Research Article

Anticoagulant activity of partially purified chitinase produced by *Citrobacter freundii* str. nov. haritD11 by fermentation of wheat bran coupled with fish scales

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Abstract
Solid phase fermentation of wheat bran coupled with powdered fish scales could yield chitinase using a novel *Citrobacter* strain. The fermentation conditions are optimized conventionally (84.14 U/gds) and statistically (94.3 U/gds). The partially purified chitinase had a specific activity of 53.41 U/mg with optimal activity at pH 8.5 and 50 °C, it was stable at 7.5–10 pH range for 24 h with more than 80% stability at 45–60 °C, and the Km value of chitinase with swollen chitin (substrate) is 8.11 mg/ml with a V_{max} of 2.43 mmol h^{-1} ml^{-1}. The partially purified enzyme was halotolerant showing maximum activity and stability up to 10% of Nacl, also possessing potential anticoagulant activity. This is the first report to date elucidating the production of halotolerant chitinase from wheat bran supplemented with fish scales using *Citrobacter freundii* haritD11 and its application as an anticoagulant agent and notable tolerance to heavy metal ions.

Keywords Chitinase · Wheat bran · *Citrobacter freundii* haritD11 · Purification · Anticoagulant · Antimicrobial

1 Objectives and background

In our prior works which pivoted around *Citrobacter freundii* str. nov. haritD11, isolation and identification of *C. freundii* haritD11, its fermentation using shrimp waste, production, purification and characterization subterfuges of chitinase were evidently reported [20–22, 24]. However, this is the first report to date elucidating the production of chitinase through solid state fermentation of wheat bran supplemented with fish scales using *C. freundii haritD11*, its partial purification, characterization and constituent anticoagulant activity. This paper also re-emphasizes that this novel strain *C. freundii haritD11* could be commercially exploited to use its enzyme preparations both in crude and purified forms as anticoagulant agents. Moreover, until now there is no known report on chitinase production from *Citrobacter freundii* through fermentation of wheat bran supplemented with fish scales and estimation of possible anticoagulant, antibiotic and metal resistance properties of obtained chitinase.

2 Introduction

As feedstock in solid state fermentation (SSF), a plethora of solid wastes such as husks of rice, wheat, green/black/red grams, cereal brans, sugarcane bagasse, vegetable and fruit peels, and chitinous wastes such as shrimp/crab shells and fish scales can be deployed [22, 33]. Agroindustrial wastes such as wheat/rice bran are used by microorganisms as finicky substrates in SSF for production of industrial enzymes and biopharmaceuticals due to their porous structure amenable for growth and capacity to hold requisite moisture [8]. Production of a multitude of industrial enzymes such as cellulases and xylanases [29], amylases [2, 23], proteases [19] using SSF and agroindustrial wastes

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has been reported by earlier researchers. Fish scales are chitin loaded and can be used as substratum to produce chitinolytic microbial enzymes [11, 14]. Chitin production in vertebrates by means of chitin synthase has also been reported [30]. Wheat bran supplemented with chitinous sources can be used as a substrate for chitinase production [5].

Marine microbes are reputed for their immense biodiversity and production of different kinds of enzymes and biochemicals of industrial grade [3, 17]. Screening and assessment of biochemical requirements of a microorganism for enzyme production is a must for bioprocess development during SSF for chitinase production; hence in this report SSF is carried out using wheat and conventional optimization studies through one-variable-at-a-time (OVAT) approach followed by statistical optimization using Box–Behnken method [6] are conducted for monitoring chitinase controlling parameters. *Citrobacter freundii* strain novel haritD11 (GenBank accession: KC344791) with chitinolytic activity isolated from marine sediment is used as inoculum for SSF studies and the chief substrate was wheat bran, procured from a local flour mill, were used throughout the experimentation. The chitinase produced is partially purified through ammonium sulfate precipitation, dialysis, gel filtration and characterized for its various properties.

3 Material and method

3.1 Chitinase activity assay

0.1 ml of chitinase to be assayed is 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 °C. Amount of reducing sugar released is measured by DNS method [25] at 540 nm using *N*-acetyl-D glucosamine standard. One unit of chitinase activity is defined as the amount of enzyme producing 1 µmol of GlcNAc per hour at specified assay conditions [3].

3.2 Microorganism and fermentation studies

Citrobacter freundii haritD11 (GenBank Accession number KC344791) was isolated from a marine sediment sample taken from beach area of the Bay of Bengal sea coast, India (17°31′51″ north latitude and 83°4′53″ east longitude), and is used as fermentation inoculum throughout. The organism's biochemical and molecular taxonomy were reported in our previous report [22]. Its chitinolytic activity is visualized by streaking over minimal salt (MS)-chitin agar plate containing 0.5% w/v colloidal chitin with MS medium [12], incubated and observed for 72 h at 30 °C. The strain showed a striking zone of clearance of nearly 0.62 cm after 24 h. It is maintained on yeast extract-malt extract agar slants and subcultured every 30 days [18]. Initial fermentation conditions for chitinase production are 24 h incubation at 30 °C, 0.5%v/w inoculum (10^9 CFU/ml), 30% v/w moisture and 8 pH using 5 g of wheat bran. Leaving other parameters constant, the only factor being studied is altered, likewise production conditions are optimized one by one; 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 are compared. Carbon source supplements such as fish scales, shrimp waste, crab shell powder, glycerol, sucrose, soluble starch; and nitrogen sources such as yeast extract, peptone, tryptone, NH_4Cl, $(NH_4)_2SO_4$ and corn steep liquor are tested for increase in chitinase production. The fermentation conditions are further optimized by RSM—Box–Behnken method and at fully optimized conditions fermentation of a 10 g batch of wheat bran is carried out through SSF. Software used was STATISTICA Version 10.

3.3 Purification studies

Purification procedures are done at 4 °C. Culture broth is centrifuged at 6000*g*, lyophilized, mixed in 0.2 M acetate buffer, stirred for an hour, centrifuged at 5000*g* for 15 min, and supernatant is dialyzed against the same buffer. Dialyzed enzyme is subjected to ammonium sulfate precipitation technique at different ranges of concentration from 50 to 80% (w/v). This process was conducted at 4 °C to maintain the biological activity of chitinase. The precipitate is centrifuged, dissolved in acetate buffer and dialyzed against the same buffer. The dialysis bag used was Himedia dialysis membrane-50 with average flat width of 24.26 mm, 7.14 mm radius and an approximate capacity of 1.6 ml/cm. The buffer used for dialysis was changed two to three times for the complete removal of salts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis technique is used to check the molecular weight of the partially purified enzyme. Fractions with enzyme are pooled to measure enzyme activity and protein content. Chitinase activity and protein content were analyzed in supernatant and the pellet [16, 18].

3.4 Characterization studies of partially purified chitinase

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

3.4.1 Effect of pH on enzyme activity and stability

Effect of pH on chitinase activity is tested by incubating the reaction mixture of 1.12 µg of chitinase and 0.5% chitin at different pH levels ranging 6.5–10 under standard assay conditions. Effect of pH on chitinase stability is determined by pre-incubating 28 µg of chitinase in 100 µl of various buffers without substrate at 30 °C, for 24 h. 100 mM each of sodium acetate (pH 4–7) and Tris–HCl (pH 7–9) are used as buffers. After pre-incubation, the reaction mixtures are tenfold diluted in a 200 µl volume and 40 µl of the diluted samples are checked for residual activity under standard assay conditions.

3.4.2 Effect of temperature on enzymatic activity and stability

Effect of temperature on chitinase activity is tested by incubating 0.4 µg of chitinase with 0.5% chitin at different temperatures up to 80 °C, at pH 8 for 5 min. Thermal stability of chitinase without substrate is determined by incubating 15 µg chitinase in 50 mM sodium acetate buffer at a range of temperatures for half hour at pH 8.5. The residual chitinase activity is measured at standard assay conditions. To measure the thermal stability of chitinase in the presence of substrates, 18 µg of chitinase in 50 µl volume is incubated with 0.5% chitin in 50 mM sodium acetate buffer pH 6.5, at 50 °C for 30 min. After incubation, the reaction mixtures are diluted 30-fold in 300 µl and 40 µl of the diluted samples are taken to determine the residual activity under standard assay conditions.

3.4.3 Effect of metal ions and compounds on chitinase activity

The influence of standard metal ions on partially purified chitinase is studied by adding 20 mM 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 is also tested by adding 10 mM of the chemical to the assay mix and assaying the activity.

3.4.4 Substrate specificity and enzyme kinetics

The substrate specificity of the enzyme is tested by reacting it with substrates such as cellulose, carboxymethyl cellulose 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 $1/V$ against $1/S$. Kinetic parameters (Km and V_{max}) were estimated by linear regression from Lineweaver–Burke plot [24].

3.4.5 Anticoagulant activity

Five test tubes with the fabricated 5 mg/ml fibrin solution are added with partially purified chitinase (2.8 mg/ml) in the amounts of 0.4 ml, 0.6 ml, 0.8 ml and 1 ml. After addition, the test tubes were incubated at 40 °C and assayed for fibrinolysis. Further, the efficacy of the enzyme as an anticoagulant was tested using an artificial blood clot. 3 ml + 3 ml of blood was taken in two 10-ml injection bottles and was left for 5 min to clot. The first test tube was added with saline and the latter with partially purified chitinase, 1 ml each and observed every 10 min. Moreover, the *C. freundii* haritD11 partially purified chitinase was found to possess anticoagulant activity comparable with the commercially available nattokinase as revealed by the fibrin plate assay [13].

4 Results and discussion

4.1 Optimization studies

4.1.1 Solid state fermentation

Laboratory scale fermentation, with initial conditions of 24-h incubation, 30 °C, 5% v/w inoculum, 30% v/w moisture and 8 pH using 5 g wheat bran, was conducted adopting OVAT approach [23], and at the corrected optimization conditions (Table 1) there was 2.52-fold increased chitinase activity compared to the unoptimized medium (34.11 U/gds). Among all the tested physico-chemical factors, temperature, moisture content and fish chitin content were found to notably influence the chitinase production. The temperature that promoted maximum chitinase yield (41.87 U/gds) was 35 °C. Temperatures below 30 °C and above 60 °C resulted

Table 1 Compendium of physico-chemical parametric optimization, effecting chitinase production from *Citrobacter freundii* haritD11 during one-variable-at-a-time method

Parameter/variable	Chitinase activity (U/gds)
Incubation time, 24 h	34.11
Temperature, 35 °C	41.87
Inoculum content, 2%v/w	42.33
Moisture content, 60%v/w	56.49
pH, 8.0	58.36
Fish scale chitin content, 30%w/w	83.57
Peptone content, 2%w/w	84.14

in enzyme inhibition [17]. Similar temperatures for chitinase production were reported by other researchers as 30 °C for *Aeromonas* [1] and 35 °C for *Citrobacter* [23]. Maximum chitinase production was shown at 60%v/w (56.49 U/gds) moisture content. Too much moisture decreased porosity, and too low decreased solubility of substrate nutrients resulting lowered enzyme activity. Similarly, chitinase production at 65%v/w moisture content using wheat bran has been reported with *Trichoderma sps.* [27]. Fish scale chitin content of 30%w/w increased chitinase activity (83.57 U/gds) being a naturally rich source of chitin. All experimentations for evaluation of physical parameters were conducted in triplicate, and average values are represented; subsequently, experiments were conducted for optimization of these selected nutrients employing response surface methodology (RSM).

4.1.2 Response surface methodology

Box–Behnken method adopting three variables with 15 runs was used for statistical optimization. To perform the 3-factor and 15-runs analysis, the lowest and highest concentrations of the selected variables were: temperature 30 °C and 40 °C, moisture content 50% v/w and 70% v/w, fish chitin content 20% w/w and 40% w/w, respectively. The application of RSM yielded the following regression equation explaining empirical relationship between chitinase yield and test variables in coded units.

$$Y = 81.56 + 2.03X_1 + 5.66X_1X_1 - 0.89X_2 - 5.09X_2X_2 + 12.38X_3 + 3.82X_3X_3 - 5.99X_1X_2 - 4.33X_1X_3 - 4.33X_2X_3;$$

where Y is enzyme yield; X_1, X_2 and X_3 are coded values of temperature, moisture content and fish chitin content, respectively (Table 2). Estimation of regression analysis and ANOVA explains determination coefficient ($R^2 = 0.8$) indicating that only very few of the total variations are not explained by the model (Table 3). The parity plot displaying clustered points around the diagonal line (Fig. 1) indicates a correlated fit between experimental and predicted values. The smaller P values and good interaction between independent variables confirm significance of each coefficient [26]. The yield values for different concentrations of the variable can also be predicted from the respective response surface plots (Fig. 2), and the maximum predicted values are indicated by the confined surface of the response surface diagram.

The critical levels of the three independent variables examined as predicted from the model are: temperature 34 °C, moisture content 61% v/w and fish chitin content 37.8% w/w, and at these conditions predicted chitinase

Table 3 ANOVA; Var.:NewVar4 and 3-level factors $R^2 = 0.8$

	SS	*df*	MS	*F*	*p*
(1)Var1 L + Q	507.009	2	253.5043	1.362331	0.337075
(2)Var2 L + Q	389.364	2	194.6821	1.046221	0.417288
(3)Var3 L + Q	1442.847	2	721.4235	3.876926	0.096232
1 * 2	125.328	1	125.3280	0.673512	0.449172
1 * 3	75.169	1	75.1689	0.403957	0.552999
2 * 3	74.736	1	74.7360	0.401631	0.554102
Error	930.407	5	186.0813		
Total SS	3613.642	14			

Table 2 The Box–Behnken design matrix employed for three independent variables with 15 runs along with observed and predicted chitinase activity values

Run no.	Temperature	Moisture content	Fish chitin content	Observed chitinase activity (U/gds)	Predicted chitinase activity (U/gds)
1	30.00000	50.00000	30.00000	75.6700	79.5438
2	40.00000	50.00000	30.00000	98.1200	94.8112
3	30.00000	70.00000	30.00000	85.6400	88.9487
4	40.00000	70.00000	30.00000	85.7000	81.8263
5	30.00000	60.00000	20.00000	63.4400	49.6950
6	40.00000	60.00000	20.00000	69.0000	62.4375
7	30.00000	60.00000	40.00000	76.5700	83.1325
8	40.00000	60.00000	40.00000	64.7900	78.5350
9	35.00000	50.00000	20.00000	64.2800	74.1513
10	35.00000	70.00000	20.00000	70.5700	81.0063
11	35.00000	50.00000	40.00000	118.0000	107.5638
12	35.00000	70.00000	40.00000	107.0000	97.1288
13	35.00000	60.00000	30.00000	86.4100	87.4267
14	35.00000	60.00000	30.00000	88.2900	87.4267
15	35.00000	60.00000	30.00000	87.5800	87.4267

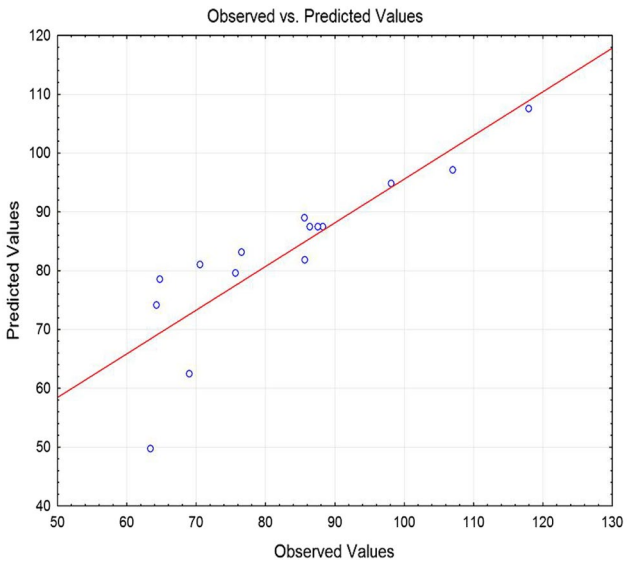

Fig. 1 The parity plot between the experimental and predicted values of chitinase activity presenting the accuracy of the model

activity was 92.117 U/gds. A verification experiment at the critical conditions confirmed that the experimental value (94.3 U/gds) was similar to the value predicted substantiating both validity and effectiveness of the model. Statistically optimized culture conditions using Box–Behnken method showed augmented chitinase production of 1.12-fold that of basic optimization (one-variable-at-a-time-approach) culture conditions (84.14 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 [23].

4.2 Purification and characterization studies

The crude chitinase extract was partially purified by ammonium sulfate precipitation and dialysis. Chitinase in the culture filtrate was extracted by 70% ammonium sulfate precipitation followed by dialysis and DEAE Sephadex A-50. The partially purified chitinase is shown as a single band in the SDS-PAGE Zymogram with molecular weight of 62.5 kDa (Fig. 3). The molecular weights of chitinases from marine bacteria are mainly around 60 kDa. Similarly, 64 kDa molecular weight of *Xanthomonas sp. strain AK* chitinase was reported by Yamaoka et al. [24, 32]. Chitinase was purified 3.9-fold with 37% yield and specific activity of 53.41 U/mg protein from 20 g of wheat bran supplemented with fish chitin (Table 4). Chitinase of *Stenotrophomonas maltophilia* isolated from rhizospheric soil was purified 2.4-fold through Sephadex gel filtration and has 50-55 kDa molecular weight [28].

4.2.1 Effect of pH and temperature on enzyme activity and stability

Partially purified chitinase showed optimum activity at 8.5 pH. A sharp decrease in enzyme activity was shown at pH values higher than 10 or lower than 7.5 showing increased activity at alkaline conditions. The enzyme was stable at 7.5–10 pH range for 1 h at 4 °C in various buffers (100 mM each of sodium acetate (pH 4–7) and Tris–HCl (pH 7–9); Correspondingly, chitinase from *Alternaria infectoria* was stable at alkaline pH [8] but some bacterial chitinases were found stable at acidic pH [9, 10].

The optimum temperature for chitinase was recorded at 50 °C; likewise 50 °C has been reported for *Aspergillus terreus* [9], *Paenibacillus pasadenensis* CS0611 and *Escherichia coli* [31]. Chitinase maintained 80% stability between 45 °C and 60 °C in 50 mM sodium acetate buffer, lower or higher temperatures lead to activity inhibition. At these conditions, chitinase was tested for its salt tolerance at various concentrations of sodium chloride and showed 85% activity at 8% and was completely stable at 8% 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.

4.2.2 Influence of metal ions and EDTA, SDS, Urea on enzyme activity

The influence of various metal ions on the chitinase activity is shown in Table 5. There was a relative increase in activity with Na^+ (66%), K^+ (31%), Mg^{2+} (40%), Mn^{2+} (65%), Fe^{2+} (32%), Cu^{2+}(13%), Urea (28%), NaCl (80%), while relatively decreased chitinase activity was detected with EDTA (13%), SDS (39%), Hg^{2+} (11%), and ethyl-acetimidate (20%). A similar kind of effect of metal ions and other compounds has also been recorded for chitinase from *Bacillus thuringiensis* subsp. *kurstaki* HBK-51 where Ni^{2+} (32%), K^+ (44%) and Cu^{2+} (56%) increased the enzyme activity, while EDTA (7%), SDS (7%), Hg^{2+} (11%) and ethyl-acetimidate (20%) decreased the activity of the enzyme [15]. *Bacillus* sp. R2 purified chitinase when subjected to the effect of metal ions, chemical agents on its activity showed that K^+, Mn^{2+}, Na^+, Mg^{2+} and Ca^{2+} stimulated chitinase activity by 2,4,7,15 and 21%, respectively, whereas Cu^{2+}, Fe^{2+}, Zn^{2+}, Ag^+ and Hg^{2+} inhibited the enzyme [7].

4.2.3 Substrate specificity and enzyme kinetics

The partially purified chitinase showed highest substrate specificity toward swollen chitin among chitin, swollen chitin, cellulose and carboxy methyl cellulose; hence, the kinetics of enzyme were studied with swollen chitin. Michaelis–Menten constants were determined

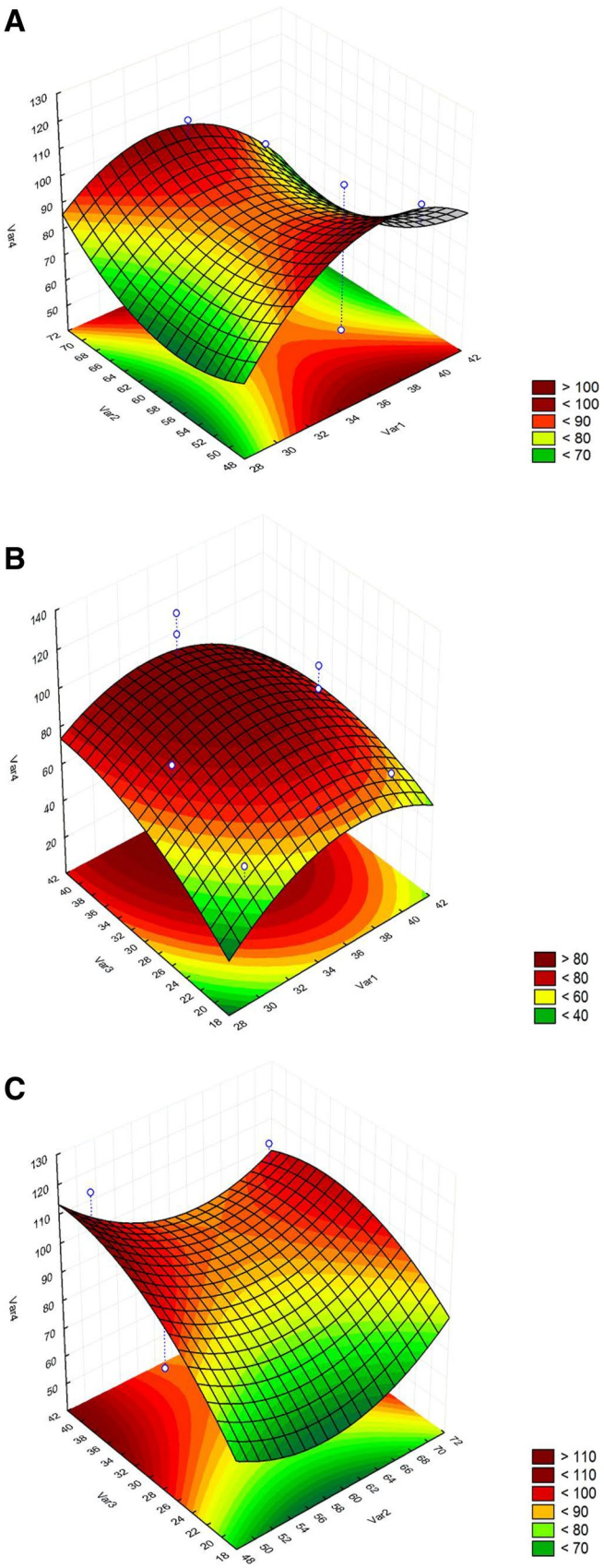

Fig. 2 Contour plots between temperature (Var 1), moisture content (Var 2), fish chitin content (Var 3) and the corresponding chitinase activity. The different colored bars shown in the right side scale represent the various levels of chitinase activity accordingly, and as the color gets darker, the response increases. The response is at its highest at the darkest region of the graph

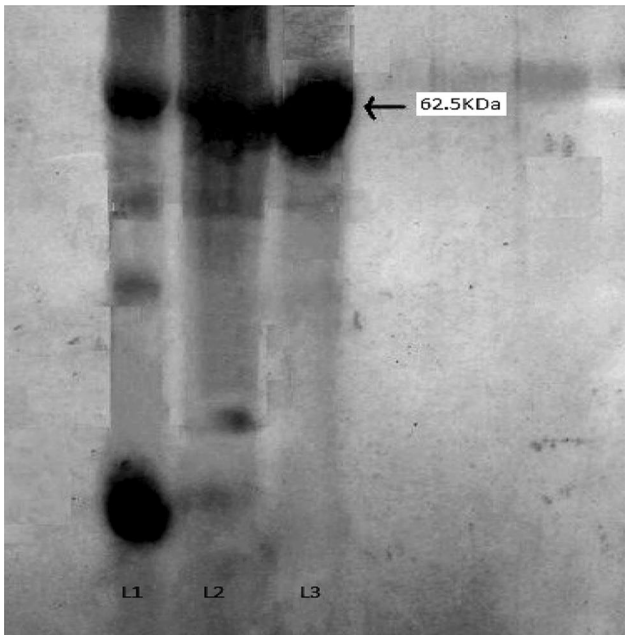

Fig. 3 SDS PAGE result (L1 marker lane; L2, L3 enzyme sample lanes). L1 is the lane with the molecular markers ladder, L2 shows the lane of bands with dialyzed chitinase, and L3 is the lane showing single band of partially purified chitinase subjected to Sephadex DEAE A-50 gel filtration

using Lineweaver–Burke plot designed to calculate reaction velocities at each substrate concentration. From the Michaelis–Menten kinetics and the Lineweaver–Burke plot (Fig. 4), the Km value of the *C. freundii* haritD11 partially purified chitinase for swollen chitin is 8.11 mg/ml with a V_{max} of 2.43 mmol h^{-1} ml^{-1}. Similarly, the value of Km for *Serratia marcescens* B4A partially purified chitinase was 8.3 mg/ml with swollen chitin as a substrate [34]. The higher the velocity of the enzyme, the faster the rate of catalysis; hence, lower the Km value, the enzyme is a favorable biocatalyst. However, the *Bacillus* sp. BG11 exhibited Km value as high as 12 mg/ml with swollen chitin have been reported [4].

4.2.4 Anticoagulant activity

Among various test tubes with the fabricated 5 mg/ml fibrin solution incubated with partially purified chitinase,

Table 5 Effect of metal ion or other chemical compounds on the activity of chitinase

Metal ion/compound	Relative activity
None	100
Na+	166
K+	131
Mg^{2+}	140
Mn^{2+}	165
Fe^{2+}	132
Hg^{2+}	73
Co^{2+}	31
Cu^{2+}	113
As^{4+}	26
Pb^{2+}	64
Zn^{2+}	90
Urea	128
SDS	61
EDTA	87
NaCl	180

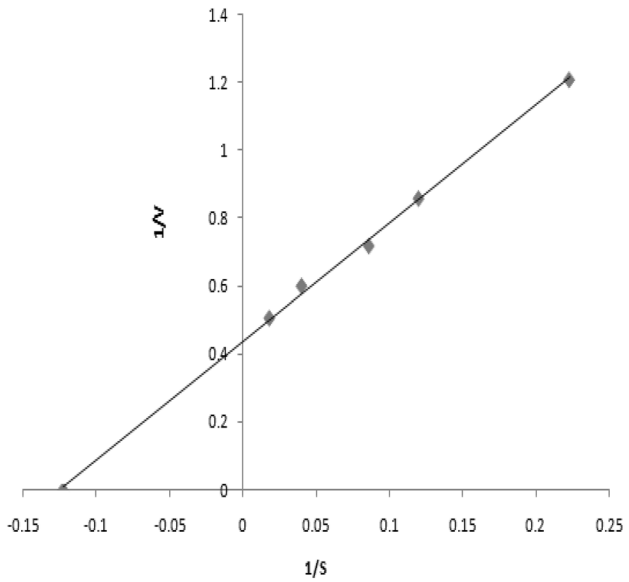

Fig. 4 Lineweaver–Burke plot

Table 4 Steps in purification of chitinase

Purification step	Total activity (U)	Specific activity (U/mg)	Folds of purification	% Yield
Culture supernatant	3813	13.7	1	100
$(NH_4)_2SO_4$ ppt	2917	24.66	1.8	78
Dialysis	2249	43.84	3.2	59
Sephadex DEAE A-50	1410	53.41	3.9	37

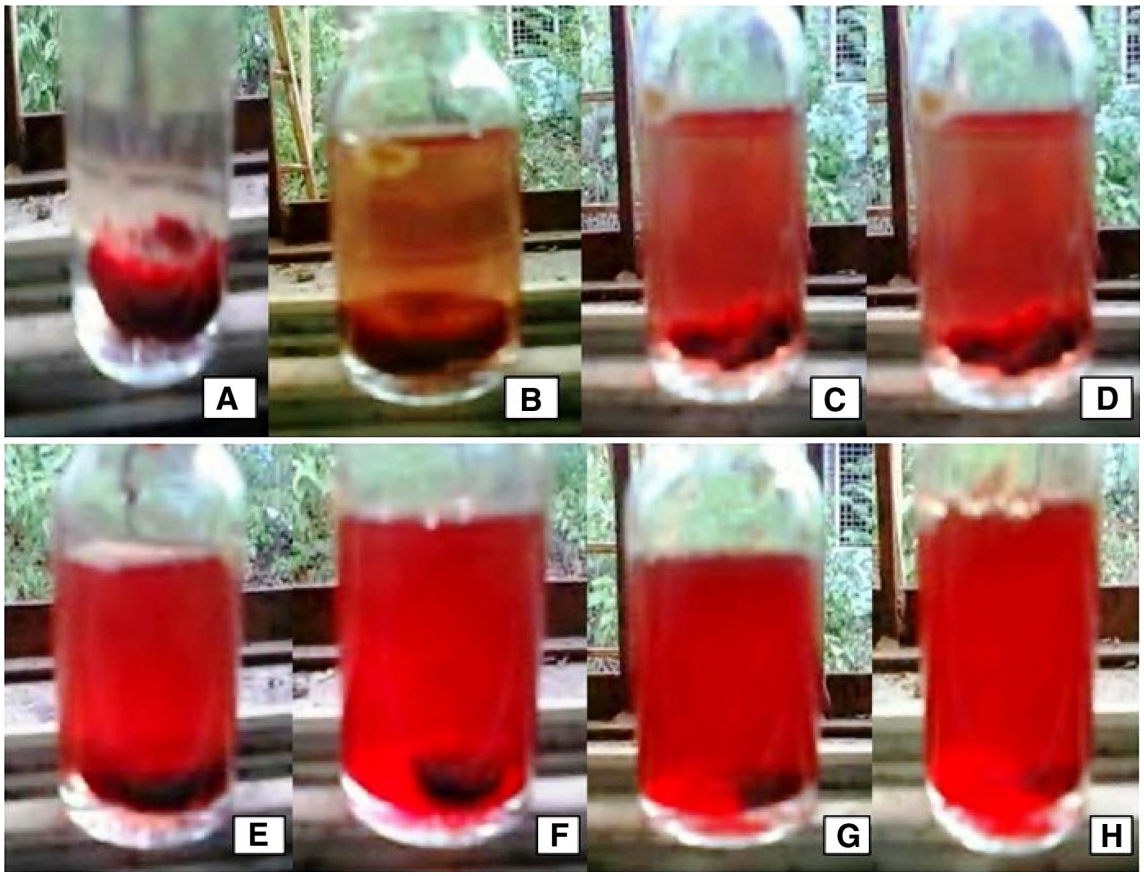

Fig. 5 Evaluation of the fibrinolysis using artificial blood clot (**a** clot with saline; **b** clot with chitinase; **c** clot with chitinase after 10 min; **d** clot with chitinase after 20 min; **e** clot with chitinase after 30 min; **f** clot with chitinase after 40 min; **g** clot with chitinase after 50 min; **h** clot with partially purified chitinase after 60 min)

maximum fibrinolysis was shown by the 1 ml amount. Further, the efficacy of the purified enzyme as an anticoagulant was tested with an artificial blood clot and apparently complete lysis was observed after 60 min (Fig. 5). Moreover, the *C. freundii* haritD11 partially purified chitinase was found to possess fibrinolytic activity comparable with the commercially available nattokinase where the radial hydrolysis zone around the chitinase well was 1.62 mm and 1.7 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 hemostatic materials [35]. However, this is the first report stating that chitinase of marine *Citrobacter freundii* haritD11 can be used as a potent anticoagulation agent due its ability to lyse a blood clot effectively within an hour. Hence, chitinases can also be used as commercial blot clot dissolving agents or hemolytic agents that find applications in myriad medical sectors.

5 Conclusion

There are numerous reports on the production of chitinase from microorganisms such as fungi, actinomycetes, bacteria and plants; however, there is no single evident report on chitinase production from *Citrobacter freundii* fermenting wheat bran supplemented with fish scales (at a monitored ratio). Our research has established the superior production of chitinase through supplementation of fish chitin with wheat bran improving the quality of an assorted substrate for solid state fermentation, contributing to cost-cutting strategies and production of chitinase with anticoagulant activity. From an industrial perspective, chitinase of *Citrobacter freundii* str. nov. haritD11 has many beneficial characteristics such as high productivity, high specific activity, easy purification, relatively high halotolerance and thermostability, and as an anticoagulant in its partially purified form. In a nutshell, this is the first report to date elucidating the production of chitinase using an assorted ratio of wheat bran and fish scale chitin with *Citrobacter freundii* haritD11, its partial purification,

characterization and application as a potential anticoagulant agent.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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