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Optimization of Process Parameters for Chitinase Production by a Marine Isolate of Serratia marcescens

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ABSTRACT
Chitinases play an important role in the decomposition of chitin and results in the utilization of chitin as a renewable resource. A total of 18 chitinolytic bacteria were isolated from the sand sample. Following primary and secondary screening in colloidal chitin medium, strain CBC-5 demonstrated the highest chitinolytic activity and was selected for further study. It was later identified as Serratia marcescens. Addition of easily metabolized sugars had inhibitory effect on the enzyme production and the highest yield was obtained with colloidal chitin as the sole source of carbon and yeast extract (1% w/w) as nitrogen source. 10.3 % increase in chitinase yield was observed when Triton X-100 was added to the medium. The optimum pH, temperature, and incubation period for chitinase production by the isolate was found to be 7.0, 30°C and 72 h respectively. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed the apparent molecular weight of the enzyme to be 42 kDa. The results obtained in the present study showed that isolated bacterium is a potent producer of chitinase and the enzyme can be exploited for the biodegradation of chitinous wastes and may find applications as biocontrol agents against fungi and insects.

KEYWORDS: *Serratia marcescens, colloidal chitin, chitinase, optimization.*

INTRODUCTION

Chitin is a long chain structural polymer composed of *N*-acetylglucosamine (GlcNAc) monomers connected via β (1-4) linkages [1]. It is the second most abundant organic polysaccharide in nature after cellulose. In nature this homopolysaccharide is present in the shells of crustaceans, exoskeleton of insects, fungal and algal cell wall, zooplankton and several phytoplankton species [1, 2]. It was estimated that almost 10% of the global landings of aquatics products come from these chitin-rich organisms [1], making chitin possibly the most abundant biomolecule in the marine environment [2]. In the terrestrial environment, chitin accumulates as a waste from shellfish production and processing industries. The accumulation of this abundant waste may prove to be an environmental hazard due to the easy deterioration and is a growing challenge for most of shellfish-producing countries.

Chitin is often tightly bound with other compounds like protein, lipids, pigments and calcium carbonate [3]. Conversion of these chitinous wastes to useful chitin and related oligomers involves processes like demineralization, deproteinization or hydrolysis, which was earlier carried out with strong acid and bases that involves high cost, low yields and corrosion problem [4]. The probable alternative to solve this problem is utilization of chitinolytic enzyme, chitinase and β-N-acetyl hexosaminidase [1].

Chitinases (EC 3.2.1.14) are produced by several bacteria, actinomycetes, fungi and also by higher plants [5, 6, 7, 8].

The production of inexpensive chitinolytic enzymes not only solves environmental problem but also reduces the production cost

of chito-oligosaccharides required in the manufacture of value added products such as sweeteners, several growth factors, and single cell protein [9, 10]. Furthermore, chitinase has also many other applications such in the biocontrol of plant pathogenic fungi and insects (biopestisides), preparation of protoplast from filamentous fungi, degradation of fish wastes [11, 12].

Microorganisms produce the chitinase primarily for assimilation of chitin as carbon and (or) nitrogen source [13]. Chitinases from marine bacteria have been isolated and properties of their chitinases reported. However, chitinase production by marine bacteria from coastal areas of India has not been studied extensively. This study primarily aims at the isolation of native chitinolytic bacterial strains from marine sediments, selection and identification of those able to produce maximum levels of chitinase, along with the optimization of the process parameters for microbial chitinase production.

MATERIALS AND METHODS

Sample collection:

Sand sample for microbiological analysis was collected during April to May 2010 from the fishermen colony situated to the north of St. Thomas Cathedral at Marina beach, Chennai Tamil Nadu, India. Besides regular marine fishes, the colony receives and processes significant amount of its shell fish catch. The sample was aseptically collected in a sterile zip lock cover and placed in an ice pack for transportation to the laboratory in Bangalore, where it was processed.

Preparation of colloidal chitin:

Colloidal chitin used as the substrate for chitinase production, was made from commercially available chitin powder (Sigma). Twenty grams of the chitin powder (Sigma) was mixed with 400 ml of concentrated HCl, and then kept overnight at 25°C. Then the pH of the resulting suspension was neutralized by adding 10 N NaOH. After keeping the suspension overnight in the refrigerator, it was centrifuged (5,000 rpm for 30 min) and washed with 2 liters of sterile distilled water until the colloidal chitin became neutral (pH 7.0). The resulting colloidal chitin was freeze dried to powder and stored at 4°C until further use [14].

Isolation of chitin degrading bacteria:

Chitin degrading bacteria were isolated from the sea sand sample using serial dilution and spread plate technique on semi synthetic media (SSM) with the following composition (g/L) along with 0.5% colloidal chitin, $(NH_4)_2SO_4$, 0.28; NH_4Cl, 0.23; KH_2PO_4, 0.0067; $MgSO4.7H_2O$, 0.04; $CaCl_2.2H_2O$, 0.022; $FeCl_3.6H_2O$, 0.005; Yeast extract, 0.2; NaCl, 0.015; $NaHCO_3$, 1.0 and 1 ml/L of trace element solution containing (g/L) $ZnSO_4.7H_2O$, 0.01; $MnCl_2.4H_2O$, 0.1; $CuSO_4.5H_2O$, 0.392; $CoCl_2.6H_2O$, 0.248; $NaB_4O_7.10H_2O$, 0.177 and $NiCl_2.H_2O$, 0.02 with glucose (1% w/v). All the chemicals used were of analytical grade and of the highest purity.

The plates were incubated at 30±2°C for 5 days and following incubation examined for the formation of clear zones (CZ) around the colonies. The size of the clear zone and colony size (CS) were both measured and colonies demonstrating zone of chitin breakdown were transferred to chitin agar slants.

Primary screening of chitin degrading bacteria:

Primary screening was performed by single line inoculation of all the above chitin degrading bacterial isolates on MSM plates (supplemented with 0.5% colloidal chitin) and incubated at 30±2°C. The zone of clearance due to chitin hydrolysis was recorded up to 5 days. The bacterial isolates which showed a

CZ/CS ratio over 3 were then subjected to secondary screening.

Secondary screening of chitin degrading bacteria:

The selected isolates from the primary screening were inoculated in 25 ml of liquid medium and incubated at 30±2°C, 200 rpm for 5 days. Following incubation, 2 ml of respective cultures were taken and centrifuged at 5000 rpm for 20 min. The chitinase activity was determined in the supernatants by performing the chitinase assay. The isolate demonstrating highest chitinase activity was selected for the further study.

Assay of chitinase:

Supernatants from the respective cultures were obtained by centrifugation at 5000 rpm for 20 min. The individual reaction mixture consisted of 1ml of individual culture supernatants, 1ml of 1% (w/v) colloidal chitin in citrate phosphate buffer pH 5.5 and incubated at 50°C for 30 min. Following incubation, all the reaction mixtures were put in boiling water bath for a period of 2 to 3 min to stop the enzyme action. The solutions were centrifuged at 5,000 rpm for 10 min. The amount of reducing sugar in the supernatants (resulting due the chitinolytic activity) was determined by dinitrosalycilic acid (DNS) method [15]. The colour development was read at 540 nm using a UV-VIS spectrophotometer (SANYO Gallenkemp, Germany). One enzyme unit is defined as the amount of enzyme that liberates 1µmol of *N*-acetyl-D-glucosamine per minute under the standard assay conditions.

Estimation of protein content:

The soluble protein content of the enzyme sample was determined by Lowry's method [16] using crystalline bovine serum albumin as the standard.

Characterization of the isolate:

The screened bacterial isolate was identified based on the morphological and biochemical characteristics. The morphological characteristics were identified by culturing the isolate on nutrient agar plates and studying the shape, size, color, opacity, texture, elevation, spreading nature and margin of the colonies, followed by gram's staining and motility test. The biochemical characterization of the isolate was performed by catalase test, oxidase test, indole, methyl red test, Vogesproskauer test, citrate utilization test, H_2S production test, urea hydrolysis test, gelatin liquefaction test, lipase production, pigment production test and carbohydrate utilization test (lactose, maltose, mannitol, sucrose, glucose and xylose). Bergey's Manual of Determinative Bacteriology (9th Edition) was used as a reference to identify the isolate.

Inoculum preparation:

The selected isolate was inoculated in 50 mL sterile medium broth taken in a 250 mL Erlenmeyer flask and incubated at 30°C for 48 h on a rotary shaker at 250 rpm. Following incubation, the culture broth was centrifuged at 8000 rpm for 15 min, washed twice, and suspended in physiological saline and stored under refrigeration for future use.

Optimization of growth conditions:

The isolate was grown at different concentrations (0.1, 0.3, 0.5, 1, 1.5, 3, 5 and 10% w/v) of colloidal chitin added to the broth to determine the optimum concentration of substrate for chitinase production. Effect of additional carbon sources (0.1% w/v: maltose, lactose, sucrose, xylose, fructose and glucose), nitrogen sources (0.1% w/v: peptone, tryptone, yeast extract, beef extract, ammonium sulphate, urea and sodium nitrate) on chitinase production, was determined by supplementing the broth medium with individual nutrients and incubated at 30°C and

200 rpm for 5 days. Flasks with no added sugar of nitrogen supplement acted as controls.

To test the effect of surfactants on the enzyme production, the medium was supplemented with 0.01% (v/v) Tween 20, Tween 80, Triton X-100 and 0.01% (w/v) sodium lauryl sulphate and sodium deoxycholate. Various physical parameters such as pH (4, 5, 6, 7, 8 and 9), effect of temperature (20, 25, 30, 35, 40, 45 and 50°C) and incubation period (24, 48, 72, 96, 120, and 144 h) were optimized by conventional methods for maximal chitinase production. All the experiments were conducted in triplicates.

Molecular weight determination:

For the purpose of molecular mass determination of chitinase, the crude enzyme was purified by 40% (w/v) ammonium sulphate precipitation method. The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate buffer (pH 7) and dialysed overnight against 0.01 M phosphate buffer at 4°C to prevent enzyme denaturation. Further, for the molecular weight determination, the dialysed enzyme sample was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using broad range pre-stained protein marker (NEW ENGLAND Biolabs, UK).

Statistical analysis:

All the optimization studies were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. P values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

In the past several years, there has been steady increase in the demand of chitin and its derivatives for the various industrial, clinical and pharmaceutical applications. The potential of developing enzymatic process for the production of chitooligosaccharides has attracted the attention of biotechnologists. Chitinolytic enzymes are gaining importance for their biotechnological applications. Since, the cost of chitinase represents an important share of the total cost of bioconservation of chitin, there is necessity to develop bioprocesses with reduction in the cost of the production.

Due to the potential applications of chitinase, it is very important to study the organisms that can produce the enzyme. Microorganisms are generally preferred to plant and animals as sources of industrial enzyme because their production cost is low and enzyme contents of microbes are more predictable and controllable. The ability of a microbial community to degrade chitin is important for recycling of nitrogen in the soil. Chitinolytic microbes occur widely in nature and prevent the polysaccharide deposited from dead animals and fungi from accumulating in land and marine sediments.

Isolation and screening of the chitinolytic bacteria:

18 chitinolytic bacteria were isolated from the sea sand sample plated on MSM fortified with 0.5% (w/v) colloidal chitin. The primary screening of these bacterial isolates on colloidal chitin agar demonstrated that 7 isolates produced a CZ/CS ratio above 3. Among these 7 highly potent chitinolytic bacteria, during the secondary screening, the culture filtrate of the isolate CBC-5 showed maximum chitinase activity (9.26 U/ml) and was selected for further studies **(Figure 1)**.

Characterization of the isolate:

Following the morphological and biochemical characteristics, isolate CBC-5 was identified as *Serratia marcescens* (**Table 1)**.

Factors influencing chitinase production:

Most of the studies on chitinases production have been carried out in liquid or submerged fermentation, although some attempts have been made in recent times on solid state fermentation (SSF). Generally, presence of chitin in the production medium is useful for the production of chitinase [17, 18]. Among different sources of chitin, colloidal chitin was found to be best for chitinase production [19]. Chitinases can be synthesised in the absence of substrates (constitutive enzyme) or in its presence (adaptive enzyme). However, addition of chitin to culture media greatly enhances enzyme production.

TABLE 1: Morphological and biochemical characterization of the screened bacterial isolate.

Biochemical tests	Results
Gram stain	-
Motility	+
Catalase	+
Oxidase	-
Indole production	-
Methyl Red	-
Voges Proskauer	+
Citrate utilization	+
H_2S production	-
Urea hydrolysis	-
Gelatin hydrolysis	+
Pigment production	+
Lipase production	+
Sugar fermentation	
Lactose	-
Maltose	+
Mannitol	+
Sucrose	+
Glucose	+
Xylose	-

Keys: +, positive; -, negative

Figure 1: Chitinase activity of the selected bacterial isolates. Data represent mean ± S.D. (n=3); P < 0.05

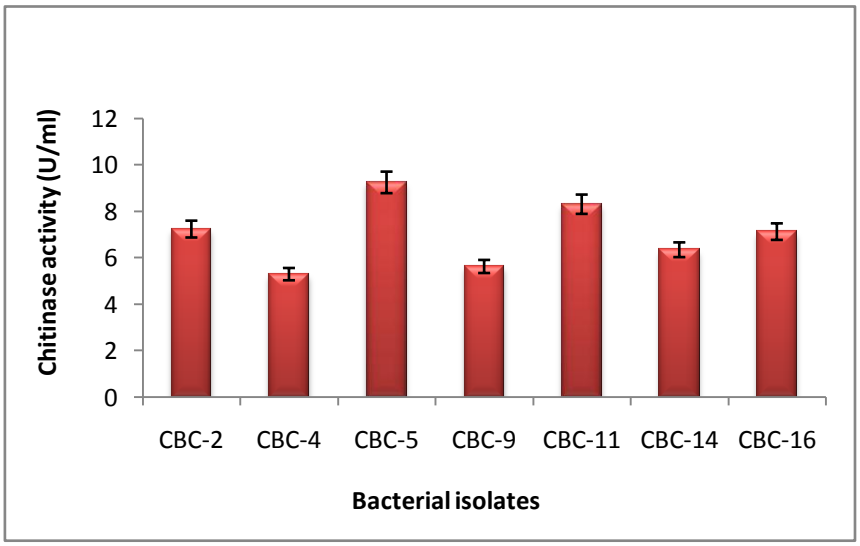


Figure 2: Effect of colloidal chitin concentration on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

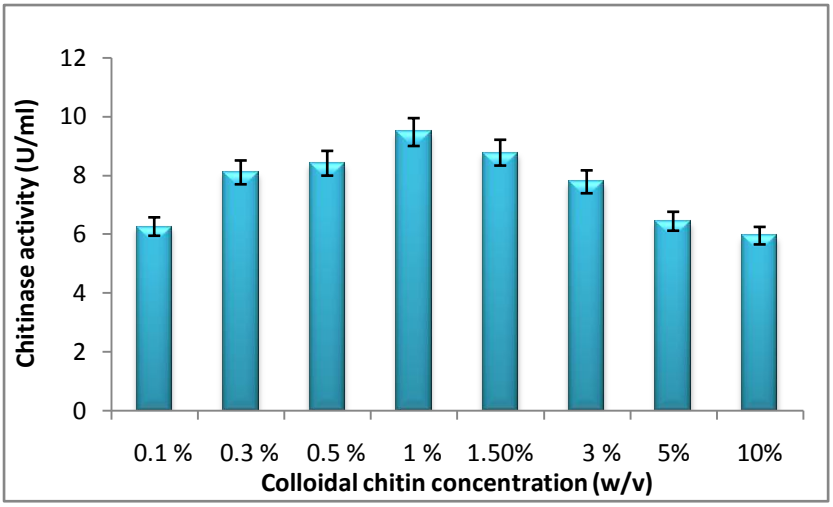


Figure 3: Effect of additional carbon source on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

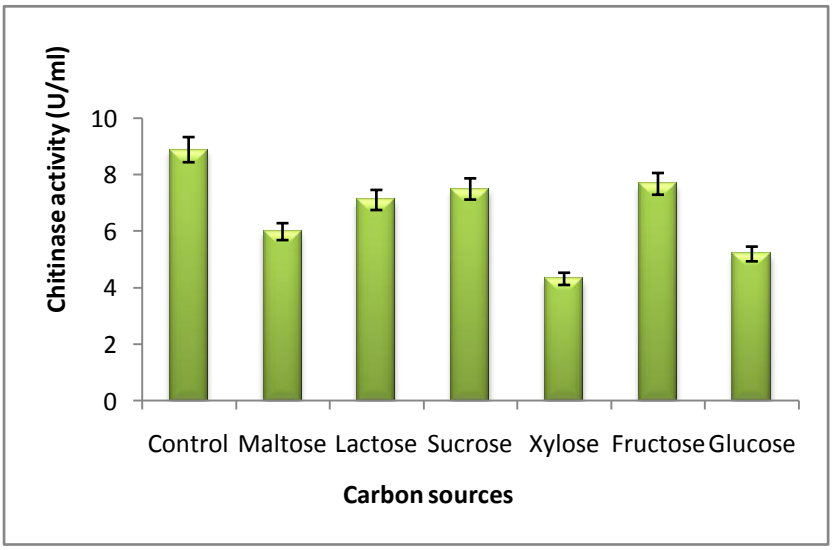


Figure 4: Effect of additional nitrogen source on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

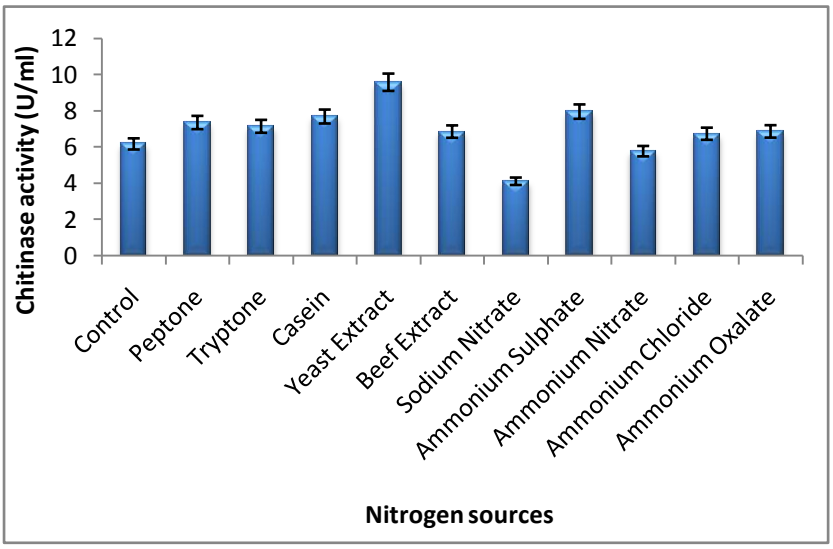


Figure 5: Effect of detergents on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

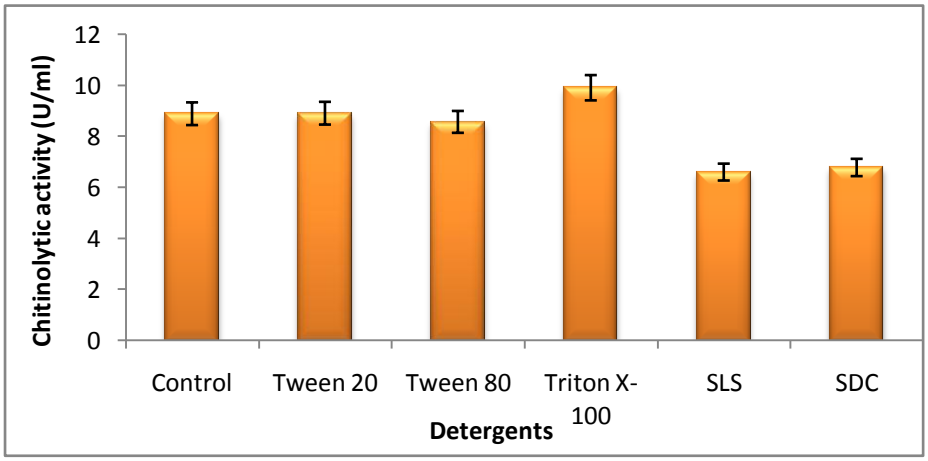


Figure 6: Effect of media pH on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

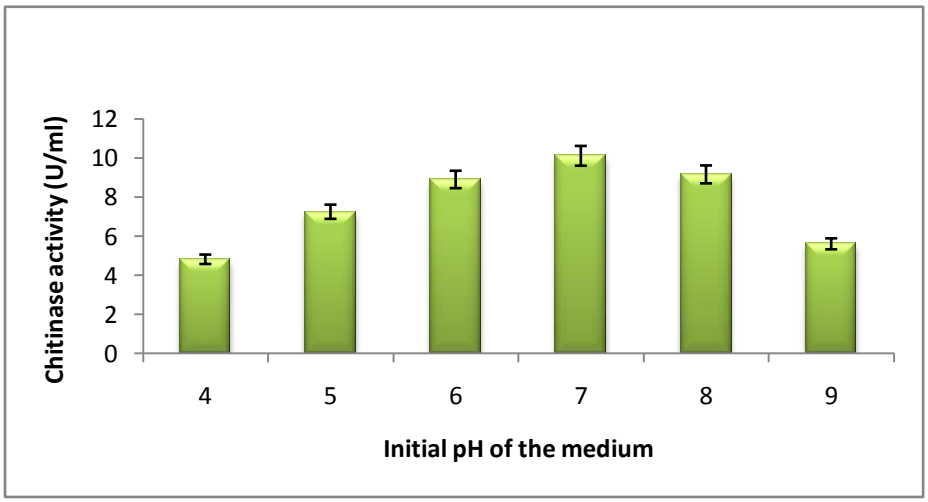


Figure 7: Effect of incubation temperature on chitinase production. Data represents mean ± S.D. (n=3); P < 0.05

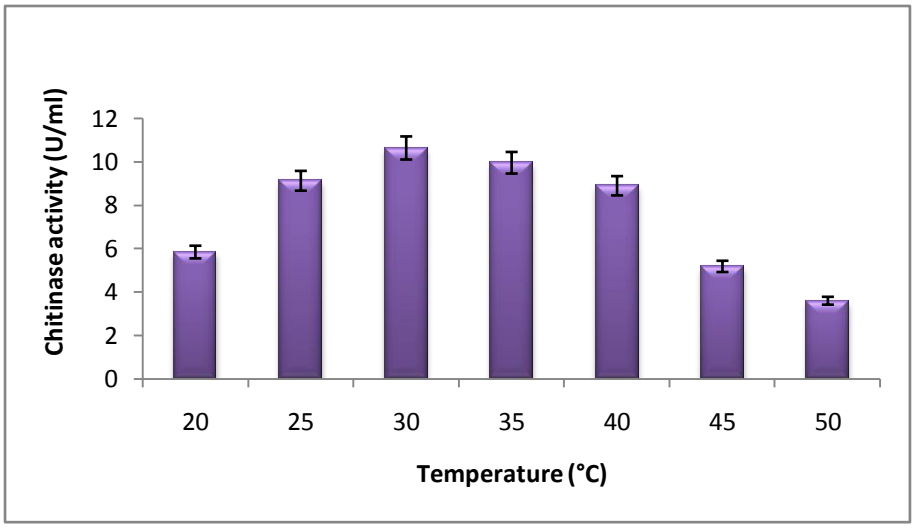


Figure 8: Effect of incubation period on chitinase production. Data represent mean ± S.D. (n=3); P < 0.05

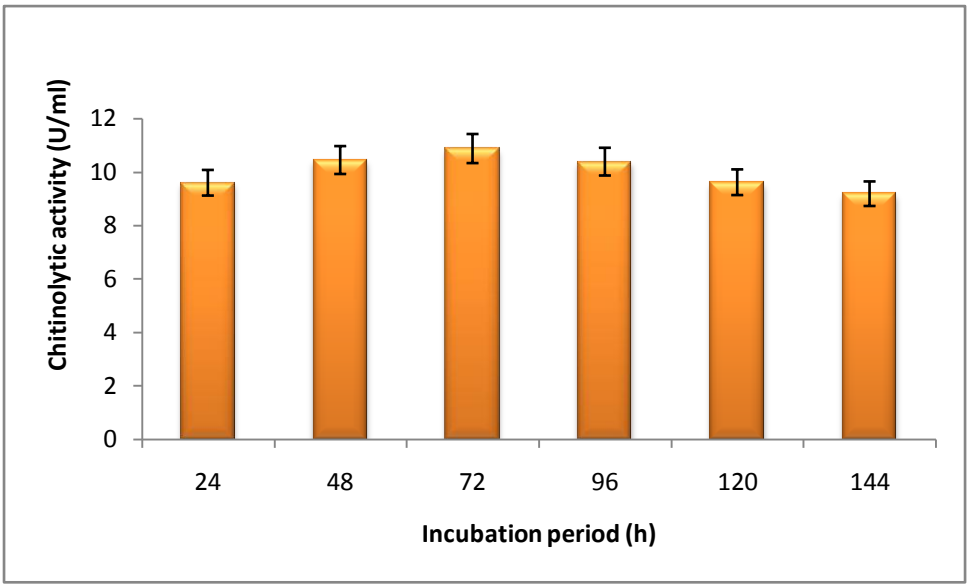
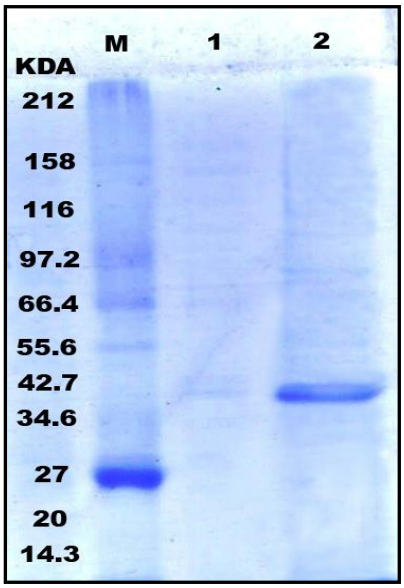


Figure 9: Protein profile of the partially purified chitinase by SDS-PAGE. Lane M, standard protein marker; Lane 1, crude chitinase from *S. marcescens*; Lane 2, dialyzed enzyme. Molecular sizes of the marker proteins (in kDa) are shown on the left



Optimization of nutrient supplements:

It has been suggested that for most microorganisms the optimum chitin concentration for chitinase induction is in the range of 1-2% (x/v) [20]. Out of the different colloidal chitin concentrations ranging from 0.1 to 10% (w/v), maximum enzyme production (9.47 U/ml) was at a concentration of 1% **(Figure 2)**. Similarly, when chitin concentrations were varied from 1.25 to 15 g/l it was observed that *A. xylosoxydans* produced maximum chitinase with 10-15 g/l chitin [21]. The type and nature of carbon source is one of the most important factors for any type of fermentation process [22]. The carbon source represents the energetic source that will be available for the growth of the microorganism. Microbial chitinases are inducible enzymes which are stimulated by chitin, chitooligosaccharides, chitobiose and/or

GlcNAc [1]. The production of chitinase by *S. marcescens* under the influence of different additional carbon sources and chitin alone was investigated. Addition of easily metabolized sugars reduced chitinase production but supported growth, whereas the control containing colloidal chitin as the sole source of carbon led to the highest chitinase activity (8.90 U/ml). Addition of maltose, lactose, sucrose, xylose, fructose and glucose decreased chitinase production **(Figure 3)**.

Among the various pentoses and hexoses studied with *Streptomyces* species, arabinose doubled enzyme production while glucose repressed enzyme synthesis [23]. No chitinase production was observed when *Stachybotrys elegans* was grown on glucose, sucrose or N-acetyl glucosamine [24].

At suboptimal chitin concentrations the addition of different carbon sources to the culture medium is reported to increase the chitinase production by some microorganisms including *Trichoderma harzianum.* However, similar to our result, at optimal concentrations, chitin when used as the sole carbon source resulted in higher chitinase yield as compared to other media containing additional carbon sources [20, 21, 25].

Nitrogen regulation is of wide significance in industrial microbiology since it affects the synthesis of enzymes involved in both primary and secondary metabolism. The results in present study indicated that in comparison to the inorganic nitrogen sources, the organic nitrogen sources served as better supplements for chitinase production by *S. marcescens*.

Among various organic and inorganic sources tested, yeast extract was identified as the best nitrogen source **(Figure 4)** producing the highest level of chitinase (9.56 U/ml). In confirmation to our finding, similar results have been reported in previous studies. Addition of yeast extract has been reported to increase chitinase activity in *S. marcescens*, *Alcaligenes xylosoxydans and Paenibacillus sabina* strain JD2 [17, 21]. Gohel *et al* [26] reported significant influence of urea, peptone and yeast extract on chitinase production by *Pantoea dispersa*. In contrast, Chitinase production by *Paenibacillus* sp. D1 was reduced in presence of organic nitrogen source. Similar observation has been reported for chitinase production by *Streptomyces* sp. Da11 [27].

Chitinase production by *T. harzianum* was studied using wheat bran-based solid medium, and the best enzyme yield was obtained with yeast extract as an additional nitrogen source[20], while another study involving the chitinase yield in wheat bran chitin medium in SSF was significantly not affected by the presence of different nitrogen additives [28].

It has been proposed that detergents enhance enzyme secretion by increasing cell membrane permeability [29]. The influence of the addition of various surfactants to the basic culture medium is shown in **Figure 5**. Non-ionic detergents like Tween 20, Tween 80 and Triton X-100 significantly enhanced the chitinase production with Triton X-100 giving the highest yield (9.93 U/ml). Addition of other detergents had no stimulating effect on chitinase production. Earlier, Triton X-100 had a positive effect on chitinase production, resulting in 12.4% increase, while Tween 20 and Tween 80 showed no statistically significant effect [30].

An observable increase in chitinolytic activity was seen following the addition of detergents (Tween 20, Tween 60, Triton X-100 and Tergitol N P 35) into the culture medium of *M. timone* and other microorganisms such as *A. xylosoxydans* [21, 31].

Optimization of physical parameters:

Microorganisms are sensitive to the concentration of hydrogen ions present in the medium. Thus pH change observed during the

growth of microbes also affects product stability during fermentation**.** Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth [32]. The obtained results demonstrated that as medium pH changed between 4 and 9, the chitinase production varied with the maximum (10.12 U/ml) at neutral pH. On the other hand, relatively low values for chitinase production were recorded at acidic and alkaline conditions **(Figure 6).**

In *S. marcescens* the optimal pH for chitinase production was reported to be 7.0 [17], while pH 6.0 was reported as optimum for chitinase production in *T. harzianum* [18]. The production of chitinase by *S. aureofaciens* was optimal at pH 6.5-7. Maximum chitinase production by the strain ANU 6277 was observed in 1% chitin amended CYS medium incubated at pH 6 [33].

Streptomyces thermoviolaceus OPC-520 produced relatively high level of chitinase activity of 1.13 U/mL of culture filtrate when grown in a medium at pH 7.0 [34]. The optimum pH for the chitinase produced by the strains *Bacillus* sp.13.26 and *Pseudomonas aeruginosa* K-187 was nearly neutral [35, 36]. The influence of temperature on chitinase production is related to the growth of the organism. Since temperature influences protein denaturation, enzyme inhibition and cell growth, it plays a significant role in the development of the biological process**.** The optimum temperature depends on whether the culture is mesophilic or thermophilic. In the present study temperature showed a significant role in chitinase production. *S. marcescens* being a mesophilic bacterium actively produced the maximum chitinase production (10.63 U/ml) at 30°C **(Figure 7)**. There was a gradual decrease in the enzyme production above and below at 30°C under controlled conditions.

The optimum growth temperature for chitinase production by *M. timonae* was found to be in the range of 25-30°C. The activity achieved at 20°C was 90% lower than the optimum temperature. The enzyme activity decreased as the temperature increased above 30°C and even at 35°C a 60% decrease in chitinolytic activity was observed [30].

On the contrary, among different temperature tested, *B. laterosporus* produced maximum chitinase activity of 42.93 units/ml at 35°C. It has been observed that in both the lower and higher temperatures (20 and 40°C), the chitinase activity was sharply decreased[37]. Similarly in previous studies maximum enzyme production of chitinase was observed from 30 to 40°C [23, 38].

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The incubation time varies with enzyme productions. After optimization of all the process parameters, the time course of maximal enzyme production was studied. Our result elucidated that the incubation period influences the enzyme production, wherein, the chitinase activity increased steadily and reached maximum (10.87 U/ml) at 72 h of incubation **(Figure 8)**. Further increase in the incubation period led to a reduction in chitinase production. This might be due to the depletion of nutrients in the medium.

Our result is at par with a previous report where chitinase production by *S. marcescens* WF when evaluated revealed that the maximal chitinase production related to process variables was obtained with the second order polynomial model: dry shrimp shell powder at 6%, pH=6.5, temperature of 28°C during fermentation for up to 72 h [39].

S. marcescens GG5 showed maximum enzyme production (0.2 U/ml) at 96 h of inoculation and 0.12 U/ml at 120 h of inoculation [40], while other studies reported that the production of chitinase by *S. marcescens* was maximum at 144 h of growth and that by *Bacillus circulans* no 4.1 was at 96 h [17, 41]. *S. marcescens* produced the highest chitinase after 2 days of incubation at 30°C on a rotary shaker (200 rpm). Enzyme levels remaining constant during the third day of incubation. However, chitinase production started to decline, thereafter, this being perhaps due to the lack of nutrients in the medium [42].

Molecular weight determination:

The molecular weights of microbial chitinases range from 20,000 to 120,000 with little consistency. The molecular weights of bacterial chitinases are mostly around 60,000 to 110,000, while those of actinomycetes are mostly 30,000 or lower, fungi are higher than 30,000. The molecular weight of plant chitinases are mostly around 30,000 [43].

SDS-PAGE analysis of the purified enzyme revealed one protein band with an estimated molecular weight of 42 kDa **(Figure 9)**. Earlier, the molecular weight of other chitinases from *Bacillus circulans, S. marcescens* and *Micrococcus* sp. AG84 were 45, 57 and 33 kDa respectively [25, 41, 43].

CONCLUSION

The isolation and utilization of chitinolytic *S. marcescens* from marine sample is rare. In course of our study it was found that cultural parameters have a profound effect on the production of chitinase under submerged condition. Colloidal chitin as the sole source of carbon and yeast extract as the best nitrogen source can prove to be economical in terms of the fermentation expenditure. Neutral pH along with a temperature around 30°C facilitates the highest yield. The current findings clearly denote that *S. marcescens* has a remarkable potency for the production of chitinase. But in order to be exploited industrially a deep understanding of its chitinolytic machinery has to be understood. We recommend genetic engineering strategies for strain improvement and evaluation of chitinase production from these new strains at laboratory bioreactor level.

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