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PRODUCTION AND OPTIMIZATION OF CHITOSANASE FROM BACTERIAL ISOLATE CHTM-12

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ABSTRACT
Chitosanase (Glycosyl hydrolases, EC 3.2.1.132) are the enzymes that catalyses the endohydrolysis of β-1, 4-glycosidic linkage of chitosan to release Chitooligosaccharides (COS). Chitooligosaccharides are the degraded products of chitosan or chitin prepared by enzymatic or chemical hydrolysis of chitosan. The greater solubility and low viscosity of COS have attracted the interest of many researchers to utilize COS and their derivatives for various biomedical applications. The health benefits of COS, possessing several beneficial biological effects including lowering blood cholesterol, lowering high blood pressure, protective effects against infections, controlling arthritis, improvement of calcium uptake, and enhancing antitumor properties. The present investigations have been made to find out the most suitable conditions to produce chitosanase from the bacterial isolate CHTM-12. The various culture parameters like Selection of media, inducer concentration, production pH, temperature, agitation rate (rpm), inoculum age, incubation time were individually optimized for the higher production of chitosanase. In light of the recent interest in the biomedical applications of chitin, chitosan, COS and their derivatives this article focuses on the optimization of the different parameters for the maximum production of the chitosanases from the bacterial isolate CHTM-12.

KEYWORDS: Chitosanase, Chitooligosaccharides (COS), CHTM-12, Optimization.

INTRODUCTION

Chitin a mucopolysaccharides is the second most abundant biopolymer in nature after cellulose. Chitin is white, hard, inelastic, and nitrogenous compound and is main building material found in the shells of crustaceans such as shrimp, crab, lobster and exoskeletons of mollusks, insects as well as in the cell walls of some fungi (Islam *et al*., 2017). Chitin is a linear polymer consisting of repeated units of N-acetyl-D-glucosamine (GlcNAc) bound together by β-1, 4-glycosidic bond (Zhu *et al*., 2012). Chitosan, a natural polycationic copolymer consisting of β-(1-4)-linked 2-acetamido-D-glucose (GlcNAc) and 2-amino-D-glucose (GlcN) units and is mainly derived from chitin by partial deacetylation of chitin (Polymer of GlcNAc) (Thadathil *et al*., 2014). Chitosan exhibits diverse applications in different areas such as pharmaceutical and medical applications, paper production, textile wastewater treatment, biotechnology, cosmetics, food processing and agriculture. Chitosan applications in biomedical fields includes tissue engineering, artificial kidney, skin, bone, cartilage, liver, nerve, tendon, wound healing, burn treatment etc. (Islam *et al*., 2017).

Chitosanases are the hydrolytic enzymes that have the capability of degrading chitosan, a copolymer consisting of variable number of N-acetyl-D-glucosamine and D-glucosamine in a linear fashion, which is commonly present on shell of crabs and prawns (Samrot *et al*., 2019). Chitosanase (Glycosyl hydrolases, EC 3.2.1.132) are the enzymes that catalyses the endohydrolysis of β-1, 4-glycosidic linkage of chitosan to release Chitooligo saccharides and can hydrolyze GlcN-GlcN, GlcN-GlcNAc and GlcNAc-GlcN bonds except GlcNAc-GlcNAc bond (Zhu *et al*., 2012). There are mainly two types of chitosanases enzymes on the basis of their mode of action: Exochitosanases (EC 3.2.1.165) and endochitosanases (EC 3.2.1.132). Endochitosanases enzyme also called exo-β-Dglucosaminidase cleaves β-1,4 bonds between glucosamine residues in the partly or fully acetylated chitosan while Exochitosanases cleaves chitosan from non-reducing end of COS or chitosan to produce Gln (glucosamine) or N-acetyl-D-glucosamine residue (NAG) (Sinha *et al*., 2016). Chitosanases enzymes have been found from different microorganisms including bacteria (*Bacillus* sp., *Serratia* sp., *Paenibacillus* sp., *Acinetobacter* sp., and *Streptomyces* sp.), fungi (*Aspergillus* sp., *Gongronella* sp., *Trichoderma* sp.), cyanobacterium (*Anabaena fertilissima* RPAN1), plants (vesicular–Arbuscular mycorrhizal colonised leek and onion root as well as chemical or pathogen stressed leaves of various plant species) (Thadathil *et al*., 2014). Chitooligosaccharides are the degraded product and prepared by the depolymerization of chitin or chitosan using acid hydrolysis, hydrolysis by physical methods and enzymatic degradation (Lodhi *et al*., 2014). Chitosan with Degree of polymerization less than 20 and an average molecular weight less than 3.9 kDa are called

chitooligosaccharides (COS), chitosan oligomers or chitooligomers (Liaqat *et al*., 2018). Chitooligosaccharides have various pharmaceutical and medicinal applications, due to their nontoxic and high solubility properties. The health benefits of COS, possessing several beneficial biological effects including lowering blood cholesterol, lowering high blood pressure, protective effects against infections, controlling arthritis, improvement of calcium uptake, and enhancing antitumor properties. COS may also be effective in enhancing systemic immune responses and in modulating the functions of immune-competent cells (Lodhi *et al*., 2014).

Keeping in view the importance of microbial chitosanase in medical sciences, the study was planned for the Selection of medium and optimization of physiochemical parameters to maximize the production of chitosanase from bacterial isolate CHTM-12.

MATERIAL AND METHODS

Chemicals

All chemical used in the present study were either procured from Sigma Aldrich (U.S.A.) or HiMedia Laboratories Pvt. Ltd., Mumbai and were of high purity analytical grade. The media constituents were of Bacteriological grade.

Microbial culture

The bacterial isolate CHTM-12 which is already isolated and identified was procured from the Department of Biotechnology Himachal Pradesh University, Shimla.

Morphological characterization of CHTM-12

To examine the morphology of strain, bacterial culture was allowed to grow on Luria broth agar plates (pH 7.0) for 24h incubation and Gram's staining was performed.

Maintenance of bacterial strain

The culture was maintained by repeated sub-culturing at 30^0C on Luria broth agar plates (pH 7.0). The media consists of components having following composition (%, w/v) 2.0% Luria broth, Chitosan 0.5%, Agar 2.0% in 100 ml distilled water. The culture was maintained on glycerol stock for future use.

Production of chitosanase by bacterial isolates CHTM-12 in shake flask

Seed culture was prepared by inoculating 50 mL of broth with a loop-full of culture. The culture was allowed to grow for 24h at 30^0C under shaking conditions at 150 rpm. Thereafter, 5% (v/v) of 24 h old seed culture was used to inoculate the production broth. The seeded production medium was incubated at 30^0C, 150 rpm for 24h.

Analytical procedures

Estimation of cell mass

The cells of bacterial isolate CHTM-12 were harvested by centrifuging the broth (24h old) at 10,000 rpm for 10 min in a refrigerated centrifuge (4^0C) and known amount of wet cell pellet was placed in oven at 80^0C for overnight and corresponding absorbance of the cell slurry was measured at 600 nm in a spectrophotometer (LABINDIA). The known dried cell weight corresponding to their optical density was recorded and a standard graph was plotted between dry cell weight and A_{600}. The cell mass in terms of dry weight (dcw) was measured from the standard curve.

Chitosan

0.5% (w/v) in sodium acetate buffer (50 mM, pH 5.5). The buffer and the reagents were stored at 4^0C. DNSA is photosensitive so it was stored in dark colored bottles and kept at room temperature.

Chitosanase assay (3, 5-Dinitrosalicylic acid, DNSA method)

Chitosanase catalyses endohydrolysis of β-1, 4 linkages between D-glucosamine residues in a partly acetylated chitosan. Chitosanase activity was determined spectro-photo-metrically by measuring the reducing sugar produced from chitosan by using the modified dinitrosalicylic acid (DNS) method (Miller 1959). The assay mixture (1ml) containing enzyme solution (100µl) and chitosan (900µl) prepared in acetate buffer (0.5mM, pH 5.5). The reaction mixture was incubated at 37^0C for 20 min. The reaction was stopped by keeping the reaction mixture in boiling water bath (100^0C) for 10 min. 1mL DNSA reagent was added to 1mL reaction mixture. The reaction mixture was kept in boiling water-bath for 20 min for color development (Yellow to Red coloration). A set of blank and control was also run. The absorbance of color developed was measured at 540 nm in a spectrophotometer (LAB INDIA). The amount of D-glucosamine was estimated from the standard curve of D-glucosamine plotted between the A_{540} nm and concentration of glucosamine.

Chitosanase activity

The enzyme activity was expressed in terms of units. One unit (U/mL) of chitosanase activity is defined as the amount of enzyme that liberates 1µmol of reducing sugar (D-glucosamine) per min per mL under standard assay conditions (Liaqat *et al*., 2018).

Selection of production media

The previous reported media were tested for the production of chitosanase by bacterial isolate CHTM-12. Initial pH of all media was adjusted to 7.0. All flasks were incubated at 30^0C, 150 rpm for 24h.

TABLE-1 The composition of medium used for the production of chitosanase from bacterial isolate CHTM-12.

Media	Medium composition (%, w/v)	References
M1	Nutrient broth, chitosan 0.5	
M2	Chitosan 0.5, $(NH_4)_2SO_4$ 0.1, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.3	Chen *et al.,* 2010.
M3	Chitosan 0.5, K_2HPO_4 0.01, $MgSO_47H_2O$ 0.05, $FeSO_4$ 0.02, $ZnSO_47H_2O$ 0.1, NaCl 0.5	Misra *et al.,* 2013.
M4	Chitosan 0.5, KH_2PO_4 0.01, $MgSO_4.7H_2O$ 0.05, $FeSO_4.7H_2O$ 0.001, $(NH_4)_2SO_4$ 0.02	Kim and Ji., 2001.
M5	Chitosan 0.5, NaCl 0.5, KH_2PO_4 0.07,$MgSO_4.7H_2O$ 0.05, $FeSO_4.7H_2O$ 0.001, $(NH_4)_2SO_4$ 1.2	Zakaria *et al.,* 2012.
M6	Chitosan 0.5, Yeast extract 0.5, KH_2PO_4 0.1, K_2HPO_4 0.2, $MgSO_4.7H_2O$ 0.07, NaCl 0.5, KCL 0.05, $CaCl_2$ 0.01	Choi *et al.,* 2004.
M7	Chitosan 0.5, glucose 2.0, Yeast extract 0.1, Peptone 1.0, K_2HPO_4 0.1, NaCl 0.1, MgSO4.7H2O 0.5, $(NH_4)_2SO_4$ 0.5, $CaCl_2$ 0.01	Shadia *et al.,* 2014.
M8	Luria broth, Chitosan	

Optimization of physiochemical parameters

Role of inducer: Four different combinations of chitosan (inducer) at concentration 0.5% (w/v) in seed and production medium (M6) were used for studying the effect of inducer for the production of chitosanase as shown in Table-2

TABLE-2 Different combination of chitosan (inducer) in seed and production medium

Seed media	Production media
C+	C+
C+	C-
C-	C+
C-	C-

C+ = Chitosan present; C- = Chitosan absent

Concentration of inducer

Different concentration of chitosan 0.1-1.0% (w/v) was added in M6 medium to determine the best inducer concentration. Production medium was inoculated with 5% (v/v) seed culture and incubated for 24h, growth and chitosanase activity were measured under standard assay conditions.

Optimization of production pH

To investigate the influence of initial pH of the medium on enzyme production by the bacterial isolate CHTM -12, the pH of the selected production medium (M6) was adjusted to various level (5.0-9.0). After 24h of incubation, chitosanase activity was measured under standard assay conditions. The optimum pH obtained in this study was used in the subsequent study.

Optimization of production temperature

To study the impact of incubation temperature on enzyme production and cell growth, the production medium (pH 7.0) was inoculated at various temperatures ranging from 20^0 to 50^0C. Sample was withdrawn after 24h of incubation and assayed enzymatic activity.

Optimization of agitation rate (rpm)

The effect of agitation (rpm) on chitosanase production was investigated by inoculating the production medium with 5% (v/v) seed (24 h age) at different agitation speeds of 100, 150, 200 and 300 rpm at 30^0C for 24h. A flask was also incubated under static condition.

Optimization of inoculum age

To study the effect of age inoculum on production of chitosanase inoculum of varying age (3, 6, 9, 12, 15, 18, 21 and 24h old) were added at 5 % (v/v) level to the flask containing production medium. The fermentations and assays were conducted as describe previously. The optimum age of inoculum obtained in this experiment was used in the subsequent studies.

Optimization of incubation time

To determine the optimum incubation time, production media (pH 7.0) was inoculated with 12h age culture of bacterial isolate CHTM -12 and incubated at 25^0C and 250 rpm for 30h. Sample were withdrawn at regular interval of 2h ad centrifuged at 10,000 rpm to obtain supernatant and assayed for enzyme activity.

RESULT AND DISCUSSION

Morphology of the isolate

Bacterial isolate CHTM-12 grow as round, smooth, yellowish colonies on Luria broth agar (Plate-1). This isolate appeared to be Gram positive, small cocci shaped as observed under microscope (100X) (Plate-1).

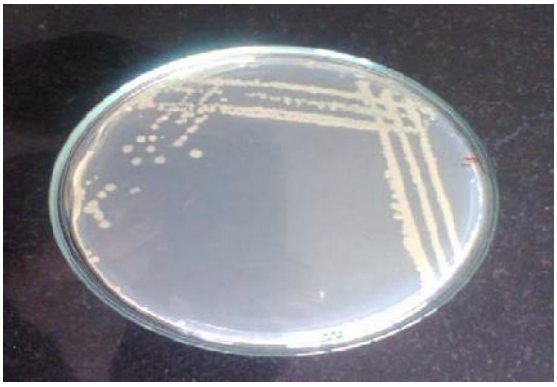

PLATE-1 Growth of bacterial isolates CHTM-12 on Luria Broth agar

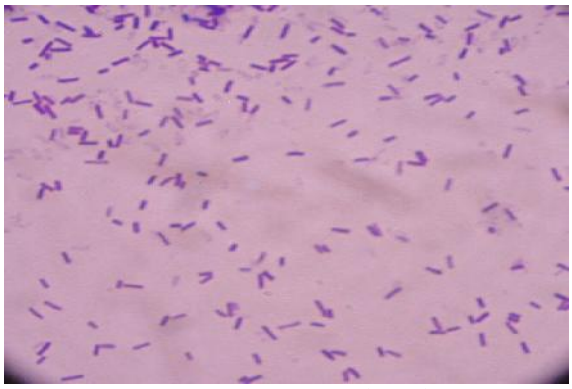

PLATE-2 Microscopic view (100X)

Optimization of production conditions for chitosanase

The optimization of culture conditions for the maximum production of chitosanase by bacterial isolate CHTM-12 was done.

Selection of media for chitosanase production

Among the eight media tested, the maximum growth and activity of chitosanase obtained was 5.6mg/ mLand 0.14 U/mL, respectively, in medium 6 (M6) containing (% w/v) chitosan 0.5, yeast extract 0.5, K_2HPO_4 0.2, Mg SO_4 .$7H_2O$ 0.07, NaCl 0.5, KCl 0.05, $CaCl_2$ 0.02. However, lowest chitosanase activity 0.02 U/mL was detected in medium 3 (Fig-1). (Cheng *et al*., 2012) reported that the medium composition for optimal production of chitosanase was as follows: 0.5 g/L chitosan, 55 g/L glucose, 1 g/L peptone, 2 g/L $(NH_4)^2SO_4$, 8 g/L urea, 1 g/L NH 4Cl, 3 g/L KH_2PO_4, 1 g/L $FeSO_4 \cdot 7H_2O$, 2 g/L $ZnSO_4 \cdot 7H_2O$, 2 g/L $CaCl_2 \cdot 6H_2O$, 2 g/L $MnSO_4 \cdot H_2O$ and 2 g/L NaCl. The chitosanase production was found to increase from 11.56 U/mL to 39.87 U/mL.(Zhang *et al*., 2013) reported that the maximum chitosanase activity (21.85 U mL^{-1}) was achieved under the optimized conditions $(NH_4)^2$ SO_4 5.05 g L–1, shrimp shell powder 23.40 g L–1, $MgSO_4 \cdot 7H_2$ O 0.88 g L^{-1}.

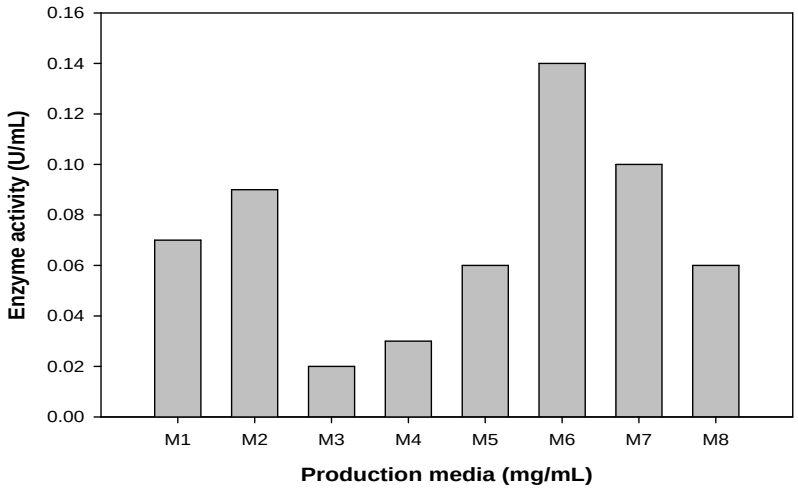

FIGURE 1 Selection of media for chitosanase production by isolate CHTM-12.

Optimization of physiochemical parameters

Role of inducer (Chitosan)

It has been reported that inducer plays an important role in the production of chitosanase. Chitosan as inducer was found to increase the cell mass as well as enzyme activity (Table-3).Maximum growth (5.8 mg/mL) and maximum activity of chitosanase (0.15 U/mL) was observed, when both the seed and production medium was supplemented with 0.5% (w/v) inducer (chitosan). Keeping these results in view, Chitosan containing seed and production media were used throughout in the present study. Moreover, all the previous reported media for chitosanase production contained chitosan as inducer.

TABLE 3 Role of inducer on chitosanase activity

S. No.	Inducer		Enzyme activity (U/mL)	Cell mass (mg/mL)
	seed	Production		
1	+	+	0.15	5.8
2	+	-	0.08	2.09
3	-	+	0.02	1.87
4	-	-	0.09	3.33

+ = with chitosan, - = without chitosan

Optimization of inducer concentration

In order to find out optimum inducer concentration different concentration of chitosan (0.1-1.0%) were added to the production medium. Maximum activity of chitosanase was observed in the production medium containing 0.7% chitosan (0.185 U/mL). As the concentration in the medium increased further from 0.7% (w/v) enzyme activity decreased (Fig- 2). Most of the chitosanases reported so far have been inducible. It was showed that in *Acinetobacter* sp. chitosanase production was maximum in basal media containing chitosan which acts as inducer to enhance its activity (Zhu *et al.*, 2002). Zhu *et al.*, (2007) reported that addition of 0.5% (w/v) chitosan had the best inducer effect for production of chitosanase by *Gongronella* sp. Dubeau *et al.*, (2013) reported that by genetic regulation of a heterologous gene (csn 106) in *Streptomyces lividans*, substantial level of chitosanase could be produced in the absence of chitosan, using inexpensive medium components.

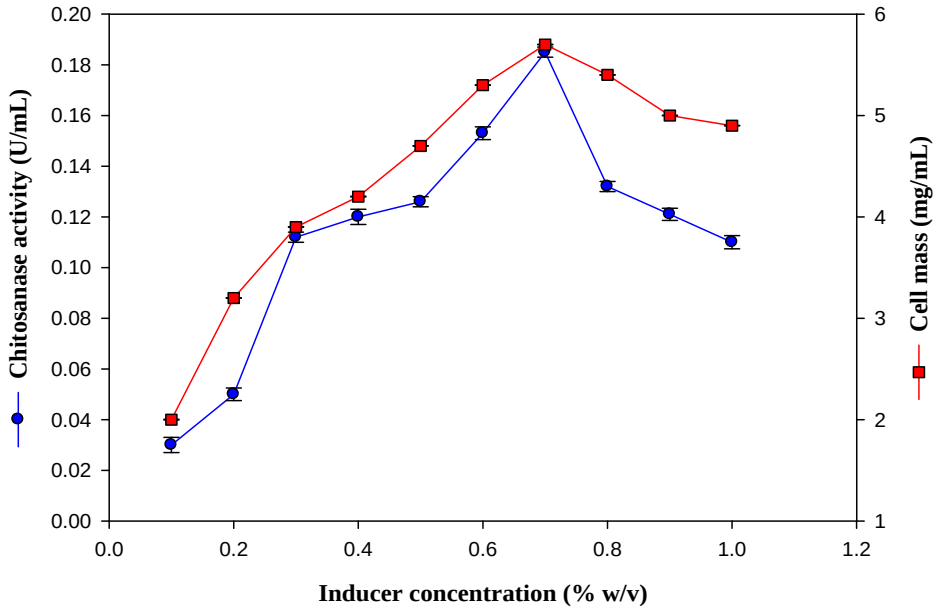

FIGURE 2 Role of inducer (chitosan) on the production of chitosanase by CHTM-12

Effect of medium pH on the production of chitosanase by CHTM-12

Enzyme production by bacteria is strongly influenced by the pH of growth medium. In the present study, pH of production media was varied from 5.0 to 9.0. Maximum biomass (6.6 mg/mL) and chitosanase production (0.190 U/mL) was obtained in production medium with pH 7.0 (Fig- 3) and on further increasing the initial pH of the production medium, enzyme activity decreased. The final pH of the fermentation broth was found to be increased. It was also evident from the result that the chitosanase production and cell growth was greatly affected at highly acidic conditions for the production medium.

Kim and Senevirathne (2011) reported that in *Bacillus pumilus*, maximum chitosanase production was achieved at pH 5.6. However, *Aspergillus fumigatus* showed maximum chitosanase production at pH 4.8 (Zhang and Zhang, 2013). Further, the *Streptomyces cyanogriseus* showed maximum production of chitosanase at pH 5.0 and enzyme was stable- in the pH range 3.0-8.0 (EI-Sherbiny, 2007).

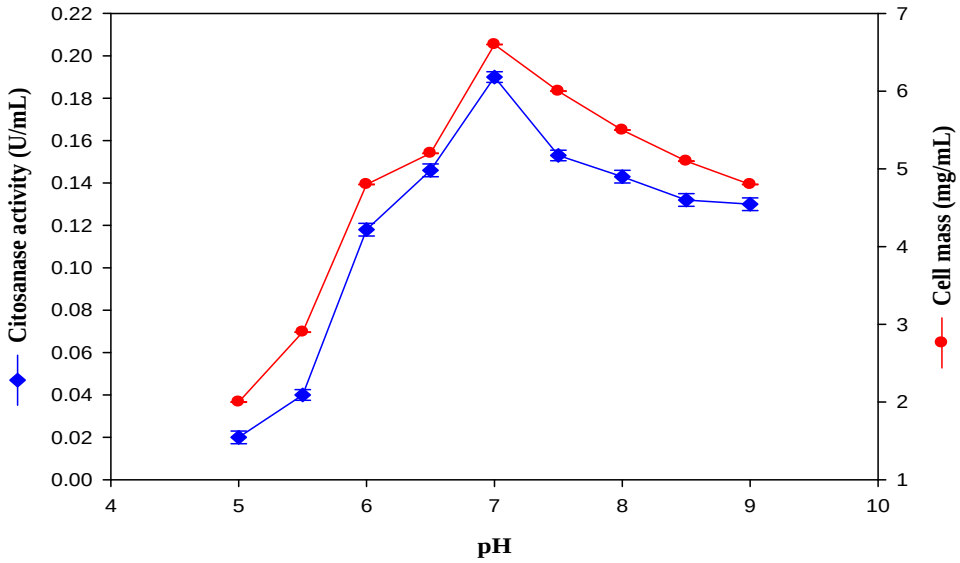

FIGURE 3: Effect of medium pH on the production of chitosanase by CHTM-12.

Effect of incubation temperature on the production of chitosanase by CHTM-12

The chitosanase production by bacterial isolate CHTM -12 was studied by incubating it at different temperature ranging from 20-50^{0}C using M6 production medium (pH 7.0) for 24h at 150 rpm . The bacterium showed narrow range of temperature for growth and enzyme production (Fig- 4). Result revealed that the maximum biomass (6.8 mg/mL) was observed at 30^{0}C and chitosanase production (0.195 U/mL) was also observed at 30^{0}C. (Kim *et al.*, 2004) found 30^{0}C as optimum temperature for the production of chitosanase enzyme of *Bacillus* sp. MET 1299. *Bacillus thuringiensis* ZJOU-010 exhibit the optimum temperature 37^{0}C for the production of chitosanase enzyme (Chen *et al.*, 2010).

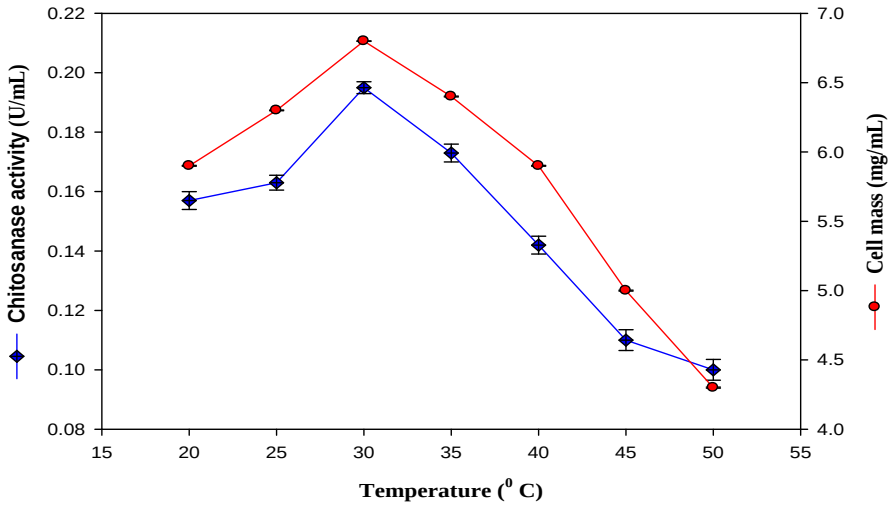

FIGURE 4: Effect of incubation temperature for production of chitosanase by CHTM-12

Effect of agitation rate (rpm) on production of chitosanase.

An experiment was conducted to determine the optimum agitation rate for the production of bacterial isolate CHTM-12 cells with high chitosanase activity. The production medium was inoculated with 24h old inoculum (5%v/v) at 30^{0}C in temperature controlled orbital shaker at varying rotation speed of 50-250 rpm. Maximum production of chitosanase enzyme was observed at 200 rpm (0.230 U/mL) with 6.9mg/mL of growth (Fig-5). However, at static the enzyme production was very poor and chitosanase activity was negligible. Further, *Paenibacillus ehimensis* showed maximum production of chitosanase at 120 rpm (Pagnoncelli *et al.*, 2010). However Zhang *et al.*, (2014) reported the agitation speed of 150 rpm at 30^{0}C as optimum for chitosanase production by *Gongronella* sp.

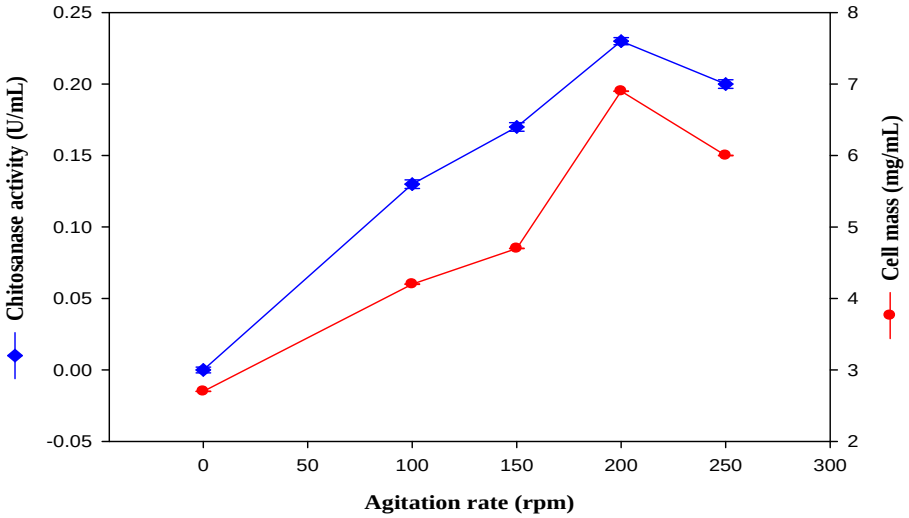

FIGURE 5 Role of agitation rate (rpm) on the production of chitosanase by CHTM-12.

Effect of inoculum age

Varying concentration of inoculum (3-24hrs.age) at 5% (v/v) concentration was used to inoculate the production medium and incubated at 25^{0}C and 250 rpm for 24h. Maximum production of chitosanase enzyme was observed at 12h old seed (0.300 U/mL) with 7.0 mg/mL of growth (Fig- 6). However, with further increase in the age of seed, the enzyme activity remains mere or less constant. Chasanah *et al*., (2011) reported the maximum chitosanase production by *Aeromonas* media KLU 11.16 with 15h old seed at 37^{0}C, 100 rpm.

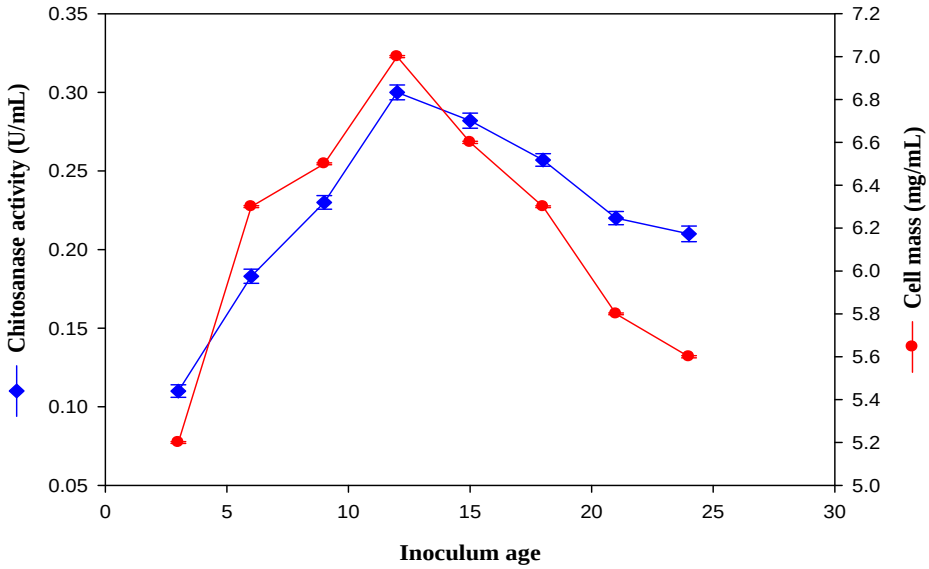

FIGURE 6: Role of inoculum age on the production of chitosanase by CHTM-12

Optimization of production time

The production medium was inoculated with 5% (v/v), 12 h old seed and flasks were incubated with 20^0 C (200 rpm). Sample were withdrawn at regular interval of 2h and assayed for chitosanase activity. The chitosanase production increased exponentially up to 24h and then remained almost constant thereafter (Fig-7). Maximum production of extracellular chitosanase was 0.389 U/mL and 7.3mg/mL biomass was obtained by bacterial isolate CHTM -12 at incubation time of 24h. Further, *Bacillus cereus* showed maximum chitosanase production at 30^{0}C after 48h of incubation (Chen *et al.*, 2006). However, Zakariya *et al*., (2012) reported that *Bacillus cereus* L1 produce chitosanase at incubation time of 24h.

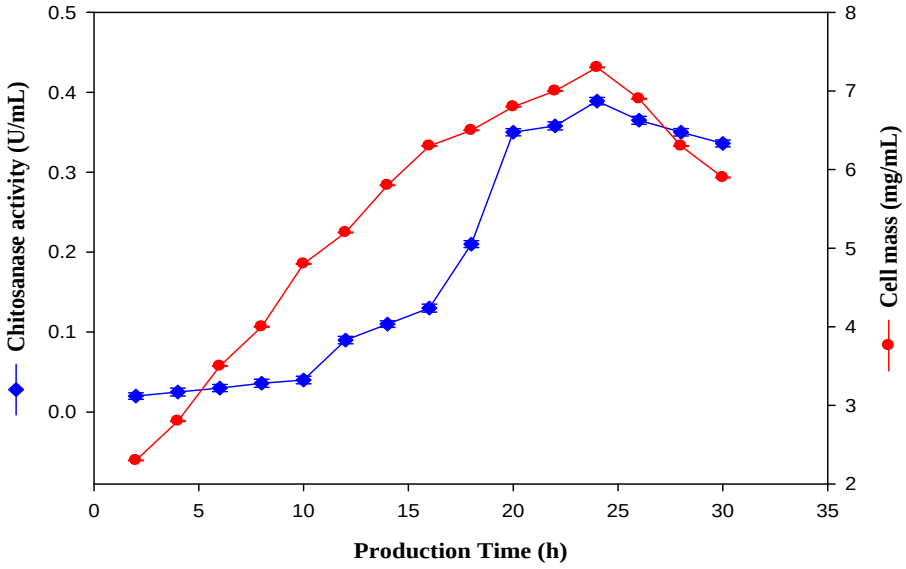

FIGURE 7: Effect of production time on the production of chitosanase by CHTM-12.

CONCLUSION

Chitosanase is the key enzyme required for the preparation of biologically active COS from chitosan. Our results demonstrate that this is a valuable enzyme for the commercial productions of the Chitooligosaccharides. Bioactive Chitooligosaccharides has significant applications especially in the food and biomedical industries. The main goal of this study was to optimize Chitosanase production by the bacterial isolate CHTM-12. The bacterial isolate CHTM-12 was found to be efficient producer of chitosanase in M6 medium containing (%, w/v) chitosan 0.5, yeast extract 0.5, KH_2PO_4 0.2, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.07, NaCl 0.05, KCL 0.05, $CaCl_2$ 0.01. The addition of inducer (chitosan) was found essential in both seed as well as production medium. The enhancement of enzyme activity was achieved in medium containing 0.7% (w/v) chitosan. Out of different physiochemical parameters optimized, initial medium pH 7.0, incubation temperature 30^0C , agitation rate 200 rpm , inoculum age 12h , incubation time 24 h were found to be optimum for production of bacterial isolate CHTM-12 cells with higher chitosanase activity.

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