie was constructed using the Takara Infusion *Bacillus* expression Kit Takara Laboratories Inc. Cat. Nos. (011614). The protocol for the construct with modification was strictly adhered to according to manufacturer's instructions by inserting an infusion of aprE signal peptide and amplified target synthase cloning gene.

### Enzymology

The successfully constructed strain was used for the synthesis of amylase, cellulase and pectinase by submerged fermentation respectively. Into a 250 ml sterilized conical flask, 1 gm of dehydrated potato starch was mixed with 100ml of distilled water (pH 7.0), thereafter a singular pure colony size of $1.67 \times 10^6$

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was inoculated into the prepared solution A. Similar colony size was inoculated separately into 250 ml conical flask containing 1 gm of cellulose mixed with 100 ml of water and another conical flask containing 1 gm of pectin mixed with 100 ml of water, labelled as solution B and C respectively. Solutions A, B and C were allowed to settle for 5 minutes enabling the inoculum to disperse adequately without enhanced agitation. The media were further incubated at 50$^{o}$C under pH 7.0 for 24 hours. At the end of the incubation period, crude synthases were recovered using a cold centrifuge at 3000 rpm for 1 minute. Crude samples were furthermore purified using a modification of [Mansoori et al. 2020]. Purified samples from A, B, and C were stored at 4$^{o}$C for 24 hours to enhance stability before application. A modification of the following protocols was carried to achieve the activities mentioned in this section: [Bakri et al. 2020] for amylase, [Pramanik et al. 2021] for cellulase, and [Echesi et al 2022] for pectinase.

**Synthase Stability**

The thermal stability of each sample was ascertained to determine functional level of compatibility within a reaction. Initial thermal stability of each sample was obtained following the protocol: into separate 50 ml conical flask labelled A, B, and C, 5 ml amylase, 5 ml cellulase and 5 ml pectinase were dispensed respectively. Into a 100 ml conical flask labelled D, a mixture of the synthases in the ratio of 5ml:5ml:5ml were mixed. All the flasks were heated at 50°C for 1 hr. At 10 min intervals, 1 mL aliquots were withdrawn from A, B, C, and D and measured using a spectrophotometer at 540 nm to ascertain optimal thermal stability.

**Infusion Technique**

7.0 g of the fine *Calotropis procera Ait Leaf* (Cp) power sample was weighed into 100 ml conical flask (Dd). Into the same flask, a 3 ml of cocktail synthase from flask D above was added and the mixture was kept in the incubator for 1 hour at 60$^{o}$C for the purpose of hydrolysis. This was used to set up the enzyme infusion technique. Same procedure was repeated using 3 ml of diethyl ether, 3 ml of 70% ethanol, and 3ml of methanol. Into separate 100 ml flasks labelled (Aa), (Bb), and (Cc). At the end of incubation period, bioactive extracts were recovered by filtration using Whatmann Filter No 1. They were concentrated using water bath @ 50$^{o}$C until molten stage was reached and furthermore used for phytochemical screen and antimicrobial studies.

**Phytochemical screening and Characterization**

Assay to determine effect of infusion technique on probable terpenoids, flavonoids, anthraquinone, alkaloids, steroids, tannins, and saponins were carried out on extracts using the methods described by (Bonathe et al. 2017, Mansoori et al. 2020).

**Antimicrobial studies**

The test strains used for this study were *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans*. A modification of [Al-Amiery et al. 2012] 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 pathogen cells. The test plates were incubated at 37$^{o}$C for 18 h. Thereafter, the least concentration of each extract showing a clear zone of inhibition was taken as the MIC.

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# RESULTS

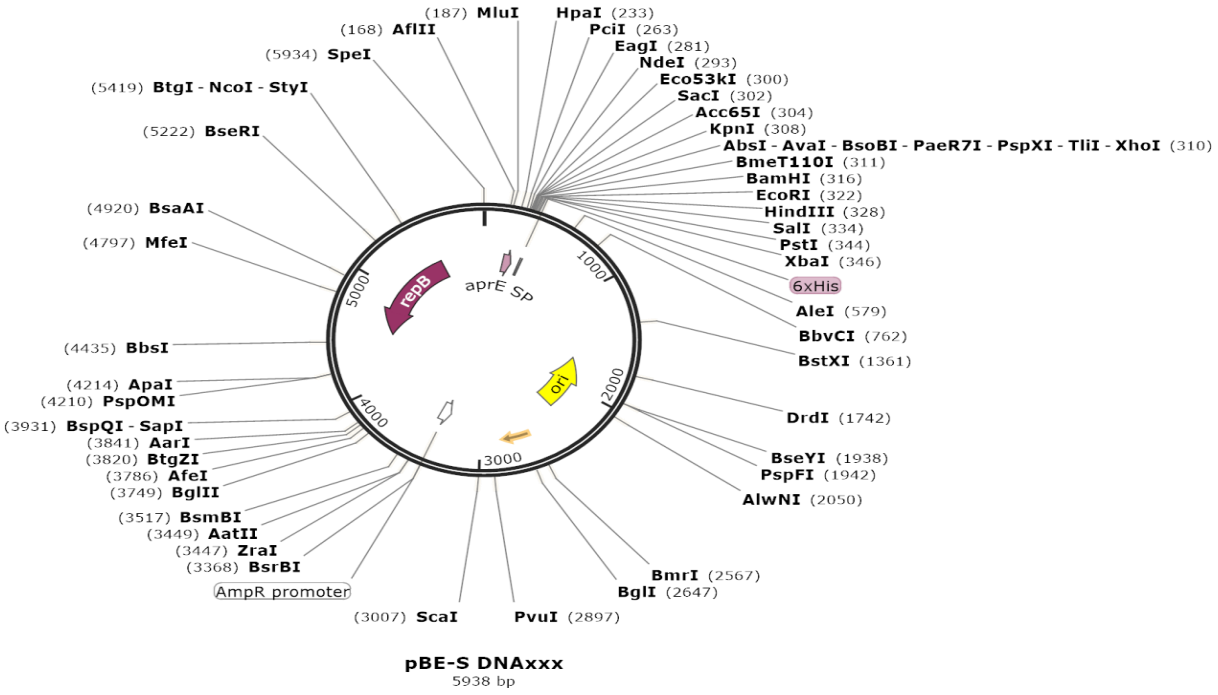


Figure 1: An illustrating of *Bacillus* expression pathway before modulation. From the above, the map constituted aprE Signal peptide, repB replication gene, digestive enzymes, and orisome. This established design above is a genetic tool for construct of *Bacillus* specie genome.

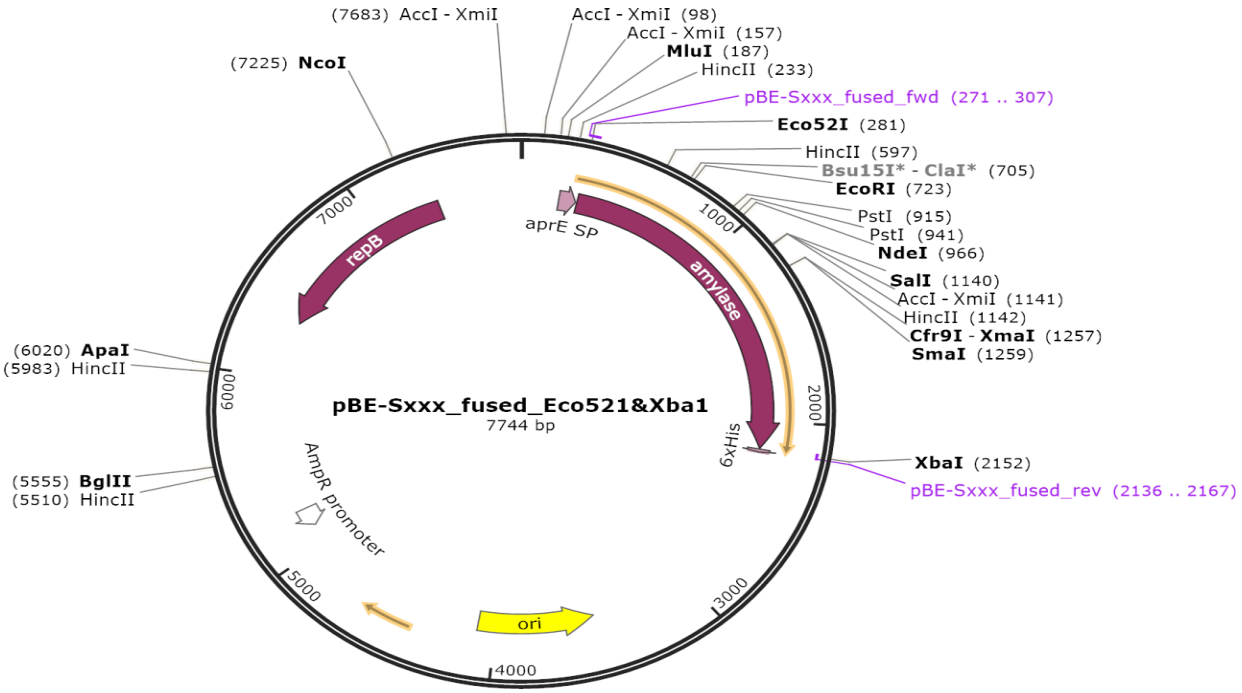


Figure 2: An illustrating of *Bacillus* expression pathway after modulation. From the above, the map constituted aprE Signal peptide, rep replication gene, AmpR promoter, 6 histidine, strict digestive enzymes, orisome, forward, and reverse primer insertions. The map also showed the length of gene in the newly constructed *Bacillus subtilis* sub specie genome. The strain was used for synthesis of amylase, cellulase, and pectinase respectively by submerged fermentation

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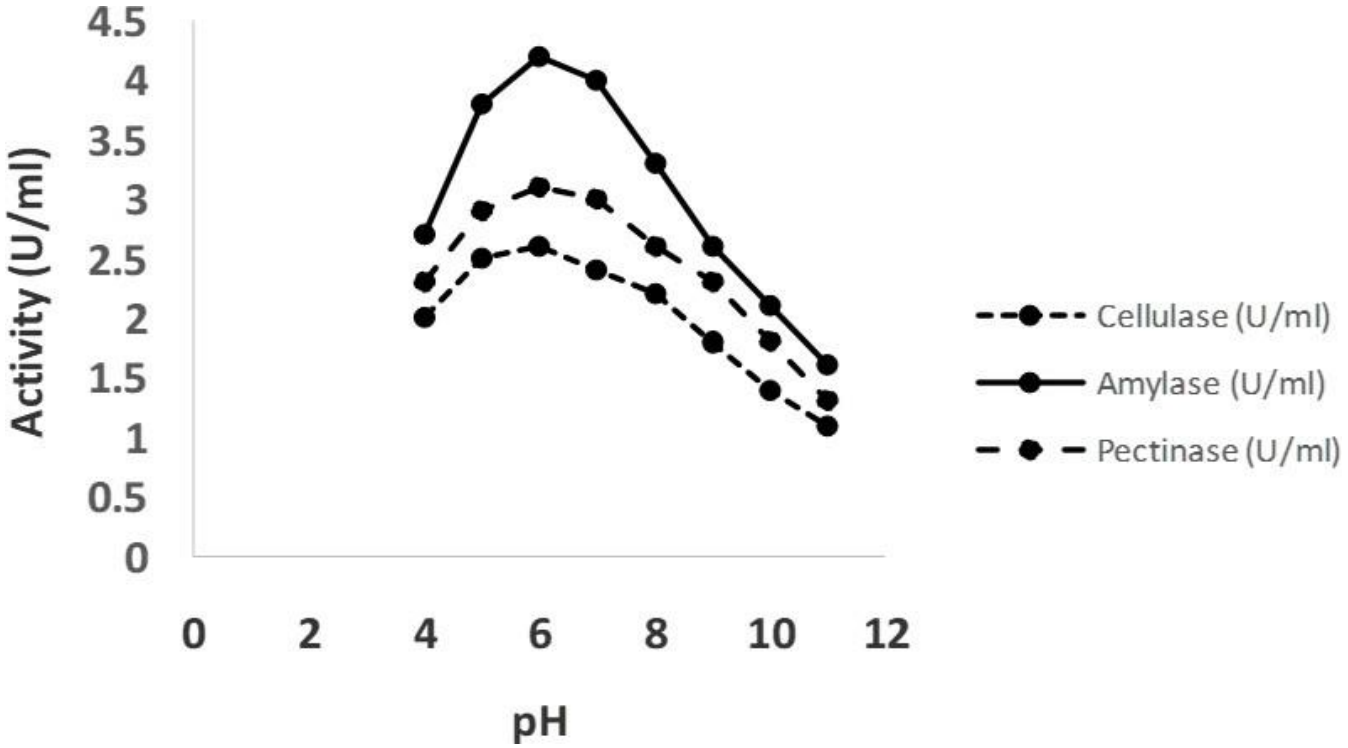


Figure 3: Optimal pH activities for cellulase, amylase, and pectinase synthases. From the Figure above all the enzymes recorded variable optimal activities at pH 6.0. Amylase activities recorded the highest activity of 4.2 U/ml, followed by 3.1 U/ml and 2.6 U/ml for pectinase and cellulase respectively.

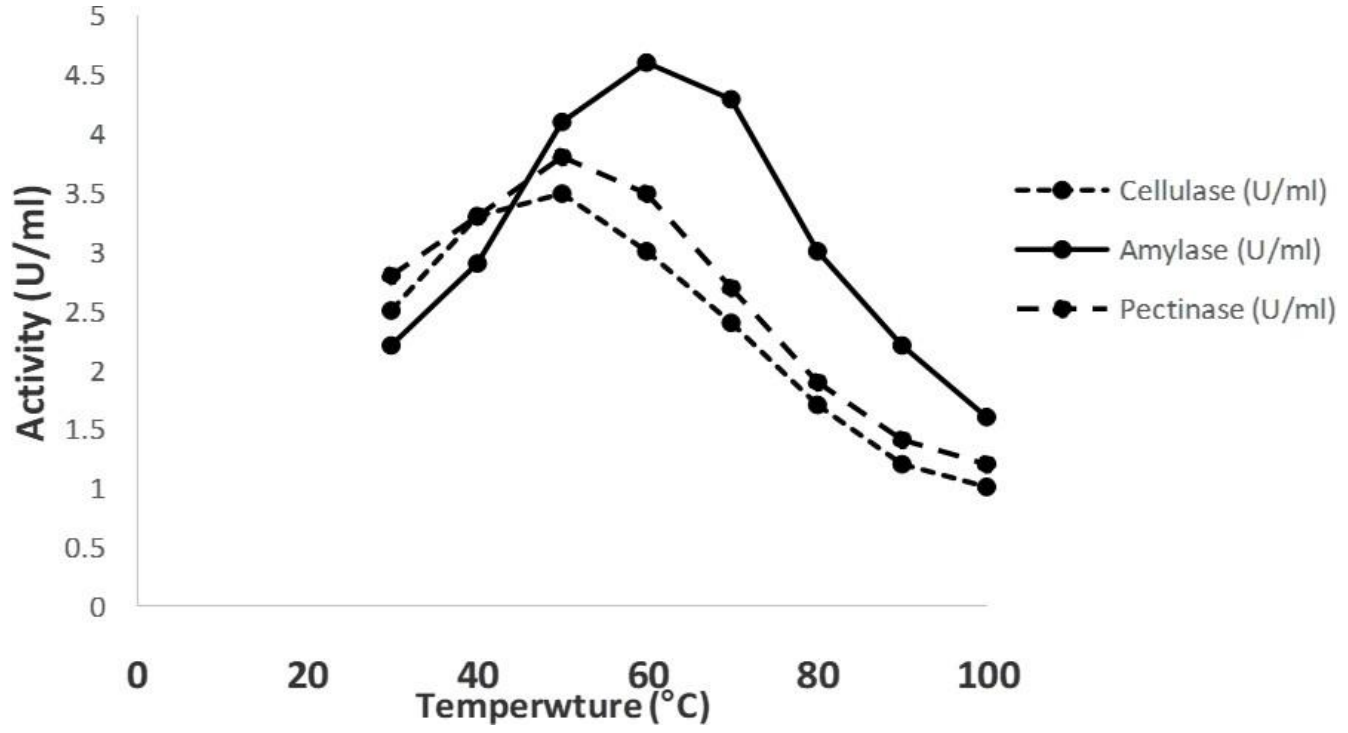


Figure 4: Optimal temperature ($^{o}$C) activities for cellulase, amylase, and pectinase synthases. From the Figure above amylase recorded the highest activity of 4.6 U/ml at 60$^{o}$C, and at 50$^{o}$C activities of 3.8 U/ml and 3.6 U/ml were recorded for pectinase and cellulase respectively.

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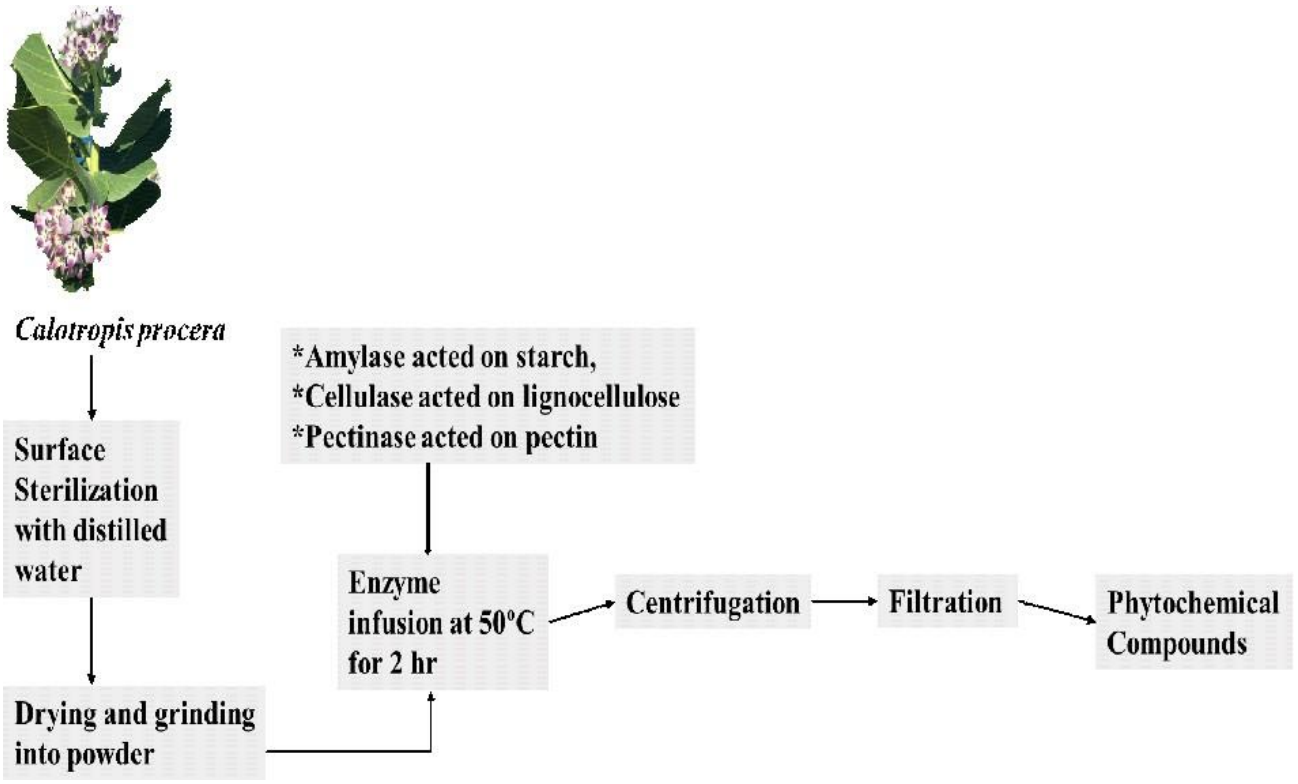


Figure 5: Application of Enzyme Infusion Technique.

The Figure showed a conventional extraction protocol involving a cocktail of enzyme-assistance towards achieving higher yield of bioactive compounds and increased expression of antimicrobial agents.

Table 1: A presentation of most probable phyto-compounds recovered from *Calotropis procera* and their expression assessment.

| Phyto-chemical Compounds | Diethyl Ether | Ethanol | Methanol | Enzyme Assisted |
|---|---|---|---|---|
| Terpenoids | + | – | + | ++ |
| Flavonoids | + | + | + | ++ |
| Anthraquinone | – | – | – | + |
| Alkaloids | + | + | + | +++ |
| Steroids | + | + | + | +++ |
| Tannins | + | + | + | ++ |
| Saponins | + | + | + | ++ |

Table 2: A presentation of minimal inhibitory concentration activities of anti-microbial agents present in recovered samples

| Test Strains | Diethyl Ether (mm) | Ethanol (mm) | Methanol (mm) | Enzyme Assisted (mm) |
|---|---|---|---|---|
| *Escherichia coli* | 21.8 | 22.4 | 20.4 | 33,3 |
| *Enterobacter aerogenes* | 19.6 | 17.4 | 14.6 | 28.6 |
| *Staphylococcus aureus* | 20.6 | 21.3 | 19.4 | 30.4 |
| *Pseudomonas aeruginosa* | 21.9 | 19.8 | 17.6 | 31.6 |
| *Klebsiella pneumoniae* | 21.8 | 20.3 | 18.6 | 30.6 |

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| | | | | |
|---|---|---|---|---|
| *Candida albicans* | 17.0 | 19.2 | 14.4 | 22.3 |

## DISCUSSION

The discovery and application of wild-type catalysts have been credited to different sources like microorganisms, creatures and plants, and microbial agents have been most often examined because of their modern advantages attributed to stability, yield, and shelf life of usability. Improvements in bioprocess activities have resulted in the discovery of novel enzymes using a variety of tools, which have had the effect of modulating genomes and creating new strains with hyperactive properties over time. These factors include an increase in by-product production, development, and meeting time-to-market demand. In a report by [Tjalsma et al. 2000], it was cited that using an effective method of gene cloning into protein expression vectors, improved protein expression from thousands of genes could be achieved. According to Adrio and Deamain [2010], one of the most important species used in the production of industrial enzymes is *Bacillus subtilis* and because it is generally recommended as a safe strain and easy to manipulate. A variety of strategies based on molecular cloning tools can be used to improve the genome of this species, which can result in an increase in expression level through the amplification of gene copy number, optimization of codon usage, strong promoters to boost gene transcription, enhancement of signal peptides, fusion to heterologous signal peptides for hyperactivity [Bornscheuer et al. 2012, Hamid and Ikram, 2012, Miao et al. 2020]. A novel *Bacillus subtilis* subspecies was created with the help of a *Bacillus* expression kit during this investigation. In order to create a strain capable of resisting antibiotics like kanamycin, modifications to the provided instructions were used to achieve the newly constructed genomic structure. In Figure 1 above, constituents such as aprE Signal peptide, repB replication gene, digestive enzymes, and orisome. were included in the design. The map representation shows a structure before construct of the strain. Figure 2 also shown above on the other hand constitutes the following: aprE Signal peptide, rep replication gene, AmpR promoter, 6 histidine, strict digestive enzymes, orisome, forward, and reverse primer insertions. This a representation of successful *Bacillus subtilis* subspecies construct.

Similar studies have reported the successful construction of reporter genes for cloning host-cell-active bacterial promoters [Yang et al. 2013, Yao et al. 2019,]. In this investigation, to approve the impact of the quality regulation, the developed construct was utilized with a convention change of [Bakri et al. 2020, Pramanik et al.2021, Echesi et al. 2022] by submerged fermentation to synthesize cellulase, pectinase, and amylase, respectively. After dilution of purified synthase extracts, analysis was performed, and for amylase, cellulase, and pectinase, optimal activities of 4.6 U/ml was recorded at 60$^{o}$C, while 3.6 U/ml, and 3.8 U/ml were recorded at 50$^{o}$C for cellulase and pectinae respectively. At pH 6, the optimal activities of all synthases were found to be 4.2 U/ml, 2.6 U/ml, and 3.1 U/ml, respectively. An illustration of optima activities was presented in Figure 3 and Figure 4 respectively and the optimal activity curves for pH range from 4 – 11, and temperature range from 30 – 100$^{o}$C for amylase, cellulase and pectinase are indicated respectively. The modulation technique effectively improved the stability of the *Bacillus* genome at increased temperature ($^{o}$C) up to 60$^{o}$C and lower ionic concentration of pH 6.0, despite the fact that the growth and effective activity of *Bacillus subtilis* have recorded optimal growth and functionality at 50$^{o}$C and pH 7. Comparable reports have shown that changed plasmids advance components that impact target quality record for hyper performance [Yang et al. 2013]. In addition, the successful development and its relevance to the environmental and industrial benefits have been extended to promote the improvement of bioactive agents found in plant materials. This development has effectively demonstrated improved bioprocess activities at extreme conditions that typically limit the stability of enzymatic reactions in terms of temperature and pH. *Calotropis procera Ait Leaf* has been accounted for as an important plant material of Phyto-chemicals, Sumczynski et al. 2016] and its properties have aided drug discovery-related research [Shobha et al. 2014]. A few reports have distinguished the presence of significant bioactive mixtures and antimicrobial specialists in *Calotropis procera Ait Leaf*. Figure 5 is a schematic illustration of isolating phytochemicals from a plant material. In the figure above, a cocktail of amylase, cellulase and pectinase

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produced were applied in the achieve enzyme assisted extraction. Moreover, the same steps were carried out using selected diethyl ether, 70% ethanol, and methanol solvents. Methods for isolating phytochemical compounds have been identified in a number of reports to overcome the health risks associated with synthetic drugs, but their limitations have consistently favoured the production of costly synthetic drugs [Gonzalez et al. 2021]. The use of mixed enzymes to boost the potency of antimicrobial properties and assist in the expression of phyto-compounds was investigated as a means of overcoming the limiting factors in biotransformation. Analysis on enzyme-assisted extracts, 70% ethanol, methanol, and dimethyl ether extracts were carried. The results revealed a 35% increase in identified compounds from enzyme-assisted extracts; terpenoids, flavonoids, anthraquinone, alkaloids, steroids, tannins, and saponins as compared to others. The outcomes recorded were lined up with the reports stated according to [Cole et al. 2019], in which enzyme-treated matrices were used to extract bioactive compounds comparison to untreated controls. Analysis of the extracts to identify the phytochemicals from various extracts were outlined and in Table 1.

In a similar experiment, application of enzyme-assisted extraction increased the phenolic content by more than 25% to 30% in comparison to the control [Maratha et al. 2017, Nadar et al. 2018, Yazdi et al. 2018]. An increase in phyto-compounds was recovered through the enzymatic pre-treatment method in a omparable study. According to a common reference from each report, bioactive compounds from various plants had higher yields and inhibitory properties than the control, which was consistent with the findings of this investigation. Valuable modern hydrolytic bioagents are being utilized by maturation methods and in view of better return from microbial sources and great ecological factors like temperature ($^{O}$C) and pH, their pertinence to modern bioprocessing has become overpowering. Because they are sensitive and enable the synthesis of active molecules from specific substrates in a relatively short amount of time, enzymes actively proceed in a single mode or consortia effectively under ideal conditions. This mode of operation is related to the pre-treatment of substrates, which ultimately results in the extraction of valuable compounds with advantages for the economy, society, and industry. Aligned with the findings of this investigation is a similar report in which a mixture of alpha-amylase and cellulase was used to infuse a powdery sample of *Combretodendron macrocarpum* for one hour at 50°C and pH 7.0. This made it possible for the synthases to isomerize the sample's cellulose and starch, respectively, and made bioactive compounds more likely to be secreted. The utilization of chemical consortia was additionally detailed report by [Shen et al. 2021] where a mix of cellulase, pectinase, and tannase was utilized to remove phenolic compounds at various extents. Antimicrobial expression was also measured in this study, and the enzymatic cocktail had a significant impact on the expression of bioactive agents in the *Calotropis procera* extract. In Table 2, effect of enzyme assisted extraction on bioactive agents' inhibitory activities against selected test strains are shown. The reports clearly justified the application of biocatalyst in the extraction of valued plant chemicals. The concentrates of diethyl ether, 70% ethanol, methanol, and catalyst helped extricates were investigated for inhibitory activities against normal pathogenic strains: *Escherichia coli*, *Enterobacter aerogene*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Candida albicans*. Compared to the inhibitory activities of the other extracts mentioned above, the enzyme-assisted extract had higher expressions of 33.3 mm, 26.5 mm, 30.4 mm, 31.6 mm, 30.6 mm, and 22.3 mm for *Escherichia coli*, *Enterobacter aerogene*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Candida albicans*, respectively These compounds have been found to be present in cell walls, but due to the plant's cellular structure, it is typically challenging to obtain yield and identify novel compounds. Plant cell wall interruption is the essential step of extraction for the vast majority bioactive mixtures existing inside the structure [Zhang et al. 2021, Mota et al. 2018]. The extraction of valuable plant phytochemicals has been somewhat enhanced through the use of enzymatic processes. When compared to untreated samples, it almost always increased the concentration and contributed to an increase in the release of target compounds [Doria et al. 2022, Rani et al. 2021, Zhang et al. 2020, Chandhirasekar et al. 2021, Gilgor, 2019, Danalache et al. 2018, Krakowska-Sieprawska et al. 2020, Kumar et al. 2021, Huy et al. 2019]. In a similar study, it was found that when enzymes and substrates bind, the shape of the enzyme molecule changes to make it work best with the interaction. This shape change could put stress and strain on the substrate, which would

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break the bonds and make the reaction go ahead. In addition, adding the enzyme can speed up the reaction when the concentration of the substrate is high until it reaches its limiting level.

The common results obtained showed that enzyme-assisted extraction led to a reduction in extraction time and solvent volume in addition to the increased yield and quality of product [Qi et al. 2021, Wilkins et al. 2007]. Investigations into enzyme-assisted extraction technology have demonstrated the role of operational conditions such as the temperature of the reaction, the time of extraction, the pH of the system, the enzyme concentration, and the particle size of the substrate. The findings of this investigation are compared to those of [Rodrigueq et al. 2020, Cole et al. 2019, Shen et al. 2021], in which plant cell wall permeability was increased through the use of a consortium of enzymes for the extraction of bioactive compounds. Similar to the study by [Catalkaya and Kahveci, 2019], which found that the best enzymatic treatment efficiency depends on enzyme activity, treatment time, substrate ratio, and particle size. Consequently, most of the investigations of novel utilizations of bio-compounds helped extraction propose the requirement for the advancement of conditions and boundaries. As a result, one of the most important steps in increasing the release of plant materials and maintaining their stability is the extraction of secondary metabolites from the plant. Therefore, extraction efficiency is influenced by the extraction parameters like time, temperature, solvent type, extract particle size, enzyme type, and enzyme concentration.

## CONCLUSION

Laboratory optimization studies have shown that the extraction of value-added bioactive compounds using enzymes alone or in combination is a rapidly developing and beneficial field. It implies that functional plant characteristics are necessary for the best expression of characteristics. Notwithstanding, environmentally friendly processes are going after more reasonable improvement of developments in an expansive range of enterprises. All in all, this study has shown that bioactive syntase application is a maintainable extraction method to be more powerful in the recuperation of important bioactive mixtures. Additionally, the utilization of a consortium will significantly increase the extraction yield of valuable compounds in the pharmaceutical and food industries.

## CONFLICT OF INTEREST

No Conflict of Interest

## SPONSORSHIP

No Sponsorship/Grant/Award was acquired

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