Carbohydrate Polymers 121 (2015) 1–9

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Chitooligomers preparation by chitosanase produced under solid state fermentation using shrimp by-products as substrate

T. Nidheesh, Gaurav Kumar Pal, P.V. Suresh*

Academy of Scientific and Innovative Research, Meat and Marine Sciences Department, CSIR-Central Food Technological Research Institute, Mysore 570020, India

ARTICLE INFO

Article history:
Received 27 October 2014
Received in revised form
17 December 2014
Accepted 18 December 2014
Available online 27 December 2014

Keywords:
Shrimp by-products
Solid state fermentation
Chitosanase
Chitooligomers
Purpureocillium lilacinum
Response surface methodology

ABSTRACT

Solid state fermentation (SSF) conditions were statistically optimized for the production of chitosanase by *Purpureocillium lilacinum* CFRNT12 using shrimp by-products as substrate. Central composite design and response surface methodology were applied to evaluate the effect of variables and their optimization. Incubation temperature, incubation time, concentration of inoculum and yeast extract were found to influence the chitosanase production significantly. The R^2 value of 0.94 indicates the aptness of the model. The level of variables for optimal production of chitosanase was 32 ± 1 °C temperature, 96 h incubation, 10.5% (w/v) inoculum, 1.05% (w/w) yeast extract and 65% (w/w) moisture content. The chitosanase production was found to increase from 2.34 ± 0.07 to 41.78 ± 0.73 units/g initial dry substrate after optimization. The crude chitosanase produced 4.43 mM of chitooligomers as exclusive end product from colloidal chitosan hydrolysis. These results indicate the potential of *P. lilacinum* CFRNT12 for the chitosanase production employing cost effective SSF using shrimp by-products.

1. Introduction

Seafood processing, one of the major agro-industry in tropical and subtropical countries based on aquaculture and marine fishing, has assumed great importance during the last couple of decades due to the ever increasing demand for processed shrimps in the international market (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014). The industrial processing of shrimp involves the removal of cephalothorax and carapace as non-edible by-products, which accounts for 50–70% by total volume of raw materials, depending upon the species and processing methods (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014; Olsen, Toppe, & Karunasagar, 2014). Annually, India produces more than 100,000 tons of shrimp by-products, of which only an insignificant quantity is used for the isolation of chitin. The rest of the produce is either discarded into the coastal and nearshore area, or under-utilized (Kumar & Suresh, 2014; Nidheesh & Suresh, 2014). Considering the potentials of shrimp by-products and society's current levels of awareness on negative impact of pollution, possible utilization of shrimp by-products as media substrate for microbial fermentation and production of economically useful products is proposed as a cost effective and ecofriendly waste management approach (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Kumar & Suresh, 2014; Suresh, 2012; Wang, Hsu, & Liang, 2010; Wang, Liang, & Yen, 2011; Wang, Peng, Liang, & Liu, 2008). In spite of number of study reports available on the utilization of shrimp by-products as fermentation media, so far no commercial venture based on such by-products is known. Thus an ideal bioprocess, at commercial scale, for efficient utilization of shrimp by-products is warranted

Chitosan, the linear cationic (1-4)-2-amino-2-deoxy-β-D-glucan produced from chitin by partial deacetylation, is a prominent polysaccharide owing to its biocompatibility (Jayakumar et al., 2010; Muzzarelli et al., 2012). Chitooligomers (COS) are readily soluble in water and exert, better than chitosan, antimicrobial, antitumor and immunomodulatory activities (Kim & Rajapakse, 2005; Muzzarelli, 2009; Thadathil & Velappan, 2014; Wee, Reddy, Chung, & Ryu, 2009). Thusly, the production of chitooligomers has received increased attention in the food and biomedical industries (Gao, Ju, Jung, & Park, 2008; Nguyen et al., 2014; Pechsrichuang, Yoohat, & Yamabhai, 2013; Wang, Wu, & Liang, 2009; Wee et al., 2009). The enzymatic production of chitooligomers has numerous advantages over the chemical methods including a high yield, and less environmental impact (Kim & Rajapakse, 2005; Sun, Han, Liu, Zhang, & Gao, 2007; Wang et al., 2009; Wee et al., 2009).

* Corresponding author. Tel.: +91 821 2514840; fax: +91 821 2517233.
E-mail addresses: drsureshpv@cftri.res.in, drsureshpv@hotmail.com (P.V. Suresh).

http://dx.doi.org/10.1016/j.carbpol.2014.12.017
0144-8617/

Chitosanases (EC 3.2.1.132, Chitosan *N*-acetyl glucosaminohydrolase) are hydrolytic enzymes acting on β-(1 → 4) glycosidic linkage of chitosan resulting in low molecular weight chitooligomers as exclusive end products (Thadathil & Velappan, 2014). Therefore, chitosanases, can be effectively used for the preparation of chitooligomers from chitosan substrates. However, the bulk production of chitosanases for commercial applications is still scanty (Lee, Xia, & Zhang, 2008; Da Silva, Honorato, Franco, & Rodrigues, 2012). Hence, screening of high yielding enzyme-productive microorganisms and development of low-cost cultivation media, as well as different bioprocessing approaches for more economic production of the chitosanases are being intensively pursued by scientific community.

Solid state (substrate) fermentation (SSF) is defined as a fermentation process in which microorganisms grow on solid materials without the need or, presence of free liquid and the moisture necessary for microbial growth exists in adsorbed state with solid matrix (Pandey, Selvakumar, Soccol, & Nigam, 1999). SSF has been reported to be economical and an effective alternative to submerged fermentation (SmF) due to its cost effective production of high titers of microbial extracellular enzymes. SSF can be carried out using cheaply available agro-industrial residues such as wheat bran, shrimp by-products, etc. (Suresh, Kumar, & Sachindra, 2011; Suresh, Sachindra, & Bhaskar, 2011; Thadathil, Kuttappan, Vallabaipatel, Kandasamy, & Velappan, 2014). Despite number of reports available on production of chitosanase by SmF, to the best of our knowledge only one report (Da Silva et al., 2012) is available on the chitosanase production from *Trichoderma koningii* under SSF using wheat bran supplemented with chitosan. Hence, the present study was aimed at developing an ideal bioprocess for the production of chitosanase by a soil isolate of *Purpureocillium lilacinum* CFRN12 through statistical optimization of process variables, employing the cost-effective SSF process using the shrimp by-products as the solid substrate. In addition the hydrolytic activity of the crude chitosanase obtained from the fungus for the production of bioactive chitooligomers from chitosan was investigated.

2. Materials and methods

2.1. Materials

Potato dextrose agar (PDA, Himedia, Mumbai, India), D-glucosamine (Sisco Research Laboratory, Mumbai, India); chitosan, glycol chitosan, ρ-nitrophenyl *N*-acetyl-β-D-glucosaminide (ρ-NPGlcNAc) (Sigma Chemical Co., St. Louis, USA), High Performance Liquid Chromatography (HPLC) standards for monomeric GlcN and oligomeric GlcN (Associates of Cape Cod Inc., East Falmouth, MA, USA), were procured. All other reagents and chemicals were of analytical grade.

2.2. Preparation of shrimp by-products substrate

Marine shrimp (*Penaeus* sp.) processing fresh by-products containing cephalothorax and carapace were collected from local seafood market and transported to the laboratory in chilled condition in an icebox. They were minced with a wet mill (Stephen Mill, UM 5 Universal, Germany) into about 1–2 cm particle size and washed twice with tap water (1:10, w/v) using a planetary mixer in order to remove the dirt, soil and sand adhering to the by-products. The washed shrimp by-product chips were dried at 55 ± 2 °C for 12 h in a hot air drying oven (Kilburn, Bombay, India), milled with an electric wearing blender, and sieved through 22 mesh sieve. The shrimp by-products powder, thus prepared, was used as the substrate for SSF studies without any further treatment.

2.2.1. Proximate and chemical analysis of shrimp by-products

The moisture, lipid and ash content were determined according to standard methods of AOAC (2000). Total nitrogen content of shrimp by-products was estimated using Kjeldahl method (AOAC, 2000). Chitin and pH of shrimp by-products were determined according to Suresh et al. (2011a).

2.3. Microorganisms and inoculum preparation

P. lilacinum CFRNT12 (GenBank accession No. KJ524453) and *Penicillium decumbens* CFRNT15 (GenBank accession No. KJ563941), used in the current investigation, were isolated from the native soil sample (unpublished data, Fig. 1) during the course of an earlier investigation. These microorganisms were maintained on PDA slants, under refrigeration, in the culture collection of the laboratory (MMS Department, CSIR-CFTRI, Mysore, India). The identity of the cultures was confirmed by the standard identification techniques for fungi and 18S DNA sequencing. Spore inocula were prepared by growing the cultures on PDA slants at 30 ± 2 °C for 5 days. Later, the spores were dispersed in distilled water containing 0.1% (w/v) Tween 80. The concentration of spore suspension was adjusted to ∼log 7.5 colony-forming units/ml, and then used as inoculum (unless otherwise mentioned).

2.4. Production of chitosanase under SSF

2.4.1. Preparation of solid substrate medium and fermentation

Five gram of solid shrimp by-product substrate taken in Erlenmeyer conical flask (100 ml) was moistened with 5.5 ml of tap water and autoclaved at 15 psi pressure for 30 min. pH of the solid shrimp by-products substrate medium was adjusted to 6.0 ± 0.2 using 0.5 M HCl solution before autoclaving. After cooling, the solid substrate medium was inoculated with 2 ml of prepared spore suspension and incubated at 32 ± 2 °C in an incubator without air moisture control. The solid substrate medium had 55% (w/w) initial moisture content after the addition of the inoculum. After incubation, the fermented solid substrate in each flask was added with 50 ml of chilled acetate buffer (pH 5.6, 0.1 M) and mixed well on an environmental shaker (150 rpm) for 20 min. The slurry was then allowed to settle for about 5 min, and the supernatant was collected by filtration through a dampened cheese cloth. Later the collected supernatant was clarified by centrifugation (12,000 rpm) at 4 °C for 20 min and used for various assays.

2.5. Assay of chitosanase

Chitosanase (EC 3.2.1.321) activity was assayed using glycol chitosan as the substrate (Da Silva et al., 2012). The enzyme assay mixture consisting of 400 μl of 0.1% (w/v) glycol chitosan, and 100 μl of enzyme solution was incubated at 32 ± 2 °C for 30 min. The reaction was terminated by heating the reaction mixture in a boiling water bath for 10 min. Heat inactivated enzyme with the substrate was used as a blank. The amount of reducing sugar produced in the supernatant was estimated using dinitrosalicylic acid reagent (Miller, 1959). D-glucosamine was used as the standard. One unit of the chitosanase activity was defined as the amount of enzyme which released one micromole of D-glucosamine per minute under the conditions mentioned. The enzyme production in SSF was expressed as units/g of initial dry substrate (U/g IDS) (Da Silva et al., 2012; Thadathil et al., 2014).

2.6. Non- specific chitosan degrading enzyme assays

Non-specific chitosan hydrolyzing enzymes like endo-chitinase (EC 3.2.1.14) using ethylene glycol chitin (Suresh, Sachindra, et al., 2011), β-*N*-acetylhexosaminidase (EC 3.2.1.52) using ρ-NPGlcNAc

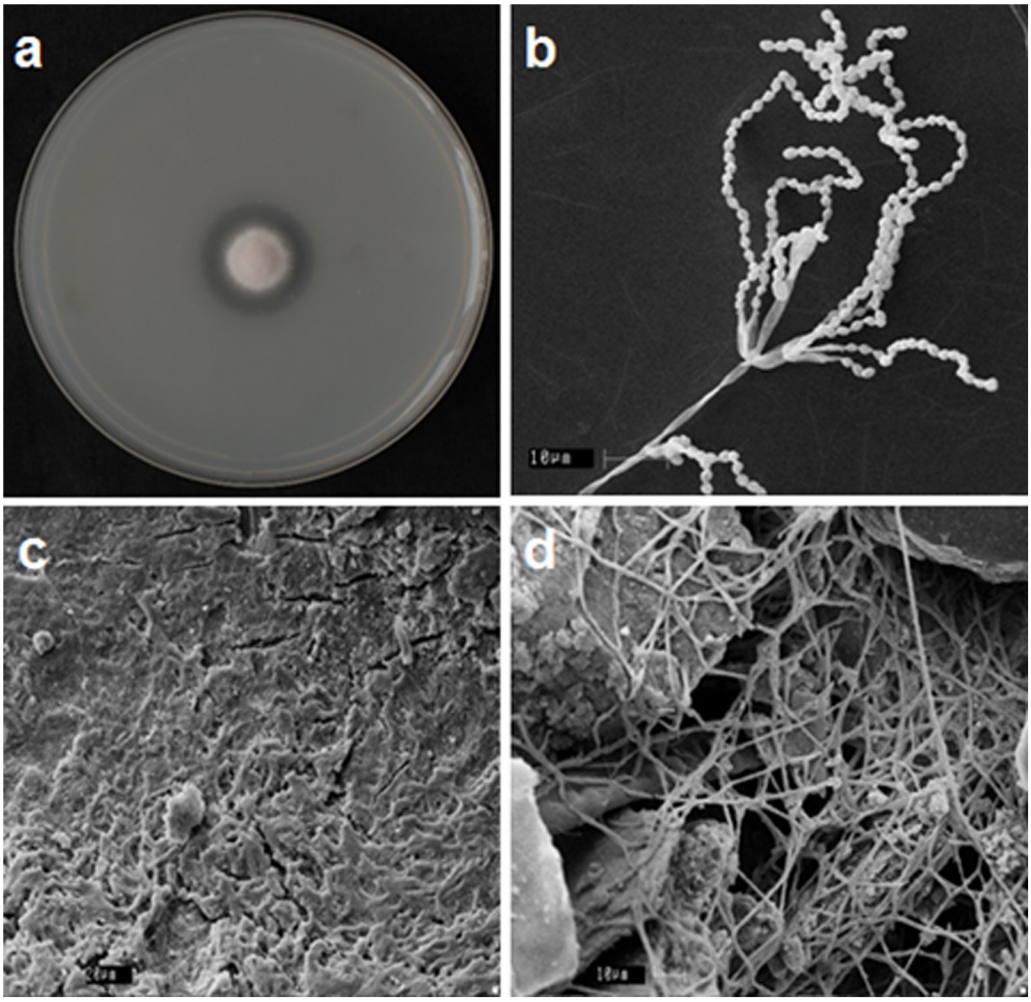

Fig. 1. Zone of clearance produced by *P. lilacinum* CFRNT12 in Chitosan – Mineral agar media (a) Scanning electron micrograph of *Purpureocillium lilacinum* CFRNT12 (b) scanning electron micrograph of shrimp by-products medium before inoculation (c) after inoculation (d).

(Suresh, Kumar, et al., 2011) and protease by the casein digestion method (Kumar & Suresh, 2014) were assayed.

2.7. Experimental designs and optimization of fermentation conditions by RSM

2.7.1. Central composite design (CCD)

Five variables, which normally influence the shrimp by-products fermentation on the production of chitosanase by the fungi, were selected and optimized using CCD and RSM. Based on the initial experiments, a CCD was developed for five variables, *viz.* incubation temperature (°C, X1), incubation time (h, X2), inoculum concentration (%, w/v, X3), concentration of yeast extract (%, w/w, X4) and initial moisture content of the substrate (%, w/v, X5). CCD matrix was formulated at five levels (−2, −1, 0 +1, and +2, coded values), with 12 central points and 18 axial points (with one variable set at extreme ±2 level), and with a total number of 28 runs. The details of experimental design with coded and actual levels of all five variables are summarized in Table 1 and Supplementary material 1a.

The responses obtained in CCD were subjected to multiple non-linear regression analysis for obtaining empirical models that relate the response to the independent factors. The results of CCD were used to derive second order polynomial model equation (Eq. (1)).

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response (chitosanase activity, U/g IDS); β_o is the intercept (regression coefficient); β_i is the linear coefficient; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient. X_i and X_j are the independent variables.

Three dimensional response surface and contour graphs were drawn to illustrate the main and interactive effects of independent variables. The response surface graphs were drawn for the variations in responses as a function of levels of two independent variables when all the other variables were kept at their central levels. The optimum values of each independent variable for maximum response variable (chitosanase activity) were determined from response surface graphs and desirability profile (Thadathil et al., 2014). The designed CCD model and the optimized conditions derived by RSM were further validated by a set of experiments using random combinations of five independent variables (Supplementary material 1b).

2.8. Scanning electron microscopy (SEM)

The growth of *P. lilacinum* CFRNT12 and degradation of the solid shrimp by-products substrate under SSF were observed using SEM. The samples were prepared by glutaraldehyde – graded alcohol method. The dried samples were examined in a scanning electron microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV after sputter coating of gold (∼100 Å).

Table 1
Central composite design matrix with independent variables in actual values and values of response variable (chitosanase activity, U/g IDS). *X*1, incubation temperature (°C); *X*2, incubation time (h); *X*3, inoculam (%, w/v); *X*4, yeast extract (%, w/w) and *X*5, initial moisture content of the substrate (%, w/w).

Run	Independent variables					Response variable	
	*X*1	*X*2	*X*3	*X*4	*X*5	Observed	Predicted
1	27	72	5.75	0.575	70	36.03 ± 0.63	34.51
2	27	72	5.75	1.525	60	24.48 ± 0.41	21.72
3	27	72	15.25	0.575	60	29.59 ± 0.43	25.92
4	27	72	15.25	1.525	70	30.81 ± 0.64	26.91
5	27	120	5.75	0.575	60	30.39 ± 0.47	30.28
6	27	120	5.75	1.525	70	32.08 ± 0.36	31.73
7	27	120	15.25	0.575	70	37.56 ± 0.56	36.31
8	27	120	15.25	1.525	60	41.78 ± 0.73	39.29
9	37	72	5.75	0.575	60	0.84 ± 0.02	2.25
10	37	72	5.75	1.525	70	2.95 ± 0.08	4.13
11	37	72	15.25	0.575	70	20.68 ± 0.23	20.95
12	37	72	15.25	1.525	60	12.66 ± 0.14	11.69
13	37	120	5.75	0.575	70	2.53 ± 0.06	6.35
14	37	120	5.75	1.525	60	10.13 ± 0.15	12.71
15	37	120	15.25	0.575	60	23.21 ± 0.33	24.88
16	37	120	15.25	1.525	70	18.15 ± 0.29	19.59
17	22	96	10.5	1.05	65	29.12 ± 0.26	36.56
18	42	96	10.5	1.05	65	6.80 ± 0.16	0.52
19	32	48	10.5	1.05	65	4.12 ± 0.14	8.53
20	32	144	10.5	1.05	65	25.03 ± 0.36	21.79
21	32	96	1.0	1.05	65	23.99 ± 0.29	21.28
22	32	96	20.0	1.05	65	32.88 ± 0.51	36.75
23	32	96	10.5	0.1	65	31.70 ± 0.48	30.80
24	32	96	10.5	2.0	65	25.32 ± 0.45	27.38
25	32	96	10.5	1.05	55	27.85 ± 0.52	29.45
26	32	96	10.5	1.05	75	32.81 ± 0.55	32.38
27 (C)	32	96	10.5	1.05	65	35.71 ± 0.23	35.42
28(C)	32	96	10.5	1.05	65	36.29 ± 0.35	35.42

2.9. Enzymatic hydrolysis of chitosan substrates

2.9.1. Concentration of enzyme

The crude chitosanase preparation obtained from the SSF by *P. lilacinum* CFRNT12 was filled into the dialysis bag and sealed. The sealed dialysis bags were placed in a container containing polyethylene glycol (mol. wt. 20,000) and stored under refrigeration for 12 h. Later the concentrated enzyme was dialyzed with several batches of acetate buffer (pH 5.6, 0.1 M) at 4 °C for 24 h in order to remove the reducing sugars present. The concentrated and dialyzed enzyme was used for chitosan hydrolysis experiments.

2.9.2. Chitosan hydrolysis

The chitosan hydrolysis was carried out at 37 ± 2 °C under stirring (~100 rpm) using a shaking water bath. Reaction mixture in Erlenmeyer conical flask (50 ml capacity) contained 0.5% of chitosan substrates [colloidal and crystalline (>22 mesh size) chitosan] in 20 ml of acetate buffer (pH 5.6, 0.1 M) and 2.5 ml of concentrated enzyme (169.92 unit/ml) preparation. Aliquots were withdrawn at different intervals and heated in a boiling water bath for 10 min to terminate the enzyme activity. After removing the undigested material by centrifugation (10,000 rpm, 20 min), the amount of chitooligomers (hydrolysate) in the supernatant was quantified (Miller, 1959).

2.9.3. Analysis of chitosan hydrolysate by thin layer chromatography (TLC)

The chitosan hydrolysates prepared under the conditions described above were spotted on silica gel G TLC plate (Merck, Darmstadt, Germany) and developed with *n*-propanol: 30% ammonia (2:1) solution. The amino sugar on the TLC plate was visualized by ninhydrin reagent. The standards used included D-glucosamine and a mixture of D-glucosamine, chitobiose, chitotriose, chitotetrose, chitopentose and chitohexose.

2.9.4. Analysis of chitosan hydrolysate by HPLC

A HPLC system containing a LC-10AT pump, UV detector SPDM-10AVP (Shimadzu, Japan) and UBondapak™ NH_2 10 μm (3.9 × 300 mm) column (Water, Ireland) was used to analyze the products released by the hydrolysis of colloidal chitosan. The clear hydrolysate obtained after centrifugation (at 12,000 rpm, 10 min) was filtered with a membrane filter (0.45 μm pore) (Whatman Inc, USA) and 20 μl of the sample was injected onto the column using a mixture of acetonitrile and water (70:30) as the mobile phase at a flow rate of 1 ml/min. D-glucosamine and a mixture of D-glucosamine, chitobiose, chitotriose, chitotetrose, chitopentose and chitohexose were used as the standards. The concentration of chitooligomers was estimated as area of sample/area of standard (Nguyen et al., 2014).

2.10. Statistical analysis

All the experiments were conducted at least in triplicate, and average values ± standard deviations are reported. The results were statistically analyzed by using the Analysis of Variance (ANOVA) techniques, in which a p value of ≤0.05 was regarded as significant. Statistica Release 7 software (Statistica, 2005) was used for the data analysis.

3. Result and discussion

The washed shrimp by-products used as the substrate for the conduct of SSF contained 36.62 ± 1.4% protein, 14.76 ± 0.8% chitin, 9.94 ± 0.2% fat and 38.87 ± 0.3% ash (minerals) on dry weight basis. pH of the dried shrimp by-products was 8.6 ± 0.2. In general, the proximate composition of shrimp by-products may vary batch to batch due to varied reasons including species specificity of the shrimp, the proportion of head to shell, age and processing methods among others (Kumar & Suresh, 2014).

Table 2
ANOVA table of central composite design model for chitosanase activity. *X*1, incubation temperature (°C); *X*2, incubation time (h); *X*3, inoculam (%, w/v); *X*4, yeast extract (%, w/w) and *X*5, initial moisture content of the substrate (%, w/w).

Factors	SS	df	MS	*F*-value	*p*-value
X1 (L, Linear)	1947.595	1	1947.595	11,348.31	0.005976*
(Q, Quadratic)	379.779	1	379.779	2212.91	0.013531*
X2 (L)	264.009	1	264.009	1538.34	0.016228*
(Q)	547.267	1	547.267	3188.83	0.011272*
X3 (L)	358.754	1	358.754	2090.40	0.013922*
(Q)	54.672	1	54.672	318.56	0.035631*
X4 (L)	17.584	1	17.584	102.46	0.062690
(Q)	53.356	1	53.356	310.90	0.036067*
X5 (L)	12.926	1	12.926	75.32	0.073034
(Q)	27.051	1	27.051	157.62	0.050601
1L*2L	1.011	1	1.011	5.89	0.248826
1L*3L	107.493	1	107.493	626.34	0.025424*
1L*4L	0.068	1	0.068	0.40	0.642359
1L*5L	10.196	1	10.196	59.41	0.082135
2L*3L	16.299	1	16.299	94.97	0.065098
2L*4L	38.061	1	38.061	221.78	0.042685*
2L*5L	90.702	1	90.702	528.51	0.027675*
3L*4L	3.502	1	3.502	20.41	0.138690
3L*5L	3.798	1	3.798	22.13	0.133337
4L*5L	19.885	1	19.885	115.87	0.058974
Lack of fit	232.164	6	38.694	225.46	0.050935
Pure error	0.172	1	0.172		
Total SS	3872.802	27			

* Statistically significant at 95% of confidence interval.

3.1. Chitosanase production by fungi using shrimp by-products under SSF

Chitosanase production under SmF has been already reported using shrimp shell powder/by-products by *Serratia* sp. TKU016 (Wang et al., 2008), *Bacillus subtilis* TKU007 (Wang & Yeh, 2008), *Acinetobacter calcoaceticus* TKU024 (Wang, Tseng, & Liang, 2011) and squid pen powder (Wang et al., 2010). However, to the best of our knowledge, no reports are available on the production of chitosanase using shrimp by-products as the solid substrate under SSF conditions by any bacteria or fungi.

3.2. Optimization of chitosanase production P. lilacinum CFRNT12 by CCD

Biosynthesis and secretion of extracellular enzymes and other metabolites by fungi during SSF is significantly influenced by the composition of the culture medium as well as the culture conditions. Nevertheless effective control of the bioprocess variables could be appropriately employed such that desirable modification is achieved in the production of the enzyme and other metabolites and their subsequent excretion by microorganisms (Suresh, Sachindra, et al., 2011). In the current investigation optimization of bioprocess variables were carried out by statistical based experimental approach using CCD and RSM.

The mean response as the chitosanase activity obtained along with the predicted response generated in CCD is presented in Table 1. The model generated was quadratic type, which accounts for the natural logarithm of the response, as a function of five independent variables and their linear, quadratic and interactive functions. Second order polynomial equations derived from the experimental data by multiple regression analysis for the chitosanase activity is presented in Eq. (2) (coefficient with less than 95% of significance was not considered in the equation). The regression equation obtained showed the R^2 value of 0.94001, and adjusted R^2 value of 0.7686, indicating a good agreement between the experimental and predicted values of the chitosanase production (a value of $R^2 > 0.75$ indicates the aptness of the model). This value ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 94.0% of the variability observed in the response could be explained by the model. The R^2 is the proportion of variability in response values explained or accounted by the model (Thadathil et al., 2014; Wee et al., 2009). The model characteristics were evaluated to determine if the models could be successfully used. The model had significant *F*-value (129.257) and "Prob > *F*" value (0.023009), non-significant lack-of-fit and standard error value of 0.172.

$$\begin{aligned} \text{Chitosanase activity } (U/g\,IDS)\ (Y) = {} & (-580.934) + (10.101 \times X1) \\ & + (-0.169 \times X1^2) + (2.948 \times X2) \\ & + (-0.009 \times X2^2) + (-0.071 \times X3^2) \\ & + (-7.009 \times X4^2) + (9.637 \times X5) \\ & + (0.109 \times X1 \times X3) + (0.135 \times X2 \times X4) \\ & + (-0.020 \times X2 \times X5) \end{aligned} \quad (2)$$

where *Y* is the predicted chitosanase activity (production) and *X*1, *X*2, *X*3, *X*4 and *X*5 are the level of incubation temperature, incubation time, inoculum, yeast extract and moisture content, respectively.

The result of the second-order response surface model is presented in Table 2 in the form of ANOVA. The quadratic model derived from CCD could effectively be used to elucidate the variables that influence the chitosanase production under a wide array of operating conditions. The results obtained by the regression analysis of CCD model indicated that incubation temperature ($p \le 0.005$ and $p \le 0.013$), incubation time ($p \le 0.016$ and $p \le 0.011$) and inoculum concentration ($p \le 0.013$ and $p \le 0.035$) have significant linear and quadratic effects; while yeast extract showed significant ($p \le 0.036$) quadratic effects on the chitosanase production by *P. lilacinum* CFRNT12. At the same time initial moisture content of the substrate did not show any significant effect on chitosanase production by *P. lilacinum* CFRNT12 in SSF using the shrimp by-products substrate. The interaction between incubation temperature and inoculum concentration ($p \le 0.025$), incubation time and inoculum concentration ($p \le 0.042$), and yeast extract ($p \le 0.027$) showed significant effects on the chitosanase production.

The optimum values of five independent variables for the maximum chitosanase activity (response variable) by *P. lilacinum*

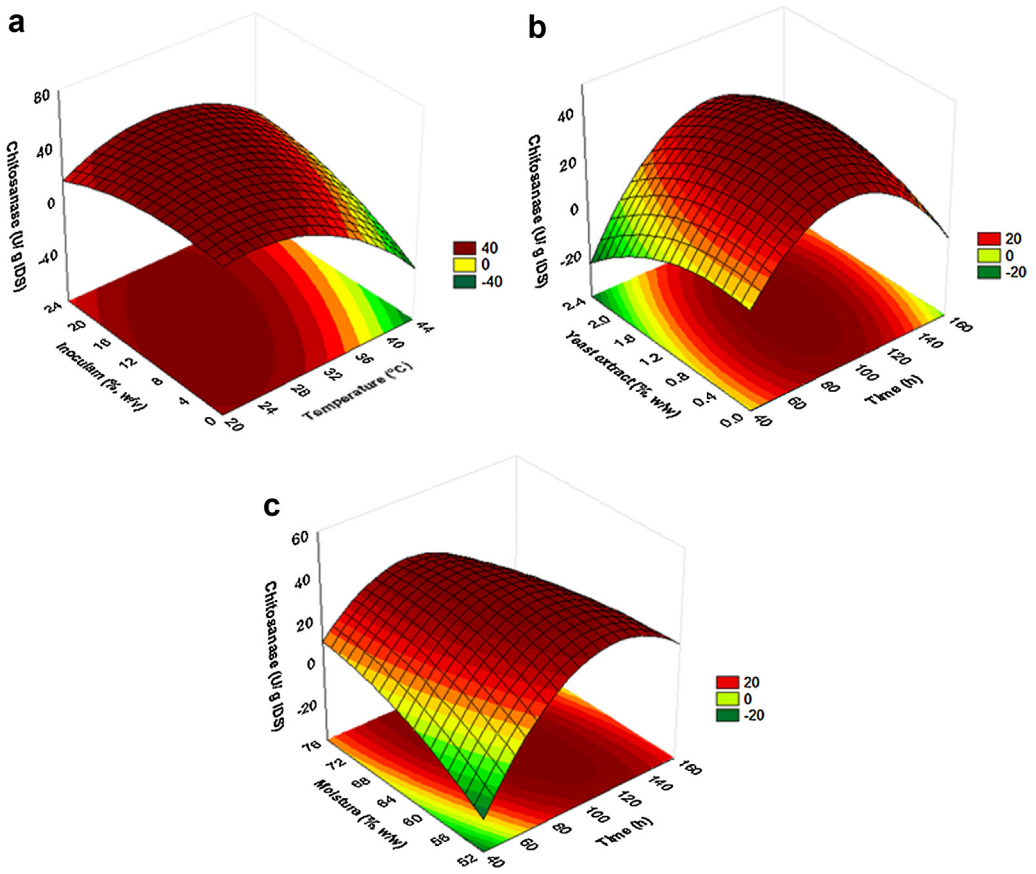

Fig. 2. Response surface and contour plots of chitosanase production by *Purpureocillium lilacinum* CFRNT12 in solid state fermentation as a function of incubation temperature and inoculum concentration (a); incubation time and yeast extract concentration (b); incubation time and initial moisture content of the substrate (c) at a central value of all other variables.

CFRNT12 in SSF using shrimp by-products substrate was elucidated from the three-dimensional response surface and contour graphs. The response surfaces and counter graphs drawn are based on the final model as a function of levels of two variables at their optimum levels, while maintaining the other variables within their experimental range. Clearly, the chitosanase activity varied significantly with the incubation time, incubation temperature, and concentrations of inoculum and yeast extract (Fig. 2). Incubation temperature is characteristic of a microorganism, and profoundly affects the production of enzymes, the duration of the synthesis phase, as well as the stability of products in SSF (Nampoothiri et al., 2004; Suresh, Sachindra, et al., 2011). Results of the current study indicated that $32 \pm 2\,^{\circ}C$ was the optimum temperature for maximal chitosanase production by *P. lilacinum* CFRNT12 under SSF using the shrimp by-products substrate. Incubation time has strong effects on growth rate of microorganisms and consequent biosynthesis of enzymes/metabolites (Thadathil et al., 2014; Wee et al., 2009). The chitosanase activity gradually increased along with increase in incubation time and finally attained a maximum value after 96 h. Further, an increase in enzyme activity was also observed along with increase in the concentration of yeast extract in the solid medium up to 1.05% (w/w) level, while higher concentrations did not favor enhanced enzyme production. The initial moisture content of the solid substrate is a critical factor in SSF since it influence microbial growth and significantly affects the hydrolytic enzyme production (Suresh, Kumar, et al., 2011). Higher titers of enzyme with a lower percentage of moisture level are characteristic of fungal SSF. However, the requirement of 65% (w/w) initial moisture content of the substrate for the maximum chitosanase activity by *P. lilacinum* CFRNT12 is agreeable with earlier reports on extracellular enzyme production by fungi (Pagnoncelli et al., 2010; Suresh, Kumar, et al., 2011). In the present study, an exponential increase in the enzyme activity was recorded with increasing concentration of inoculum and the optimum inoculum size was observed as10.5% (w/v). This might be due to the increased production of biomass. The increase in the number of spores in the inoculum would ensure a rapid proliferation of biomass and subsequent biosynthesis (Nampoothiri et al., 2004).

Statistical optimization resulted in an overall increase in chitosanase production by *P. lilacinum* CFRNT12 from 2.34 ± 0.07 to 41.78 ± 0.73 U/g IDS as compared to the medium before optimization of process variables. The optimum levels of five variables predicted for the maximum chitosanase activity were $32 \pm 2\,^{\circ}C$ of incubation temperature, 96 h of incubation, 10.5% (w/v) of inoculum, 1.05% (w/w) of yeast extract and 65% (w/w) of initial moisture content of the solid substrate (Fig. 2 and Supplementary material 1c). However, it is difficult to compare the results obtained in this investigation with other published works on chitosanase production under SSF due to lack of published reports on SSF particularly on using the shrimp by-products as a solid substrate. However, chitosanase production from *P. lilacinum* CFRNT12 was found to be significantly high (41.78 ± 0.73 U/g IDS) when compared with the chitosanase production (4.84 U/g IDS) by *Trichoderma koningii* under SSF using wheat bran supplement with colloidal chitosan (Da Silva et al., 2012).

Most of the researchers have reported that microorganisms (bacteria and fungi) require chitosan (colloidal or crystalline) for inducing the production of chitosanases (Sun et al., 2007; Wee et al., 2009) and use of colloidal chitosan as a supplement in the culture media for chitosanase production is a common strategy

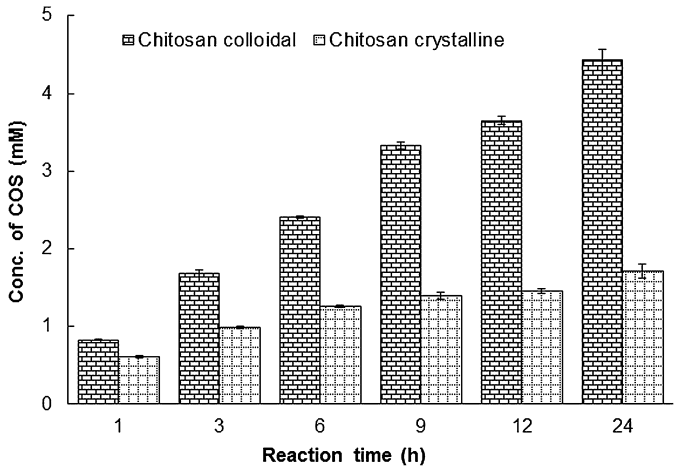

Fig. 3. Time scale production of chitooligomers from colloidal and crystalline chitosan substrates using crude chitosanase preparation of *Purpureocillium lilacinum* CFRNT12.

(Thadathil & Velappan, 2014). Whereas, the antimicrobial activity of chitosan against various groups of microorganisms including bacteria, fungi and yeasts have been documented (Xia, Liu, Zhang, & Chen, 2011). Therefore, the incorporation of chitosan as an inducer in the medium for chitosanase production may result in possible inhibition or poor growth with respect to some organisms. In this context microorganism that could produce chitosanase without chitosan as an inducer have technical advantage during fermentation due to the high viscosity, antimicrobial activity of chitosan (Xia et al., 2011; Thadathil & Velappan, 2014). The fungal culture *P. lilacinum* CFRNT12 used in the present investigation could produce maximal levels of chitosanase in the solid shrimp by-products medium without addition of the chitosan under SSF conditions. These results indicate the potential of *P. lilacinum* CFRNT12 for the possible commercial production of chitosanase under economically viable SSF using the cheap shrimp by-products as the solid substrate.

To confirm the acceptability of the CCD model for predicting the chitosanase production by *P. lilacinum* CFRNT12 by SSF using the shrimp by-products, validation experiments were conducted (Supplementary material 1b). The R^2 values for the experimental versus predicted response (chitosanase activity) was observed as 0.983. This high value indicates the usefulness of the model for chitosanase production.

3.3. Chitosan hydrolysis and production of chitooligomers

Time scale formation of chitooligomers from different chitosan substrates (colloidal and crystalline chitosan) by crude chitosanase preparation of *P. lilacinum* CFRNT12 is presented in Fig. 3. Maximum yield of chitooligomers was obtained with the colloidal (4.43 mM) and crystalline chitosan (1.7 mM) after 24 h of hydrolysis. From results presented in Fig. 3 and Table 3 it was noted that the hydrolysis rate of colloidal chitosan was very high at initial period of reaction up to 6 h, recording about 37.9% (1.68 mM) and 54.27% (2.4 mM) of total yield after 3 and 6 h of reactions, respectively. Whereas, the rate of crystalline chitosan hydrolysis recorded were about 58.31% (0.99 mM) and 74.18% (1.26 mM) of total yield after 3 and 6 h of reaction, respectively (Table 3). The slow rate of reaction observed after 6 h of hydrolysis by the enzyme preparation of *P. lilacinum* CFRNT12 might be due to either binding of the enzyme to the chitosan substrate or possible substrate inhibition. The protein binding/immobilization properties of chitin and chitosan (Ilankovan, Hein, Ng, Trung, & Stevens, 2006; Suresh, Kumar, et al., 2011) and the inhibitory effect of higher concentrations of oligosaccharides as short substrates for chitosanase activity (Honda et al., 1999; Lacombe-Harvey et al., 2013) has been documented earlier.

3.4. Analysis of the chitosan hydrolysates

From the results obtained for the hydrolysis of colloidal chitosan by the crude chitosanase and analysis by TLC and comparison with standard D-glucosamine and chitooligomers (Fig. 4) it was inferred that the hydrolyzed products consist only chitooligomers $(GlcN)_{2-6}$. This was further confirmed by HPLC analysis of products (Fig. 4). The chitooligomers formation was found to be increased along with increase in the reaction time and also varied in the composition of different chitooligomers with reaction time. HPLC analysis revealed that the main product of hydrolysis released was chitosan-trimer and chitosan-tetramer after 6 h (22.81 and 76.23%), 12 h (41.87 and 52.61%) and 24 h (43.91 and 50.36%) of reaction (Table 3). This indicates that the chitosanase produced by *P. lilacinum* CFRNT12 in SSF using the shrimp by-products substrate is 'endo' type of chitosanase that formed only chitooligomers by 'endo'-hydrolysis. The TLC and HPLC analysis clearly evidence the absence of D-glucosamine in the hydrolyzed products.

Application of chitosanase for the hydrolysis of chitosan into chitooligomers is a highly attractive method for the synthesis of specific chitooligomers (Nguyen et al., 2014; Pechsrichuang et al., 2013). However the use of chitosanase in hydrolysis is limited due to its high cost and unavailability in bulk quantity (Lee et al., 2008; Thadathil & Velappan, 2014). Chitooligomers production using microbial chitosanase has been reported by a few research groups (Cheng & Li, 2000; Nguyen et al., 2014; Pechsrichuang et al., 2013). Cheng and Li (2000) reported the production of chitooligomers using the chitosanase produced by *Aspergillus* sp. Y2K in which chitotriose, chitotetraose and chitopentaose were the major products in the chitosan hydrolysate. Recently, Pechsrichuang et al. (2013) reported the potential of recombinant *B. subtilis* chitosanase (crude culture supernatant) in the preparation of chitooligomers ($GlcN_{2-6}$) from different chitosan substrates. It is not possible to correlate and compare the yield of chitooligomers obtained in this investigation using the chitosanase from *P. lilacinum* CFRNT12 with other works on the chitosanase production due to paucity of similar reports on quantitative production (yield) of chitooligomers by enzymatic chitosan hydrolysis. In the present study, the use of crude enzyme

Table 3
Relative rate of hydrolysis of different chitosan substrates by chitosanase from *Purpureocilium lilacinum* CFRNT12 and HPLC profile of enzyme reaction product of colloidal chitosan and crystalline chitosan.

Chitosan substrate	Reaction time (h)					
	1	3	6	9	12	24
Relative rate of hydrolysis of different chitosan substrates						
Chitosan (Colloidal)	18.7	37.9	54.3	75.2	82.4	100
Chitosan (Crystalline)	35.8	58.3	74.2	82.4	85.8	100

	Reaction time (h)		
	3	6	12
HPLC profile of colloidal chitosan (S) hydrolysate (%)[a]			
$(GlcN)_2$	0.05[b]	1.53[b]	3.80[b]
$(GlcN)_3$	22.81[c]	41.87[c]	43.91[c]
$(GlcN)_4$	76.23[d]	52.61[d]	50.36[d]
$(GlcN)_5$	0.38[e]	0.22[f]	0.46[e]
$(GlcN)_6$	0.52[f]	1.13[b]	0.77[f]

[a] The concentrate was analyzed by HPLC as described in the materials and methods. $(GlcNc)_2$ = chitobiose, $(GlcNc)_3$ = chitotriose, $(GlcNc)_4$ = chitotetrose, $(GlcNc)_5$ = chitopentose and $(GlcNc)_6$ = chitohexose.
[b–f] Means with different letters within a column indicate significant differences.

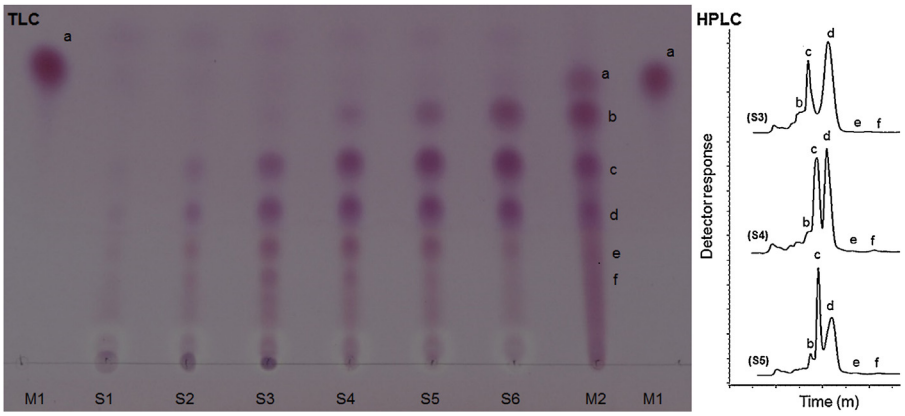

Fig. 4. TLC and HPLC chromatograms of enzyme reaction product of colloidal chitosan. S1, S2, S3, S4, S5 and S6 are sample taken at different time (1, 3, 6, 9, 12 and 24 h) interval from the hydrolysate. M1 = Standard D-glucosamine (GlcN). M2 = Standard GlcN and chitooligomers $(GlcN)_{2-6}$ mixture. a = GlcN, b = $(GlcN)_2$, c = $(GlcN)_3$, d = $(GlcN)_4$, e = $(GlcN)_5$, f = $(GlcN)_6$.

preparation instead of purified form has commercial relevance and economic concerns since the enzyme purification processes are costly in commercial enzyme production (Pagnoncelli et al., 2010).

4. Conclusions

Statistical design experimental approaches using the CCD and RSM proved as valuable for the optimization of the extracellular chitosanase production by *P. lilacinum* CFRNT12 under SSF using the shrimp by-products. Based on the results obtained in the present investigation it is concluded that *P. lilacinum* CFRNT12 has immense potential for the possible commercial production of chitosanase under SSF using shrimp by-products as the cheap solid substrate. Statistical optimization of bioprocess ensured enhanced production of chitosanase from 2.34 ± 0.07 to 41.78 ± 0.73 (U/g IDS) by *P. lilacinum* CFRNT12. Utilization of the cheap and abundant shrimp by-products as the solid substrate for SSF production of chitosanase have commercial significance besides contributing to effective solid waste management in shrimp processing industries. Furthermore, the chitosanase of *P. lilacinum* CFRNT12 has the potential for the production of specific bioactive chitooligomers.

Competing interests

The authors declare that they have no conflict of interest.

Acknowledgements

NT thanks the University Grants Commission (UGC), and GKP thank the Department of Science and Technology (DST), New Delhi, India for the award of Research Fellowship. SPV thanks Director, CSIR-CFTRI, Mysore, India for the funding under WELFO – Supra Institutional project (BSC 0202). The authors would like to thank Dr. Mahendrakar NS (Former Editor-in-Chief, Journal of Food Science and Technology), Ms. Angela Haws (Life Science Major, Penn State Harrisburg, Middletown, PA 17057, USA) and Prof. M. Chandrasekaran (King Saud University, Riyadh, Saudi Arabia) for valuable comments during the revision of this manuscript and their help with English.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.12.017.

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