A Stable, Sustainable and Edible Chitinase Formulation for Biocontrol of Fungal Rot

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Abstract

Emerging resistance to chemical fungicides and negative environmental impacts have compelled researchers to evaluate alternative methods to control disease-causing fungi. Among various food safety methods, chitinolytic enzymes provide a safe and cost-competitive natural (biological) approach to controlling fungi as compared to chemical fungicides, but the main disadvantage of enzymes relies on their instability, which decreases their half-life in the case of temperature and pH fluctuations. Enzyme immobilization can offer an opportunity to enhance enzyme stability and reusability. In this scenario, waste-derived nanoparticles (NPs) are considered ideal for synthesizing sustainable, green, highly stable nano-biofungicides. Enzyme immobilization using biopolymer strategies is considered efficient in enhancing chemical and physical properties, high surface specificity, and stability of enzyme formulation. In this study, we are synthesizing low-cost, nontoxic, environmentally friendly chitinase immobilized (NPs) based on (1) Poly (ethylene glycol)-poly(caprolactone) (PEG-PCL) and (2) Agro-waste-derived polymers (Pullulan).

Introduction

Chitinase is the most promising biocatalyst, widely used in research and industrial applications. However, their shelf-life and structural stability have limited their practical application. Enzyme immobilization techniques are employed to synthesize amphiphilic block copolymers (BCs) and then make their conjugation with the enzyme(s) (Fig.1,2) to afford an advanced and stable biofungicide for the agro-industry to control pathogenic fungi (Fig.3).

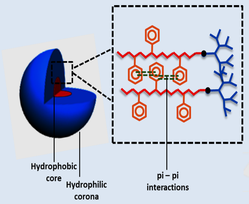

Fig.1: Structure of block copolymers (with modification Chandrasiri et al., 2022).

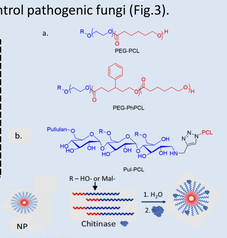

Fig.2: PEG-PCL polymer amphiphiles and their self-assembled NPs (a), Agro-waste derived (Pullulan, Mal- (maltotriose units) NPs conjugated with chitinase enzyme (2.) (b)

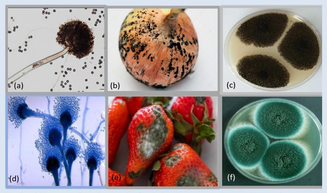

Fig.3: Microscopic view of Conidiophore of *Aspergillus niger* and *Aspergillus fumigatus* respectively at 40x (a)(d).Damages of Vegetables and fruit (such as Onion and strawberries) due to *Aspergillus niger* and *Aspergillus fumigatus* respectively (b)(e). Growth of pathogenic fungi (*Aspergillus niger, and Aspergillus fumigatus* respectively) on PDA media (c)(f) respectively.

Objectives

- Screen, identify, and optimize the culture of bacterial strains for the highest yields of chitinase using agricultural wastes (rice straw) in a positive and environmentally friendly way.
- Evaluate the antifungal potential of selected chitinase-producing bacteria against relevant pathogenic fungi (*Aspergillus fumigatus* and *Aspergillus niger*) and directly apply on strawberries and onions as a bioshield to provide a novel strategy for food protection against foodborne fungi.
- Synthesize and characterize nano-enzyme conjugation and determination of chitinase shelf-life stability in the presence of different temperatures up to the period of one month.

Materials and Methods

Isolation and screening of the bacteria based on chitinase potential.

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Phenotypic and genotypic characterizations of the select isolates.

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Agro-industrial waste (rice straw) incorporate medium was statistically optimized maximum production of chitinase as compared to already reported chitinase media (1).

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Qualitative and Quantitative optimization of medium components by statistical design (27 experiments were conducted).

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Partial purification of chitinase (ammonium sulfate precipitation with fraction of 60%) and then purified by ionic exchange chromatography.

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Chitinase antifungal efficiency assay against two pathogenic fungi (*Aspergillus fumigatus* and *Aspergillus niger)* and pathogenicity test against fungal infected strawberries and onion were also estimated (2).

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Nanoenzyme synthesis, characterization assay (2) and chitinase shelf-life stability test in the presence of different temperatures up to the period of one month were carried out.

Results

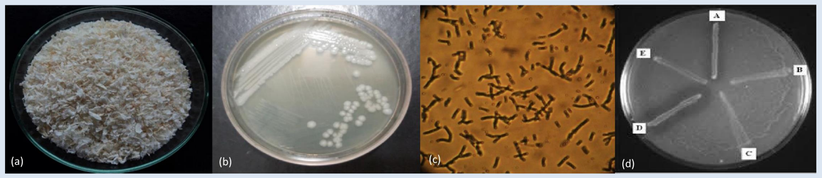

Fig.4: Chitin flake (a) Growth of *Bacillus subtilis* on agar medium (b) Microscopic view of the *Bacillus subtilis* (rod shape) at 40x (d) Clear zone formation on colloidal chitin-agar plate by *Bacillus subtilis* (D) as compared to other bacterial isolates (e).

- **Five among twenty bacterial isolates gave positive hydrolysis zone for chitinase production. On the basis of high chitinase activity only one bacterial isolate (D) was further processed for identification and optimization (Fig.4 (d)).**
- **Molecular identification of selected bacterial isolates (D) by 16S rRNA gene sequencing identified as *Bacillus subtilis*.**
- **Optimization of medium components showed prominent effect of five medium-selected components (Chitin, rice straw, peptone, $CaCl_2$, and yeast extract) out of 27 factors (Fig.5).**
- **Chitinase production was estimated about 191.30 (U/ml) in reported CPM 1 by *Bacillus subtilis*. While in self design the agro-industrial waste (rice straw) medium chitinase production was estimated upto 567.7 (U/ml) (Table.1).**
- **Among partial purification, ammonium sulfate 60% fraction illustrated chitinase activity 737 (U/ml). The ionic exchange chromatography further purified chitinases and 47 kDa band was observed using 10 % gel (SDS-PAGE) (Fig.6).**
- **Antifungal activity of partially purified chitinase illustrated 90%, and 83% inhibition in the mycelial growth of pathogenic fungi: *Aspergillus niger,* and *Aspergillus fumigatus* respectively (Fig.7,8).**
- **Efficiency of chitinase formulation for inhibiting growth of Aspergillus fumigatus estimated on onion upto 7 day at room temperature (25°C). Significant inhibitory effect in pathogenic fungal growth estimated upto 72 hours with 16% chitinase concentration apply as a biocoating on strawberries and onions (Fig.9).**
- **Nanochitinase bioconjugation exhibited enzyme-specific activity 203.1 (U/mg) with an increase in molecule size upto 337.2 nm as compared to free enzyme and nanoparticles (Table.2).**
- **Shelf-life stability of partially purified chitinase (ammonium sulfate 60%) illustrates stability upto 18 days at room temperature (25°C) (Fig.10).**

Table.1: Comparison between the composition of already reported chitinase medium (CPM 1) and agro-wastes incorporated self-design medium (CPM 2).

Sr. No.	Chitinase Medium Composition	Reported medium (CPM 1) (g/L)	Self-designed medium (CPM 2) (g/L)
1	Chitin	10.0	5.0
2	Calcium Chloride	0.01	0.3
3	Rice straw	-----	0.5
4	Yeast extract	-----	0.2
5	Sodium chloride	0.50	-----
6	Magnesium sulfate	0.12	-----
7	Disodium hydrogen phosphate	6.00	-----
8	Potassium dihydrogen phosphate	3.00	-----
9	Ammonium chloride	1.00	-----
10	Glucose	5.00	-----

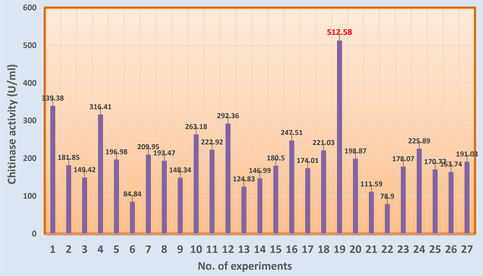

Fig.5: Experimental design for 27 runs showing results of the statistical design for chitinase activity.

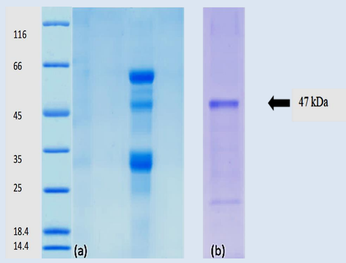

Fig.6: Partial purification of chitinase with ammonium sulfate precipitation (60% fraction); purification of chitinase using ionic exchange chromatography (b).

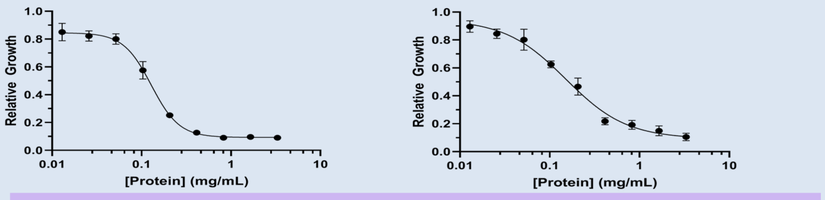

Fig.7: Antifungal assay illustrated relative growth inhibition of *Aspergillus niger, and Aspergillus fumigatus* in the presence of partially purified chitinase.

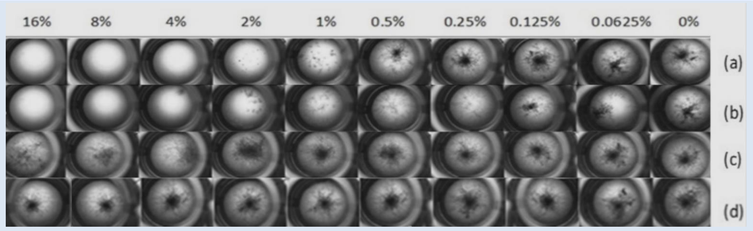

Fig.8: Microscopic view (40X) shows pathogenic fungi mycelium growth lysis in the presence of different concentrations of chitinase formulation higher to lower (16% to 0%) in horizontal line. Mycelium growth pattern of *Aspergillus fumigatus* inhibited in the presence of chitinase (a), Mycelium growth pattern of *Aspergillus niger* inhibited in the presence of chitinase (b), Negative control showed normal mycelial growth pattern of *Aspergillus fumigatus* without chitinase (c), Negative control showed normal mycelial growth pattern of *Aspergillus niger* without chitinase (d).

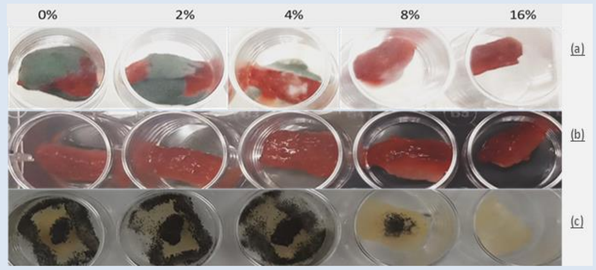

Fig.9: Macroscopic view shows pathogenic fungi mycelium growth lysis in the presence of different concentrations of partially purified chitinase formulation lower to higher (0% to 16%) in horizontal line. Mycelium growth pattern of *Aspergillus fumigatus* inhibited in the presence chitinase using surface coating method after 7 days (a), Only sterilized distilled water used as a negative control treatment (b), Mycelium growth pattern of *Aspergillus niger* inhibited in the presence of chitinase after 7 days using well coating method (c).

Table 2: Estimation of enzyme activity and characterization of enzyme-nanoparticles bioconjugation.

Sr. No.	Name of Particles / molecules	Type	Z-Average particles size (nm) (DLS)	Chitinase activity (U/ml)	Protein activity (mg)	Specific activity (U/mg)
1	Chitinase	Enzyme	205.5±0.3	737	3.3	223.3
2	Mal-PEGPCL	Nanoparticle	96.44±0.1	0	0	0
3	Meo-PEG-PCL-Mal PEG-PCl (1-1)	Nanoparticle	65.86±0.1	0	0	0
4	Meo-PEG-PCL (Negative control)	Nanoenzyme	66.2±0.1	0	0	0
5	Meo-PEG-PCL-Mal PEG-PCl (1-1) and chitinase	Nanoenzyme	337.2±1.0	589	2.9	203.1

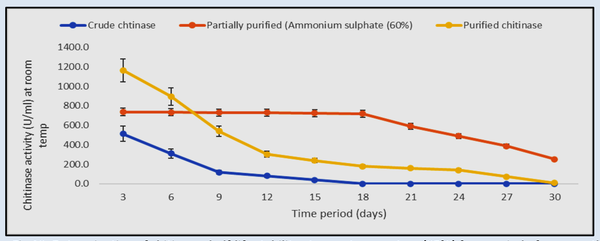

Fig.10: Determination of chitinase shelf-life stability at room temperature (25°C) for a period of one month.

Conclusion

The isolated *Bacillus subtilis* showed maximum chitinase yield and growth on rice straw wastes incorporated self design medium and showed more than 2 folds increase in chitinase production as compared to the already reported chitinase production medium (1). Partial purification by ammonium sulfate 60% fraction was found very effective and promising for inhibiting pathogenic fungi growth with 4 folds increase in chitinase activity. The antifungal potential of chitinase showed substantial potency for biocontrolling the growth of pathogenic fungi upto 72 hours on strawberries and onions, can be further utilized for field experiments to prevent post-harvest fungal damage to crops. To enhance enzyme properties, immobilized chitinase can be used as a stable, reliable platform for sustainable environmentally friendly biofungicides. Our future goal will be further characterization and screening of nanoenzyme formulation for highly stable fungal biocontrol agent, especially on commercially important fruits and vegetables.

References

1. Singh, R. K., et al. (2013). Optimization of media components for chitinase production by chickpea rhizosphere associated *Lysinibacillus fusiformis* B-CM18. *Journal of basic microbiology*, *53*(5), 451-460.
2. Paul, M. K., Mini, K. D., Benn, A. R., & Mathew, J. (2022). *Kurthia gibsonii* Mb126 immobilised chitinase against *Aspergillus flavus*, a fungal pathogen linked to lemon postharvest deterioration. *Indian Journal of Science and Technology*, *15*(3), 136-145.