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Original Research Article

Biosynthesis and Characterization of an Anticoagulant Chitinase from Fermented Wheat Bran & Shrimp Shells' Substratum

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HIGHLIGHTS

- Optimization of solid-state fermentation of *Citrobacter freundii* str. nov. *haritD11.*
- The halotolerant chitinase was produced from wheat bran supplemented with shrimp shellfish waste.
- The enzyme had notable tolerance to heavy metal ions.
- Chitinase was purified 4.24-fold with 31% yield and specific activity of 64.87 U/mg.
- The enzyme can be applied as an antifungal and anticoagulant agent.

ABSTRACT

Solid state fermentation (SSF) of wheat bran coupled with shrimp shellfish waste *Citrobacter freundii* str. nov. *haritD11* was optimized conventionally (112.43 U/gds) and statistically (124.73 U/gds). Chitinase was purified 4.24-fold with 31% yield and specific activity of 64.87 U/mg protein. The purified chitinase had a specific activity of 64.87 U/mg with optimal activity at pH 9 and temperature 45 ºC. The enzyme was stable at 8.0–9.5 pH range with 90% stability and between 45 °C – 60 °C for 1 hour. The Km value of the *Citrobacter freundii haritD11* purified chitinase with swollen chitin (substrate) is 7.53 mg/mL with a V_{max} of 2.27 mmol $h^{-1}mL^{-1}$. The purified chitinase was halotolerant showing maximum activity and stability up to 9% Sodium chloride, it also possessed potential antifungal and anticoagulant activity. This is the first report to date elucidating the production of halotolerant chitinase from wheat bran supplemented with shrimp shellfish waste using *Citrobacter freundii haritD11* with notable tolerance to heavy metal ions, its application as an antifungal and anticoagulant agent.

Keywords:
Anticoagulant
Antifungal
Chitinase
Citrobacter freundii harit D11
Purification
Shrimp waste
Wheat bran

Introduction

Organic solid wastes like gram husk, wheat/rice bran, vegetable/fruit peelings or chitinous wastes of household/ agricultural /industrial origins can be employed as feedstock in Solid state fermentation (SSF) (Meruvu and Donthireddy, 2014a; Yazid et al., 2017; Sadh et al., 2018). In particular, agroindustrial wastes like husks/ brans (which are of trivial economic importance) can be used in SSF for producing of industrially essential chemicals, biopharmaceuticals, enzymes etc. (Li et al., 2016; Chiriboga et al., 2017). Production of myriad enzymes like amylases (Almaki, 2018), proteases (Meruvu et al., 2011a; Meruvu et al., 2011b), chitinases (Meruvu and Donthireddy 2012; Meruvu and Donthireddy, 2014a), cellulases and xylanases (Sousa et al., 2018) etc. using SSF and agroindustrial wastes has been reported by

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earlier researchers.

Chitinase production using wheat bran when supplemented with chitin by many microorganisms like *Beauveria bassiana*, *Fusarium oxysporum*, *Penicillium aculeatum*, *Trichoderma harzianum*, *Oerskovia xanthineolytica* under SSF has also been reported earlier (Suresh and Chandrashekaran, 1999; Gkargkas et al., 2004; Sandhya et al., 2004; Binod et al., 2005; Waghmare et al., 2011). Microbial conversion of chitinous substrates through chitinolysis resolves environmental problems and diminishes the manufacture costs of microbial fermentation. The biochemical diversity of marine microbes makes them benign sources to produce enzymes that find applications in food, medical and other biotechnological industries (Beygmoradi and Homaei, 2017; Bahrami et al., 2018; Bissaro et al., 2018; Meier et al., 2018).

In this report SSF is carried out to observe chitinase production at different combinations of wheat bran (vegan) and shrimp waste (non-vegan) as a mixed substratum. Screening and evaluation of biochemical needs of the microorganism is a requirement for bioprocess development during SSF for optimal enzyme production. Hence in this work, traditional optimization studies through one-variable-at-a-time (OVAT) approach followed by statistical optimization using Box Behnken method is adopted for monitoring chitinase production parameters. *Citrobacter freundii str. nov. haritD11* with chitinolytic activity isolated from marine sediment is used for the SSF studies throughout experimentation. In our previous works which pivoted around *Citrobacter freundii* str. nov. *haritD11*, isolation and identification of *C. freundii haritD11*; fermentation of shrimp waste with *Citrobacter freundii haritD11* and the consequential purification and characterization subterfuges were evidently reported (Meruvu and Donthireddy, 2012; Meruvu and Donthireddy, 2013; Meruvu and Donthireddy, 2014a; Meruvu and Donthireddy, 2014b;). However, this is the first report to date elucidating the production of chitinase through solid state fermentation of wheat bran and shrimp waste using *C. freundii haritD11*, its partial purification, characterization and constituent antifungal activity. Moreover, there is no known report on chitinase production from *Citrobacter freundii* through fermentation of wheat bran-shrimp waste combination and estimation of possible antifungal and anticoagulant properties of produced chitinase.

Materials and Methods

Chitinase activity assay

0.1 mL of chitinase was mixed with 0.1 ml of substrate solution (10% colloidal chitin in 0.2 M phosphate buffer, pH 8.0) and incubated for an hour at 35 ºC. Amount of reducing sugar released was measured by DNS method (Miller, 1959) at 540 nm using N-acetyl-D glucosamine standard. One unit of chitinase activity was defined as the amount of enzyme producing 1 µmol of GlcNAc per hour at specified assay conditions (Meruvu and Donthireddy, 2014b).

Microorganism and fermentation studies

Citrobacter freundii str. nov. haritD11 (GenBank Accession number KC344791) was isolated from a marine sediment sample taken from NTPC beach area of the Bay of Bengal sea coast, India (17°31′51″north latitude and 83°4′53″ east longitude) and was used as fermentation inoculum throughout. The organism's biochemical and molecular taxonomy were reported in our previous report (Meruvu and Donthireddy, 2013). Its chitinolytic activity was visualized by streaking over minimal salt (MS)-chitin agar plate containing 0.5% w/v colloidal chitin with MS medium (Heravi et al., 2015) and incubated for 72 hours at 30 °C. The strain showed a striking zone of clearance of nearly 0.62 cm after 24 hours. It was maintained on Yeast extract Malt extract agar slants and subcultured every month (Meruvu, 2017).

Both the substrates wheat bran and shrimp waste are procured from local vendors. Initial fermentation conditions for chitinase production were 24 hours incubation at 30 °C, 0.5% v/w inoculum (10^9 CFU/ml), 40% v/w moisture and 8 pH using 5 grams (1:1 ratio of wheat bran and shrimp waste). Leaving other variables constant, the only factor being studied was altered, likewise production conditions were optimized one by one; substrate ratio, temperature 25-50 °C; pH 6.0-10.0; inoculums content 0.5-3.0% w/v; moisture content 30-80%; and levels of chitinase production were compared. The crude chitinase broth was obtained at the end of every fermentation step by mixing the fermented substrate with distilled water (1:20 ratio) and filtering with Whatmann no.1 Filter paper. The fermentation conditions were further optimized by RSM –Box Behnken method (Box et al., 1978; Srikanth et al., 2018) and at fully optimized conditions, fermentation of a 20 gram batch of mixed substrate was carried out through SSF. Software used was STATISTICA Version 10.

Purification studies

100 mL Culture broth was centrifuged, lyophilized, mixed in 20 mL 0.2 M acetate buffer, centrifuged and dialyzed. Dialyzed enzyme was subjected to ammonium sulfate precipitation technique at different

ranges of concentration from 40–70 % (w/v) at 4 °C to maintain the biological activity of chitinase. The dialyzed protein sample was suspended in 3 mL of 0.1 M citrate-phosphate buffer (pH 6.0). The suspension was eluted through Sephadex G-100 column. The elution buffer is 50 mM Tris–HCl, and the volumetric flow rate was 0.4 mL/min. Every 8 mL from the effluent of gel filtration was collected in a fraction and assayed for activity against the substrate colloidal chitin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis technique was used to check the molecular weight of the purified enzyme. Chitinase activity and protein content were analyzed in supernatant and the pellet (Lowry et al., 1951; Meruvu and Donthireddy, 2014b; Meruvu, 2017).

Characterization studies of purified chitinase

The purified chitinase was tested for its activity and stability at different ranges of temperature, pH and substrate specificities.

Effect of pH on enzyme activity and stability

Effect of pH on chitinase activity was tested by incubating the reaction mixture of 1.12 µg of chitinase and 0.5% chitin at different pH levels ranging 5–10 under standard assay conditions. Effect of pH on chitinase stability, was determined by pre-incubating 30 µg of chitinase in 500µL of various buffers without substrate at 35 ºC, for 36 hours. Post pre-incubation, the reaction mixtures were diluted 12-fold in a 200µL volume and 40µL of the diluted samples were checked for residual activity under standard assay conditions.

Effect of temperature on enzymatic activity and stability

Effect of temperature on chitinase activity was tested by incubating 0.4 µg of chitinase with 0.5% chitin at different temperatures up to 80 °C, at pH 8 for 5 minutes. Thermal stability of chitinase without substrate was determined by incubating 15µg chitinase in 50mM sodium acetate buffer at a range of temperatures for half hour at pH 8. The residual chitinase activity was measured at standard assay conditions. To measure the thermal stability of chitinase in the presence of substrates, 18 µg of chitinase in 100 µL volume was incubated with 0.5% chitin in 50mM sodium acetate buffer pH 8, at 50 ºC for 30 minutes. After incubation, the reaction mixtures were diluted again and 40 µL of the diluted samples were taken to determine the residual activity under standard assay conditions.

Substrate specificity and enzyme kinetics

The substrate specificity of the enzyme was tested by reacting it with substrates like cellulose, chitin flakes, and swollen chitin. The effect of swollen chitin on the chitinase activity of *Citrobacter freundii haritD11* was evaluated by ranging the swollen chitin concentration from 0.5 to 10 mg/mL. A Lineweaver-Burk plot was obtained by plotting 1/S against 1/V. Kinetic parameters (Km and Vmax) were estimated from Lineweaver-Burk plot (Nagpure and Gupta, 2013; Meruvu and Donthireddy, 2014b; Rahman et al., 2014).

Effect of metal ions and compounds on chitinase activity

The influence of standard metal ions on purified chitinase was studied by adding 25mM metal ions: K^{+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+}, As^{4+}, Pb^{2+}. The effect of EDTA, SDS and urea was also tested by adding 10mM of the chemical to the assay mix and assaying the activity.

Antifungal activity

Different fungi were tested for vulnerability to purified chitinase by disc diffusion method. Sterile and freshly prepared Potato Dextrose agar plates were taken and spread with 100 µL of inoculum containing nearly 10^5 conidia/ml of the test fungi. The discs were impregnated with 5 µL of 10 mg/mL purified chitinase enzyme were placed on the inoculated PDA plates and incubated for 3–4 days at 30 °C, then observed for the extent of fungal clearance zone.

Anticoagulant activity

Five test tubes with the fabricated 5 mg/mL fibrin solution were added with the purified chitinase (2.8mg/mL) in the amounts of 0.4, 0.6, 0.8 and 1 mL. After that, the test tubes were incubated at 40 ºC and assayed for fibrinolysis. Moreover, the *C. freundii haritD11* purified chitinase was tested for anticoagulant activity in comparison to the commercially available nattokinase as revealed by the fibrin plate assay (Jespersen and Astrup, 1983).

Results and Discussion

Optimization studies
Solid state fermentation

Laboratory scale fermentation, with initial conditions of 24 hours incubation, 30 °C, 5% v/w inoculum, 30%

Table 1. The Box-Behnken design matrix employed for three independent variables with 15 runs along with observed and predicted chitinase activity values.

Run no.	Temp. (ºC)	Wheat bran: shrimp waste substrate ratio	Moisture content (%w/w)	Observed chitinase activity (U/gds)	Predicted chitinase activity (U/gds)
1	30.00	0.250	45.00	108.0	116.8
2	40.00	0.250	45.00	102.0	100.6
3	30.00	0.750	45.00	121.0	122.3
4	40.00	0.750	45.00	118.0	109.1
5	30.00	0.500	40.00	104.0	101.5
6	40.00	0.500	40.00	88.00	95.75
7	30.00	0.500	50.00	110.0	102.2
8	40.00	0.500	50.00	76.00	78.50
9	35.00	0.250	40.00	122.0	115.6
10	35.00	0.750	40.00	110.0	111.1
11	35.00	0.250	50.00	97.00	95.87
12	35.00	0.750	50.00	108.0	114.3
13	35.00	0.500	45.00	110.8	111.7
14	35.00	0.500	45.00	112.3	111.7
15	35.00	0.500	45.00	112.0	111.7

v/w moisture and 8 pH using 5 grams 'wheat bran plus shrimp waste' (1:1), was conducted adopting OVAT approach and from the corrected optimization conditions the variables temperature, substrate ratio and moisture content were found to notably influence the chitinase production; hence, were further investigated statistically by Response surface methodology (RSM).

Response surface methodology (RSM)

Box Behnken method adopting 3 variables with 15 runs was used for statistical optimization. The application of RSM yielded the following regression equation explaining empirical relationship between chitinase yield and test variables in coded units.

Table 2. ANOVA test; 4 variable and 3-level factors, R^2 =0.78.

	SS	df	MS	F	P
(1)Var1 L + Q	622.743	2	311.3714	4.108772	0.088013
(2)Var2 L + Q	312.485	2	156.2427	2.061737	0.222344
(3)Var3 L + Q	514.894	2	257.4468	3.397198	0.117013
1*2	2.250	1	2.2500	0.029690	0.869952
1*3	81.000	1	81.0000	1.068854	0.348594
2*3	132.250	1	132.2500	1.745135	0.243715
Error	378.910	5	75.7821		
Total SS	2084.849	14			

ANOVA: analysis of variance; Var1: Temperature; Var2: Wheat bran: shrimp shell waste substrate ratio; Var3: Moisture content; SS: Sum of squares; df: Degrees of freedom; MS: mean squares.

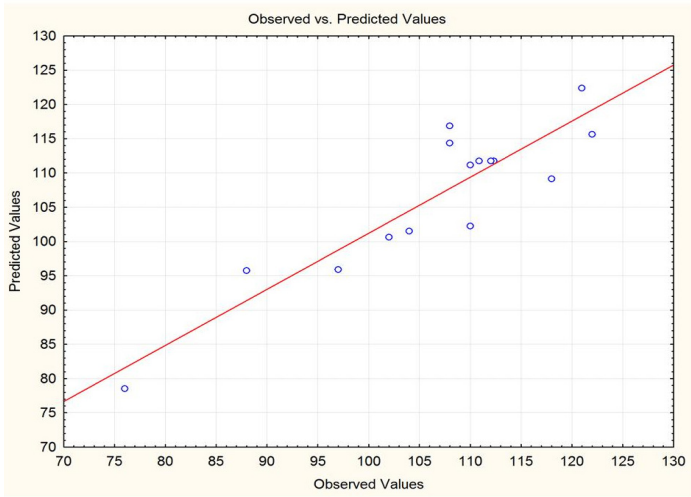


Figure 1. The parity plot between the experimental and predicted values of chitinase activity presenting the accuracy of the model (R^2 =0.8).

$Y = 110.4 - 7.37X_1 + 3.56X_1X_1 - 4.12X_2 - 7.62X_2X_2 - 4.12\ X_3 + 5.06\ X_3X_3 + 0.75\ X_1X_2 - 4.5\ X_1X_3 + 5.75\ X_2X_3$;

Where, Y is chitinase yield; X_1, X_2 and X_3 are coded values of Temperature, Substrate ratio and Moisture content, respectively (Table 1). Estimation of regression analysis and ANOVA explained coefficient (R^2= 0.78) (Table 2) and a parity plot displaying clustered points around the diagonal line (Fig. 1) indicate a correlated fit between experimental and predicted values.

The smaller P values and good interaction between independent variables confirm significance of each coefficient (Mourabet et al., 2017) and the yield can be predicted from the respective contour plots (Fig. 2) chiefly from the confined surface of the response surface diagram.

The critical levels of the three independent variables examined as predicted from the model are: temperature 32 °C, Substrate ratio 0.44 (wheat bran: shrimp waste) and Moisture content 44 % v/w and at these conditions predicted chitinase activity was 112.117 U/gds. A verification experiment at the critical conditions confirmed that the experimental value (124.73 U/gds) was similar to the value predicted (123.68 U/gds) substantiating both validity and effectiveness of the model. Statistically optimized culture conditions using Box Behnken method showed augmented chitinase production than that of basic optimization (one-variable-at-a-time-approach) culture conditions (112.43 U/gds). A similar report following RSM optimization with 1.1-fold increase in enzyme activity for chitinase production from *Parapeneopsis hardwickii* (spear shrimp) exoskeleton by solid-state fermentation was reported (Meruvu and Donthireddy, 2014a).

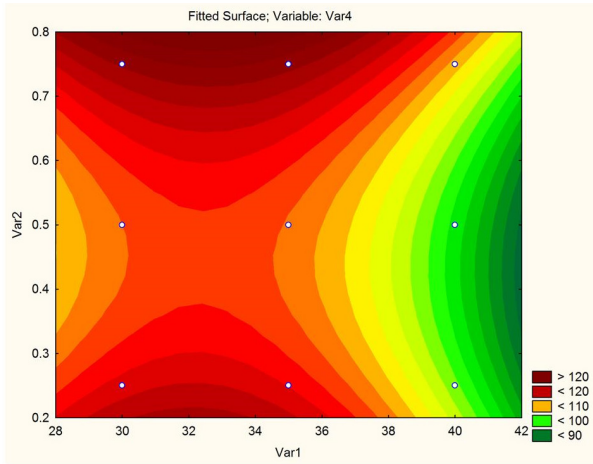
Purification and characterization studies

Chitinase in the culture filtrate was extracted by 70% ammonium sulfate precipitation followed by dialysis and gel chromatography. Chitinase was purified 2.7-fold with 31% yield and specific activity of 64.87 U/

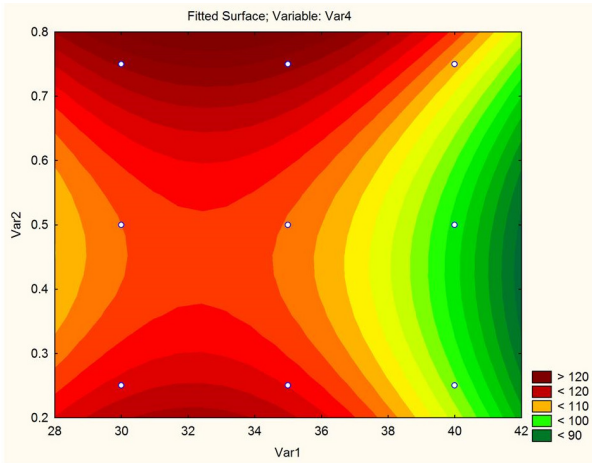
Table 3. Steps in purification of chitinase.

Purification step	Total activity (U)	Specific activity (U/mg)	Folds of purification	% Yield
Culture supernatant	4987	15.3	1	100
$(NH_4)_2SO_4$ Precipitation	4039	29.07	1.9	81
Dialysis	2643	41.31	2.7	53
Sephadex-G100 Filtration	1546	64.87	4.24	31

(a).



(b).



(c).

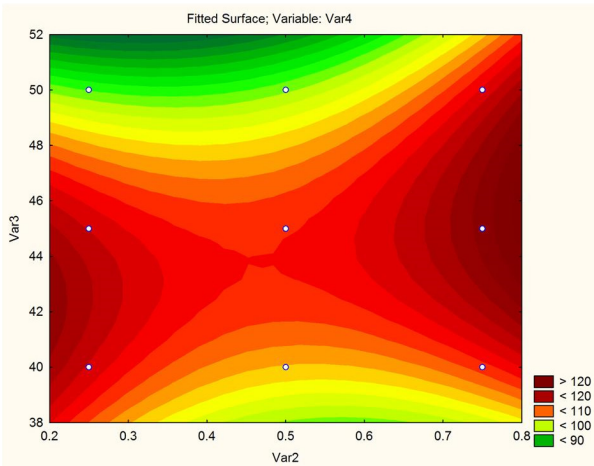


Figure 2. Contour plots between temperature (Var 1), substrate ratio (Var 2), moisture content (Var 3) and the corresponding chitinase activity. The different coloured bars shown in the right side scale represent the various levels of chitinase activity accordingly and as the colour gets darker, the response increases. The response is at its highest at the darkest region of the plot. (The optimal values of the three variables are- temperature 32°C, substrate ratio 0.44 [wheat bran: shrimp waste] and moisture content 44 % v/w).

Table 4. Effect of metal ion or other chemical compound on the activity of purified chitinase.

Metal ion/ Compound	Relative activity
None	100
Na+	172
K+	139
Mg^{2+}	143
Mn2+	178
Fe^{2+}	152
Hg^{2+}	79
Co^{2+}	43
Cu^{2+}	124
As^{4+}	31
Pb^{2+}	71
Zn^{2+}	93
Urea	148
SDS	91
EDTA	94
NaCl	260

mg protein from 30 grams of wheat bran and shrimp waste mixed substrate (Table 3).

Effect of pH and temperature on enzyme activity and stability

Purified chitinase showed optimum activity at 9 pH. A fall in enzyme activity was shown at pH values above 10 or below 8 showing increased activity at alkaline conditions. The enzyme was stable at 8-9.5 pH range for 1 hour at 4°C in various buffers; Correspondingly, chitinase from *Alternaria infectoria* was stable at alkaline pH (Chiriboga et al., 2017) but some bacterial chitinases were found stable at acidic pH (Farag et al., 2016; El-Shora et al., 2017). The optimum temperature for chitinase was recorded at 40°C, likewise 40-50°C has been reported for *Aspergillus terreus* (El-Shora et al., 2017), Paenibacillus pasadenensis CS0611 (Zing et al., 2016) and *Escherichia coli* (Thimote et al., 2017). Chitinase maintained 80% stability between 45 °C and 60 °C, other temperatures variation lead to activity inhibition. At these conditions, chitinase was tested for its salt tolerance at various concentrations of sodium chloride and showed 100% activity at 9% and was complete stability for an hour. Chitinases with alkaline pH optima and stability have applications in biological control of insect pests and can be used in synergism with other biocontrol agents.

Substrate specificity and enzyme kinetics

The purified chitinase showed highest substrate specificity towards swollen chitin among chitin, swollen chitin, cellulose, and carboxy methyl cellulose; hence, the kinetics of the purified enzyme were studied with swollen chitin. Michaelis Menten constants were determined using Lineweaver Burke plot designed to calculate reaction velocities at each substrate concentration. From the Michaelis Menten Kinetics, the Lineweaver Burke plot the Km value of the *C. freundii haritD11* partially purified chitinase for swollen chitin is 7.53 mg/mL with a V_{max} of 2.38 mmol $h^{-1}mL^{-1}$. Similarly, the value of Km for *Serratia marcescens* B4A chitinase was 8.3 mg/mL with swollen chitin as a substrate (Zarei et al., 2011).

Influence of metal ions, EDTA, SDS, and urea on purified chitinase activity

The influence of various metal ions on the chitinase activity is shown in Table 4. There was a substantial increase in relative activity with Na^{+} (72%), K^{+} (39%), Mg^{2+} (78%), Mn^{2+} (78%), Fe^{2+} (52%), Cu^{2+}(24%), urea (48%), NaCl (160%), while, relatively decreased chitinase activity was detected with EDTA (6%), SDS (9%) and Hg^{2+} (21%). A similar effect of metal ions and other compounds has also been recorded for chitinase from Bacillus thuringiensis subsp. kurstaki HBK-51 (Kuzu et al., 2012).

Antifungal activity of chitinase

The antifungal activity of this enzyme was investigated using various strains of pathogenic fungi. The purified

Table 5. The antifungal activity of purified *chitinase* represented in terms of zone of inhibition of fungal growth in millimeter.

Micro-organism	Zone of inhibition (mm)
Aspergillus flavus	31 ± 0.30
Aspergillus awamori	24± 0.17
Aspergillus terreus	28 ± 0.22
Rhizopus oryzae	21 ± 0.54
Aspergillus niger	27 ± 0.50
Shigella sonnei	9 ± 0.43

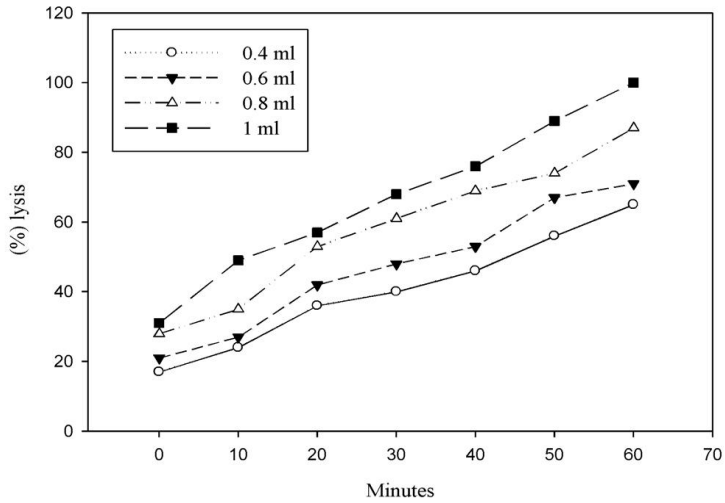


Figure 3. Graph showing evaluation of the fibrinolysis using fabricated fibrin solution.

chitinase showed excellent inhibition zones after incubation with fungi like *Rhizopus oryzae*, *Aspergillus flavus*, *Aspergillus awamori* and *Aspergillus terreus* could be used as a potent antifungal agent (Table 5). Furthermore, purified chitinase produced from mixed substrates (wheat bran plus shrimp waste) showed improved quality antimicrobial activity compared to purified chitinase produced from shrimp waste alone using the same *Citrobacter freundii str. nov. haritD11* (Meruvu and Donthireddy, 2014b).

Anticoagulant activity of chitinase

Among various test tubes with the fabricated 5 mg/ml fibrin solution incubated with partially purified chitinase, maximum fibrinolysis was shown by the 1mL amount (Fig. 3). Moreover the *C. freundii haritD11* purified chitinase was found to possess fibrinolytic activity comparable with the commercially available nattokinase where the radial hydrolysis zone around the chitinase well was1.57 mm and 1.4 mm for the latter. There are reports stating that chitin derivatives and chitosanases have been used for clinical applications as wound-healer, blood anticoagulants and haemostatic materials (Zhu et al., 2007). However, this is the first report stating that chitinase from 'wheat bran coupled with shrimp waste' fermented with *C. freundii haritD11* can be used as a potent anticoagulation agent.

Conclusion

There are numerous reports on the production of chitinase from microorganisms like fungi, actinomycetes, bacteria and plants; however, there is no single evident report on chitinase production from *Citrobacter freundii* fermenting wheat bran supplemented with shrimp waste (0.44 ratio). Our research has established the superior production of chitinase through mixed substrates improving the quality for solid state fermentation, contributing to cost-cutting strategies and production of chitinase with antifungal activity. From an industrial perspective, chitinase of *Citrobacter freundii* str. nov. haritD11 has many beneficial characteristics like high productivity, high specific activity, easy purification, relatively high halotolerance & thermostability, and as an antifungal agent in its purified form. In a nutshell, this is the first report to date elucidating the production of chitinase fermenting an assorted ratio of wheat bran and shrimp waste chitin with *Citrobacter freundii str. nov. haritD11*, its purification and characterization, and potent biotechnological applications as both an antifungal and anticoagulant agent.

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Competing Interests

As the corresponding author I declare that there are no

conflicts of interest and the project was in compliance with ethical standards.

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