New Materials, Compounds and Applications
Vol.2, No.3, 2018, pp.261-273

ANTIMICROBIAL STUDIES OF HALOTOLERANT CHITINASE PRODUCED BY *CITROBACTER FREUNDII STR. NOV. HARITD11*, FERMENTING *LATES CALCARIFER* SCALES AND ITS GROWTH PROFILE

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Abstract. There is a quintessential need to produce industrially useful enzymes like chitinases, with organic solvent tolerance in high titres for commercial applications. This report is an illustration of antimicrobial properties of Chitinase produced from *Lates calcarifer* scales when fermented with a novel chitinolytic strain '*Citrobacter freundii* haritD11, its growth profile and Monad kinetics'.

Keywords: *Chitinase, Lates calcarifer, optimization, purification, antimicrobial.*

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Received: *28 October 2018;* ***Accepted:*** *12 November 2018;* ***Published:*** *17 December 2018.*

1. Introduction

The augmented generation of organic solid waste exemplifies persistent problems related to its disposal in recent years due to the rapid progress towards modernization of the society. Commonly used inexpensive feedstock for fermentation are these organic wastes of household, agricultural and industrial origins like gram husk, wheat/rice bran, vegetable/fruit peelings and chitinous wastes etc (Sadh *et al*., 2018; Yazid *et al*., 2017; Macias *et al*., 2017; Meruvu & Donthireddy, 2014a). In particular chitinous wastes of lesser intrinsic value can be used per se in the production of industrially important chemicals, biopharmaceuticals, enzymes etc. or as feedstock substrates aiding their fermentative production. Chitin is a tough yet flexible biopolymer that profusely belongs to the marine environments and occurs in the exoskeletons of arthropods like spiders and insects, the cell walls and cysts of numerous fungi, diatoms and algae (Chiriboga *et al*., 2017; Li *et al*., 2016).

All-pervading chitinous waste sources upon our planet include fish scales, shrimp and crab shells from sea food processing industries; usual solid waste disposal methods include land filling, aerobic/anaerobic composting and/or dumping having negative environmental effects (Yazid *et al*., 2017; Kazemi *et al*., 2017). Chitinolytic bacteria participate in recycling marine wastes maintaining energy flow by degradation and/or utilization of chitin thereby resolving environmental problems through cost-effective and ecofriendly means. The biochemical diversity of marine chitinolytic microbes makes them reasonable producers of enzymes with high halotolerance and thermo-alkali stable tolerance (Bahrami *et al*., 2018; Bissaro *et al*., 2018; Beygmoradi & Homaei, 2017; Meier *et al*., 2018).

In this report solid phase fermentation (SSF) (Castañeda-Casasola *et al*., 2018) is carried out using fish scales as substrate since they were chitin-rich, budget friendly and easily available in our neighbourhood precincts. Vetting and analysis of biochemical requirements of microbial growth and enzyme production is a prerequisite for bioprocess development during fermentation, hence in this study, conventional optimization studies by one-variable-at-a-time (OVAT) approach followed by statistical optimization using Box Behnken method were done for monitoring chitinase controlling parameters. The subject microorganism chitinolytic *Citrobacter freundii* str. nov. *haritD11*, isolated from marine soil sediment is used as inoculum to ferment *Lates calcarifer* scales and its growth curve is studied in synch chitinase production conditions to study Monod growth kinetics. The enzyme is also purified, its tolerance to variation in temperature, pH, salt concentration, and antimicrobial activity is verified.

Our previous works which pivoted around *Citrobacter freundii str. nov. haritD11* included, Isolation and identification of *C. freundii haritD11*; Fermentation of *C. freundii haritD11* using shrimp waste and the consequential Purification and Characterization subterfuges (Meruvu and Donthireddy, 2014a; Meruvu and Donthireddy, 2014b; Meruvu and Donthireddy, 2013; Meruvu and Donthireddy, 2012). However, this is the first report to date elucidating the production of chitinase through solid state fermentation of *Lates calcarifer* scales with *C. freundii haritD11*, its purification, characterization and antimicrobial activity, and furthermore the Monod growth kinetics of *C. freundii haritD11* at optimized culture conditions were studied constituently. 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 biocontrol agents. Moreover, till now there is no known report on chitinase production from *Citrobacter freundii* through SSF of *Lates calcarifer* scales.

2. Material and method

2.1 Materials

Indigenous fish scales of *Lates calcarifer* (Barramundi) gathered from local vendors across the coastline of Bay of Bengal beach, Visakhapatnam are washed, oven dried, soaked in 1%HCl solution for 2-3 days and treated with 50%w/v NaOH for the deacetylation process (Vasquez *et al*., 2017; Suneeta & Pradip, 2014) resulting in raw chitin which is the substrate for SSF. Other chemicals used are commercial crab shell chitin (assay purpose), sodium carbonate, sodium chloride, potassium ferricyanide, bovine serum albumen and buffer solutions. Software used was STATISTICA Version 10.

2.2 Chitinase Activity Assay

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

2.3 Fermentation studies

2.3.1 Microrganism

Citrobacter freundii haritD11 (GenBank Accession number KC344791) was isolated from a marine sediment sample procured from beach area of the Bay of Bengal sea coast (Visakhapatnam, India) and is used as fermentation inoculum throughout this experimentation. The organism's biochemical and molecular taxonomy were detailed in our previous report (Meruvu & Donthireddy, 2013). Its chitinolytic activity is visualized by streaking over minimal salt (MS)-chitin agar plate containing 0.5% (w/vol) colloidal chitin with MS medium (Heravi *et al*., 2015) and incubated for 72 hours at 30°C. The strain showed a salient zone of clearance of nearly 0.62 cm after 24 hours (Meruvu, 2017).

2.3.2 One-Variable-at-a-Time Method (OVAT)

Initial fermentation conditions for chitinase production are 24 hours incubation at 30°C, 0.5%v/w inoculum (10^9 CFU/ml), 30%v/w moisture and 8 pH using 5g of fish scales. Only factor being studied is varied leaving other parameters constant, in this way production conditions are optimized one by one; temperature 25-50°C; pH 6.0-9.0; inoculums content 0.5-2.5%w/v; moisture content 30-80% and levels of chitinase production are compared. Carbon sources like glycerol, glucose, glucosamine, sucrose, soluble starch, and chitin; and nitrogen sources like yeast extract, peptone, tryptone, NH_4Cl, $(NH_4)_2SO_4$, and corn steep liquor are tested for chitinase production. All experimentation is done in triplicate.

2.3.3 Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is primarily used for developing mathematical and statistical models to elaborate the relationships among the several independent variables and one or more dependent variables. In this study, Box Behnken method was utilized comprising 15 experimental runs was used to develop a second degree statistical model for the optimisation of the fermentation conditions. Each factor in the design can be studied at three different levels (-1, 0, +1) yielding a set of 15 experiments. In this study the three fermentation factors which significantly affected chitinase production are extra optimized by RSM using Box Behnken '3-factors and 15 runs' method. Software used was STATISTICA Version 10 for RSM and data analysis.

2.3.4 Organism Growth Kinetics

Growth kinetic models attribute the control over the specific growth rate of microbial cells, cell doubling time and yield coefficient with respect to the substrate concentration. Monod growth kinetics of *Citrobacter freundii haritD11* are calculated by fermenting a 20 gram batch of processed fish scale powder at the OVAT optimized fermentation conditions and at RSM optimized conditions to study the growth patterns comparatively. The various growth parameters like specific growth rate (μ_{max}), yield co-efficient ($Y_{x/s}$) and cell doubling time (t_d) are calculated.

2.4 Purification studies

Purification procedures are done at 4°C. Culture broth centrifuged at 6000g is lyophilized, crude lyophilizate mixed in 0.2M acetate buffer and stirred for an hour, centrifuged at 5000g for 15 minutes and supernatant is dialyzed against the same buffer. Dialyzed enzyme is subjected to 80% $(NH_4)_2SO_4$ fractionation, precipitate centrifuged,

dissolved in acetate buffer and dialyzed against the same buffer. Fractions with enzyme are pooled to measure enzyme activity and protein content (Meruvu, 2017; Lowry *et al*., 1951).

2.5 Characterization studies

The purified chitinase is tested for its activity and stability at myriad ranges of temperature and pH.

2.5.1 Effect of pH on enzyme activity and stability

Effect of pH on chitinase activity is tested by incubating the reaction mixture of 1.12µg 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 28µg of chitinase in 100µl of various buffers without substrate at 30ºC, for 2 hours. 100mM each of sodium acetate (pH 4–7) and Tris–HCl (pH 7–9) are used as buffers. Post pre-incubation, the reaction mixtures are 10 fold diluted in a 200µl volume and 40µl of the diluted samples are checked for residual activity under standard assay conditions.

2.5.2 Effect of temperature on enzymatic activity and stability

Effect of temperature on chitinase activity is tested by incubating 0.4µg 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 is determined by incubating 15µg chitinase in 50mM sodium acetate buffer at a range of temperatures for 2 hours 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, 18µg of chitinase in 50µl volume is incubated with 0.5% chitin in 50mM sodium acetate buffer pH 6.5, at 50ºC for 2 hours. After incubation, the reaction mixtures are diluted 30 fold in 300µl and 40µl of the diluted samples are taken to determine the residual activity under standard assay conditions.

2.6 Antimicrobial studies

Aspergillus awamori, Aspergillus flavus, Candida albicans, Rhizopus oryzae, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Shigella sonnei, Pseudomonas aeruginosa, Escherichia coli and *Streptococcus agalactiae* are procured from 'Microbial Type Culture Collection and Gene Bank, India' and are tested for their susceptibility to purified and crude chitinase by the disc diffusion method (Kumaran *et al*., 2012). Petriplates with sterilized Potato Dextrose agar (antifungal activity) and Mueller Hinton Agar (antibacterial activity) are spread with 100ml inoculum containing approximately 10^4-10^5 conidia.ml^{-1}/CFU.ml^{-1} of the test fungi/bacteria. The discs (5mm radius) impregnated with 10ml of 5mg/ml purified/crude chitinase enzyme are positioned upon the inoculated plates, incubated for 4-5 days at 30°C, observed for microbial growth inhibition and documented.

3. Results and discussion

3.1 Optimization studies

3.1.1 Solid state Fermentation

Laboratory scale fermentation, with initial conditions of 24 hour incubation, 30°C, 5% v/w inoculum, 30%v/w moisture and 8 pH using 5g processed fish scales, was conducted adopting OVAT approach and at the corrected optimization conditions (Table 1) there was 1.73 fold increased chitinase activity compared to the unoptimized medium (34.2U/gds).

Table 1. Compendium of physico-chemical parametric optimization, effecting Chitinase production from *Citrobacter freundii* haritD11 during one-variable-at-a-time method. When one variable is optimized the other are kept constant and the optimized parametric value is sequentially adopted while optimizing the next parameter

Parameter optimized	Parameters kept constant	Chitinase activity (U/gds)
Incubation time 20 hours	30°C, 0.5% v/w inoculum, 30% v/w moisture, 8 pH	34.01±1.50
Temperature 40˚C	20 hours, 0.5% v/w inoculum, 30% v/w moisture, 8 pH	39.43±0.87
Inoculum content 1.5 % v/w	20 hours, 40˚C, 30% v/w moisture, 8 pH	42.80±1.90
Moisture content 50 % v/w	20 hours, 40˚C, 1.5% v/w inoculum, 8 pH	43.49±1.77
pH- 8.5	20 hours, 40˚C, 1.5% v/w inoculum, 50% v/w moisture	49.33±2.35
Soluble Starch content 2 % w/w	20 hours, 40˚C, 1.5% v/w inoculum, 50 % v/w moisture, 8.5 pH	50.57±2.11
Peptone content 3% w/w	20 hours, 40˚C, 1.5% v/w inoculum, 50% v/w moisture, 8.5 pH, 2 % w/w starch	59.19±3.80

Temperature, pH and Peptone concentration were found to notably influence the chitinase production; hence were further investigated statistically by Response surface methodology (RSM).

3.1.2 Response surface methodology

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.

$$Y = 38.18 + 10.75X_1 + 14.01X_1X_1 + 7.94X_2 + 8.77X_2X_2 + 8.21X_3 + 8.47X_3X_3 - 1.38X_1X_2 - 1.98X_1X_3 + 1.59X_2X_3;$$

where Y is enzyme yield; X_1, X_2 and X_3 are coded values of the temperature, pH and peptone concentration, respectively (Table 2).

Estimation of regression analysis explains determination coefficient (R^2=0.988) indicating that only 1.2% of the total variations are not explained by the model. The adjusted determination coefficient (Adj. R^2=0.962) is very high indicating a high model significance.

Table 2. The Box-Behnken design matrix employed for three independent variables with 15 runs along with observed and predicted chitinase activity values. X_1, X_2 and X_3 are coded values of the temperature, pH and peptone concentration

Run no.	X_1	X_2	X_3	Observed chitinase activity (U/gds)	Predicted chitinase activity (U/gds)
1	40.00	8.00	3.00	25.60±0.34	26.89
2	50.00	8.00	3.00	38.10±0.72	37.64
3	40.00	9.00	3.00	35.76±0.13	34.83
4	50.00	9.00	3.00	45.50±1.77	45.58
5	40.00	8.50	2.50	26.31±1.09	27.05
6	50.00	8.50	2.50	38.67±1.88	37.80
7	40.00	8.50	3.50	36.38±1.12	35.26
8	50.00	8.50	3.50	44.78±1.94	46.01
9	45.00	8.00	2.50	34.86±2.11	33.70
10	45.00	9.00	2.50	40.37±2.05	41.64
11	45.00	8.00	3.50	41.60±2.60	41.91
12	45.00	9.00	3.50	50.29±2.33	49.85
13	45.00	8.50	3.00	59.21±2.00	59.02
14	45.00	8.50	3.00	58.89±2.45	59.02
15	45.00	8.50	3.00	58.98±2.29	59.02

The parity plot displays clustered points around the diagonal line (Fig. 1) indicate a fitting correlation between experimental and predicted values.

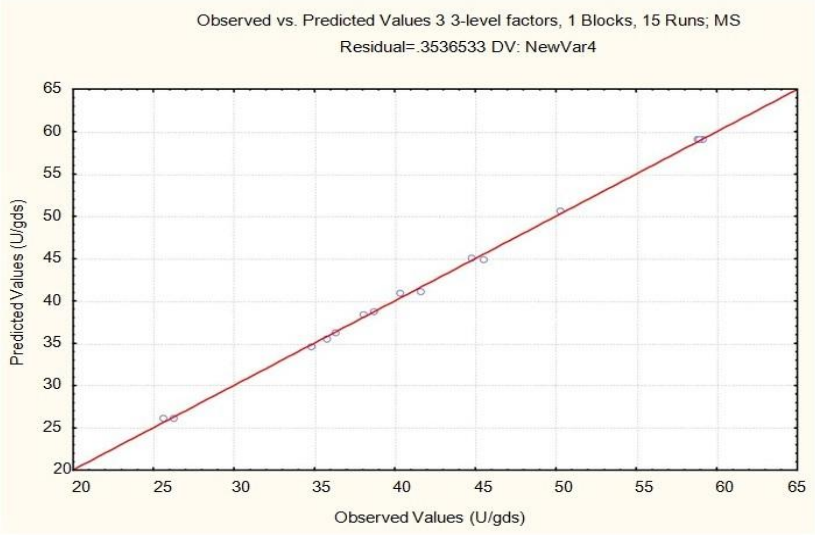
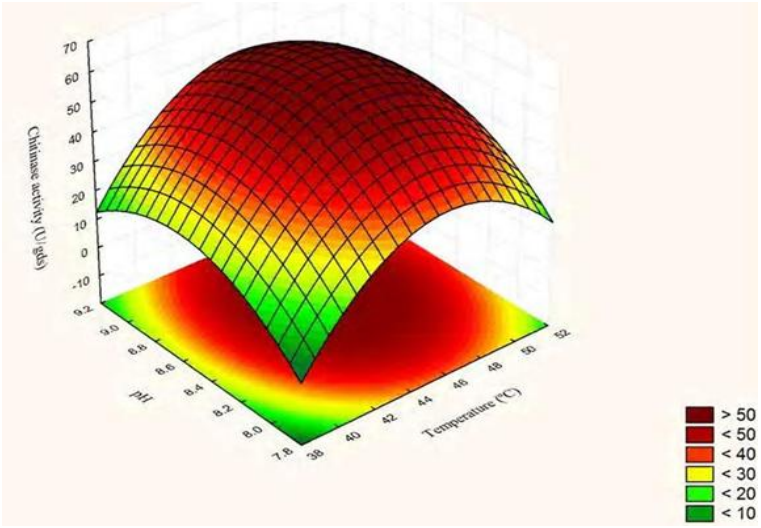


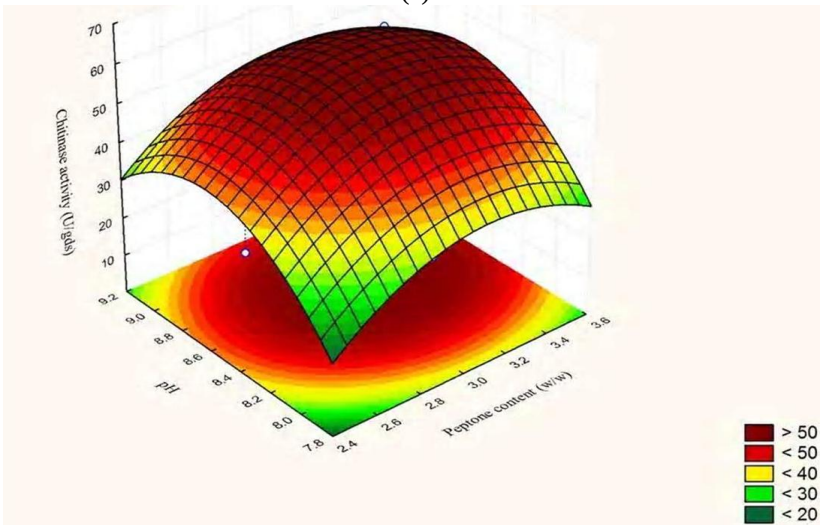
Figure 1. The parity plot between the experimental and predicted values of chitinase activity presenting the accuracy of the model

The smaller P values and strong interaction between independent variables confirm significance of each coefficient (Mourabet *et al*., 2017) (Table 3).

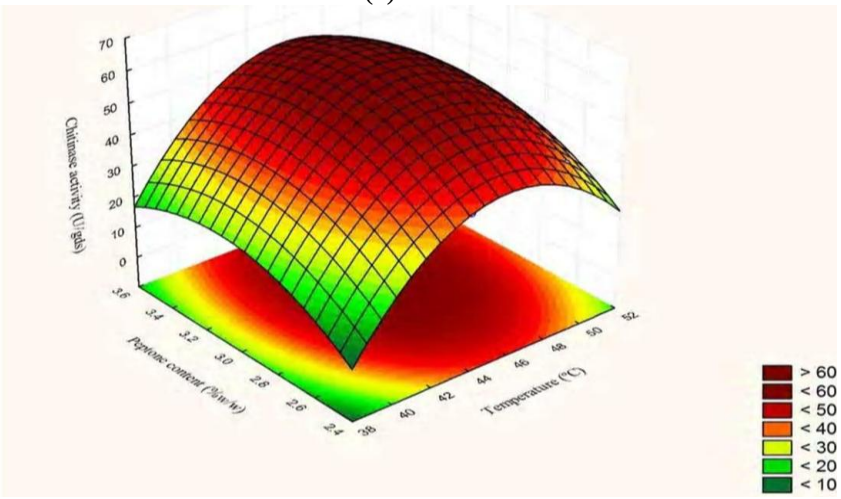
The yield values for different concentrations of the variable can also be predicted from the respective response surface plots (Fig. 2) and the maximum predicted values are indicated by the confined surface of the response surface diagram.



(a)



(b)



(c)

Figure 2 (a, b, c). Contour plots between pH, Temperature, Peptone content 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 (greater than 50) at the darkest region of the graph

Table 3. ANOVA; Var.:NewVar4 and 3-level factors

	SS	df	MS	F	p
(1) NewVar1 L+Q	956.4	2	478.2	378	0
(2) NewVar2 L+Q	410.1	2	205.06	162	0
(3) NewVar3 L+Q	400.0	2	200.03	158	0
Error	10.12	8	1.265		
Total SS	1622	14			

The critical levels of the three independent variables examined as predicted from the model are: temperature 45.95°C, pH 8.6 and Peptone concentration 3.12% w/w and at these conditions predicted chitinase activity was 60.44U/gds. A verification experiment at the critical conditions confirmed that the experimental value (64.6U/gds) was similar to the proposed value (60.448U/gds) predicted substantiating both validity and effectiveness of the model. Amendment of peptone as a nitrogen source for enhanced chitinase produced from *Streptomyces rubiginosus* was also reported by Jha *et al.*, (2016). Statistically optimized culture conditions using Box Behnken method showed augmented chitinase production of 1.10-fold that of basic optimization (one-variable-at-a-time-approach) culture conditions (59U/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 & Donthireddy, 2014a).

3.2 Growth Kinetics

The growth of *Citrobacter fruendii haritD11* was studied at optimized conditions using fish scales as a growth limiting substrate. The resultant growth curve shows an initial lag phase until 5th hour and exponential growth was charted for 6-20 hours. This means chitinase production is correlated with substrate uptake and the growth of the organism (Fig. 3).

The most plausible explanation for why the growth and enzyme produced were substandard after the 18th hour is the fact that the substrate chitin metabolized by the organism is broken down by the chitinase into simpler units for assimilation and resultant growth of the bacterium; hence as the substrate levels are diminished after the 18th hour, both chitinase production and growth are decreased alongside. Monod type microbial growth models adequately describe bio-processes under certain favourable conditions when micro-organisms actively produce specific enzymes for simultaneous degradation and consumption of nutrient substrates whilst growing at the maximum possible rate (Alt & Markov, 2012; Tzintzun-Camacho *et al.*, 2016). Applying Monod Growth kinetics, the specific growth rate is 0.369 h^{-1}**,** yield co-efficient with respect to substrate is 0.1099 mg/g and the cell doubling time is 129 minutes.

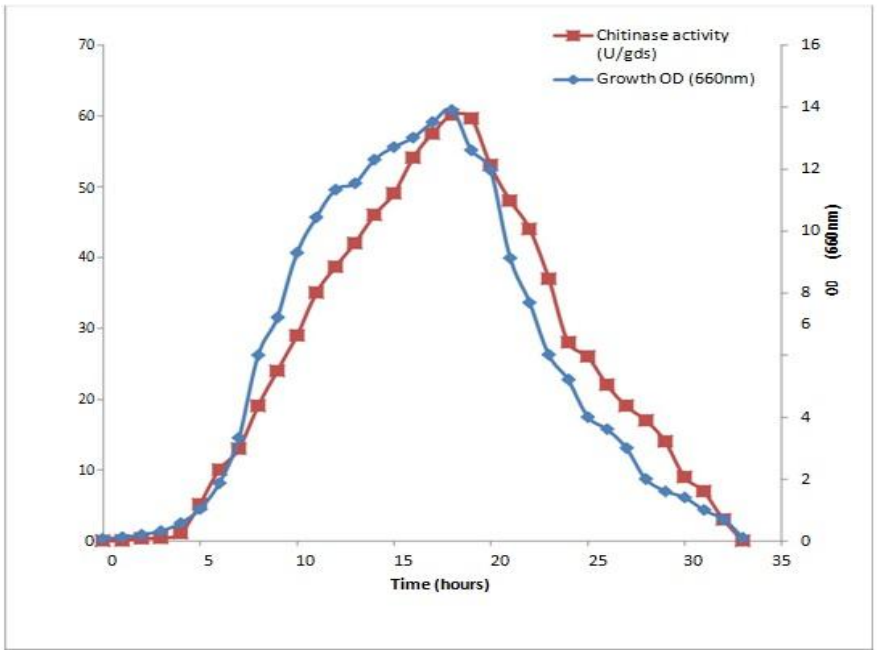


Figure 3. Growth curve of *Citrobacter freundii* haritD11 with respect to fish scales as a growth limiting substrate and concomitant chitinase activity

3.3 Purification and Characterization studies

The crude chitinase extract was purified by ammonium sulfate precipitation, dialysis and Sephadex-100 gel chromatography. Chitinase in the culture filtrate was extracted by 80% ammonium sulfate precipitation followed by dialysis and chromatography. Chitinase was purified 3.66 fold with 26% yield and specific activity of 505.81U/mg protein from 20g of processed fish scale powder (Table 4).

Table 4. Steps in purification of chitinase

Purification step	Total Activity (U)	Specific activity (U/mg)	Folds of purification	% Yeild
Culture supernatent	5880	138.2	1	100
$(NH_4)_2SO_4$ ppt	4988	345.5	2.5	67
Sephadex G-100	3512	505.8	3.66	26

3.3.1 Effect of pH and temperature on enzyme activity and stability

Purified chitinase showed optimum activity at 9.0 pH. A gradual decrease in enzyme activity was shown at pH values higher than 9 or lower than 6 showing amplified activity at alkaline conditions. The enzyme was completely stable at 7-10 pH range for 24 hours 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 (El-Shora *et al*., 2017; Farag *et al*., 2016).

The optimum temperature for chitinase was recorded at 50°C, likewise 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 100% stability between 45°C and 65°C, lower or higher temperatures showed comparatively reduced stability. At these conditions, chitinase was tested for its salt tolerance at various concentrations of Sodium chloride and showed notable activity up to 10% and was completely stable at 8% for 24 hours.

3.4. Antimicrobial activity

The antimicrobial activity of purified chitinase was tested against four fungi, *Aspergillus awamori, Aspergillus flavus, Candida albicans, Rhizopus oryzae*; seven bacteria, *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Shigella sonnei, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus agalactiae*; and the antibiotic effect in terms of inhibition zones were recorded (Table 5).

Table 5. The antimicrobial activity of purified *and crude chitinase* represented in terms of zone of inhibition of microbial (fungal/bacterial) growth in millimeter. (ND/N means not defined/negligible)

Micro-organism	**Zone of inhibition (mm)**	
	Purified	**crude**
Aspergillus flavus	16 ± 0.6	47±2
Aspergillus awamori	20± 0.14	58±1
Candida albicans	21± 0.73	52±4
Rhizopus oryzae	18± 0.52	50±2
Bacillus subtilis	4 ± 0.22	ND/N
Staphylococcus aureus	3 ± 0.13	
Klebsiella pneumoniae	4 ± 0.14	
Shigella sonnei	6± 0.19	
Pseudomonas aeruginosa	3± 1.8	
Escherichia coli	5 ± 0.07	
Streptococcus agalactiae	2± 0.73	

The antifungal activity was most predominant compared to antibacterial activity. Nonetheless the antimicrobial activity was noteworthy and chitinase could be used as a green broad spectrum antimicrobial entity. Antifungal activity of many bacterial chitinases has been reported, but there are few reports on antimicrobial activity of *Citrobacter* derived chitinase (Bhattacharya *et al*., 2016; Shehata *et al*., 2018). The antifungal activity of the crude supernatant was about 3-fold greater than purified chitinase and is assumed due to presence of chito-oligosaccharides or chitin degradation products. The use of crude preparations for biocontrol is advantageous because the presence of other hydrolytic enzymes and/or low molecular-weight antibiotic or antimicrobial substances in such crude mixtures promotes the activity.

4. Conclusion

There are numerous reports on the production of chitinase from microorganisms like fungi, actinomycetes, bacteria and plants; however there is not evident report on chitinase production from *Citrobacter freundii* fermenting fish scales. Our research has established the usage of fish scales as a potent substrate for solid state fermentation, contributing to cost-cutting strategies and production of a valuable enzyme with broad antimicrobial potential. 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 & thermo-alkali-stability, and broad antibiotic spectrum even in its crude form. In a nutshell, this is the first report to date elucidating the production of chitinase from fish scales using *Citrobacter freundii str. nov.* haritD11, its purification and characterization, and the organism's growth kinetics are reported in conjunction to the chitin utilization.

Acknowledgements

Funding: Financial assistance by Andhra University as Institutional PhD Scholar (2010–2015) is deeply acknowledged by recipient Dr. Haritha Meruvu.

Acknowledgements: My sincere thanks to the Department of Chemical engineering, Andhra University for serving as the host organization for my Research work.

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