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Isolation of a Novel *Bacillus amyloliquefaciens* KJ 782424 with Chitosanase Activity

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Abstract: A novel *Bacillus amyloliquefaciens* KJ 782424 with chitosanase activity was isolated from forest soil on the basis of zone of hydrolysis of chitosan. The identification of the isolate was done on the basis of molecular characterization using 16S- rDNA sequence analysis. This isolated *B. amyloliquefaciens* produced the highest amount of chitosanase on LB medium supplemented with 0.7 % (w/v) chitosan. Maximum chitosanase production (6.49 IU) was achieved at initial medium pH of 8.0 at incubation temperature 30°C after 22 h of incubation. This is the first report which shows that *B. amyloliquefaciens* also has the ability to produce chitosanase enzyme.

Key words: *Bacillus amyloliquefaciens*, chitosan, chitosanase, isolation.

Introduction

Chitosanases (EC. 3.2.1.132) are hydrolytic enzymes which act on chitosan to produce chitosan oligosaccharides. Chitosan is the low acetyl substituted forms of chitin and comprised mainly of glucosamine. Chitosan also occurs naturally in the cell walls of fungi (*Zygomycetes*), in algae [10] (*Chlorella* sp.) and in exoskeleton of insects [2]. Chitosanases have been screened, isolated, purified and characterized from different sources, mainly from bacteria, fungi and plants, where they play an important role in nutrition and defense. Chitosanases are further divided into three subclasses according to their hydrolytic activity of the β-glycosidic linkages in partially *N*-acetylated chitosan molecules; subclass I chitosanases hydrolyse the GlcNAc (N-acetyl-D-glucosamine)-GlcN (D-glucosamine) and the GlcN-GlcN bonds, subclass II chitosnases split only the GlcN-GlcN bonds, subclass III chitosanases split the GlcN-GlcNAc and the GlcN-GlcN bonds. Chitosanases have been classified into five glycosidehydrolase families; GH-5, GH-8, GH-46, GH-75 and GH-80. GH-46 family chitosanases, especially those from *Bacillus* and *Streptomyces*, has been studied extensively in terms of their structure, catalysis and enzymatic mechanisms [1,4,7,8,9,18]. Chitosan and its oligosaccharides possess multiple functional properties and attracted considerable interest due to their biological activities and potential applications in the pharmaceutical, food, agricultural and environmental industries. Their biological activities include antimicrobial, hypocholesterolemic, immunostimulating [15], antioxidant, anti-inflammatory, antitumor and anticancer effects [16]. Their effects are correlated with their structures and physicochemical properties [13,17,19]. Fungal chitosanases utilizes sugar as their major carbon source and break it down to various chitosan oligomers/oligosaccharides. Recently a fungal chitosanase was induced from a squid pen powder (SPP)-containing *Penicillium janthinellum* D4 medium [12]. The success in using chitosanase for diverse applications depends on the production of highly active enzyme at a reasonable cost. The

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present work focused on isolation of a novel chitosanase producer to fulfill the diverse applications requirement of this enzyme.

Materials and methods

Chemicals

All the chemicals used in the present study were of analytical grade. Chitosan from shrimp shell (>75 % deacetylated) was procured from Hi Media, India.

Isolation of potential chitosanase producing microorganisms

Chitosanase producing microorganisms were isolated from the samples collected from forest soils of different districts of Himachal Pradesh, India. The soil samples were serially diluted (upto10^8 dilutions) in the sterile physiological saline and 100 µL of each dilution was spreaded on the semi-solid medium (pH 7.0) containing (%, w/v): chitosan powder 1.0, Na_2HPO_4 0.13, KH_2PO_4 0.3, NaCl 0.05, NH_4Cl 0.1, $MgSO_4$ 0.024 and agar 2.0. The pure culture of the positive isolates were prepared and used for subsequent studies.

Screening and selection of potent isolate

Screening and selection of chitosanase producing microorganisms was done on the basis of growth in the semi-solid medium and by formation of clear zone of chitosan hydrolysis around the colonies. The positive isolates were grown in liquid medium (pH 7.0) containing (%, w/v): chitosan powder 1.0, Na_2HPO_4 0.13, KH_2PO_4 0.3, NaCl 0.05, NH_4Cl 0.1, $MgSO_4$ 0.024 at 30°C for 48 h in an orbital shaker at 150 rpm. The centrifugation (4°C) was done at 10,000g for 20 min to remove cell mass and the supernatant was used as crude enzyme. The chitosanase activity in the supernatant was measured spectrophotometrically.

Preparation of substrates for enzyme assays

Chitosan substrate (0.5 %, w/v) was prepared by suspending 0.5 g of powder chitosan in 50 mL of distilled water; it was dissolved while being stirred with an addition of 5 mL of 1.0% (v/v) acetic acid. The solution was made up to 100 mL with distilled water.

Chitosanase assay and protein estimation

Chitosanase activity was determined spectrophotometrically by measuring the reducing sugar released from glucosamine [11]. The reaction mixture (1 mL) contained enzyme solution (50 µL) and chitosan (950 µL) prepared in sodium acetate buffer (50 mM, pH 5.5). The reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by keeping the reaction mixture in boiling water bath (100°C) for 10 min. Now 1 mL DNSA (dinitrosalacylic acid) reagent was added to reaction mixture. The reaction mixture was kept in boiling water bath for 20 min for color development (Yellow to Red). A set of blank and control was also run. The absorbance of color developed was measured at 540 nm in a spectrophotometer (LABINDIA). One IU of chitosanase activity was defined as the amount of enzyme required to liberate one µmol of glucosamine ml^{-1} min^{-1} under standard assay conditions. The concentration of protein was estimated by Bradford method [5].

Selection of medium for maximum production of chitosanase by *B. amyloliquefaciens*

Since the chitosanase activity of *B. amyloliquefaciens* was found to be extracellular in nature, medium producing high amount of chitosanase activity in fermentation broth was needed to be selected and optimized. A total of ten previously reported media (pH 7.0) were tested (Table 1) for the production of chitosanase by *B. amyloliquefaciens* by using 2 %, (v/v) of 24 h age seed. The culture of *B. amyloliquefaciens* was incubated at 30°C for 24 h (150 rpm). The cell mass and chitosanase activity were evaluated in each case.

Optimization of medium pH for maximum production of cell mass and chitosanase by *B. amyloliquefaciens*

To find out the optimum pH for the maximum growth and production of chitosanase by *B. amyloliquefaciens* M-6 medium supplemented with 0.5 % (w/v) chitosan with different initial pH (4.0-8.0) were prepared and inoculated with 2.0 % (v/v) 24 h old seed. The cultivation was carried out as described previously.

Optimization of incubation temperature for maximum growth and chitosanase production by *B. amyloliquefaciens*

The M-6 production medium (pH 8.0) containing chitosan 0.5 % (w/v) was inoculated with *B. amyloliquefaciens* seed culture (2.0 %, v/v) and incubated at different temperatures (25 to 55°C) for 24 h to find out the optimum temperature for efficient cell mass and chitosanase production by *B. amyloliquefaciens*.

Optimization of incubation time for maximum production of chitosanase by *B. amyloliquefaciens*

Production medium was inoculated with 2.0 % (v/v) 6 h old seed culture and incubated at 30°C (150 rpm). Samples were taken at regular interval of 2 h and analyzed for growth and chitosanase activity till the maximum growth and production of chitosanase was observed. Final pH of the fermentation broth was also noted.

Results and discussion

Screening for chitosanase-producing isolate and its identification

Various microbial strains were isolated from different soil samples through enrichment culture using chitosan as the sole carbon source. It was observed that 60 isolates were capable of utilizing chitosan and formed transparent zones around the colonies and they were marked for further evaluation for estimation of chitosanase activity by DNS method. Among these, the most potent isolate CHT-32 showed the highest chitosanase activity and was observed as Gram positive, rod shaped with catalase positive capable of growing in aerobic environments. Based on 16S rDNA, the bacteria was identified as *Bacillus amyloliquefaciens* and a phylogenetic tree was constructed by the neighbor-joining method [14] (Fig. 1).

Selection of the medium for the production of *B. amyloliquefaciens* with high chitosanase activity

The isolated *B. amyloliquefaciens* was grown on ten different media reported for the production of chitosanase. The results suggested that the

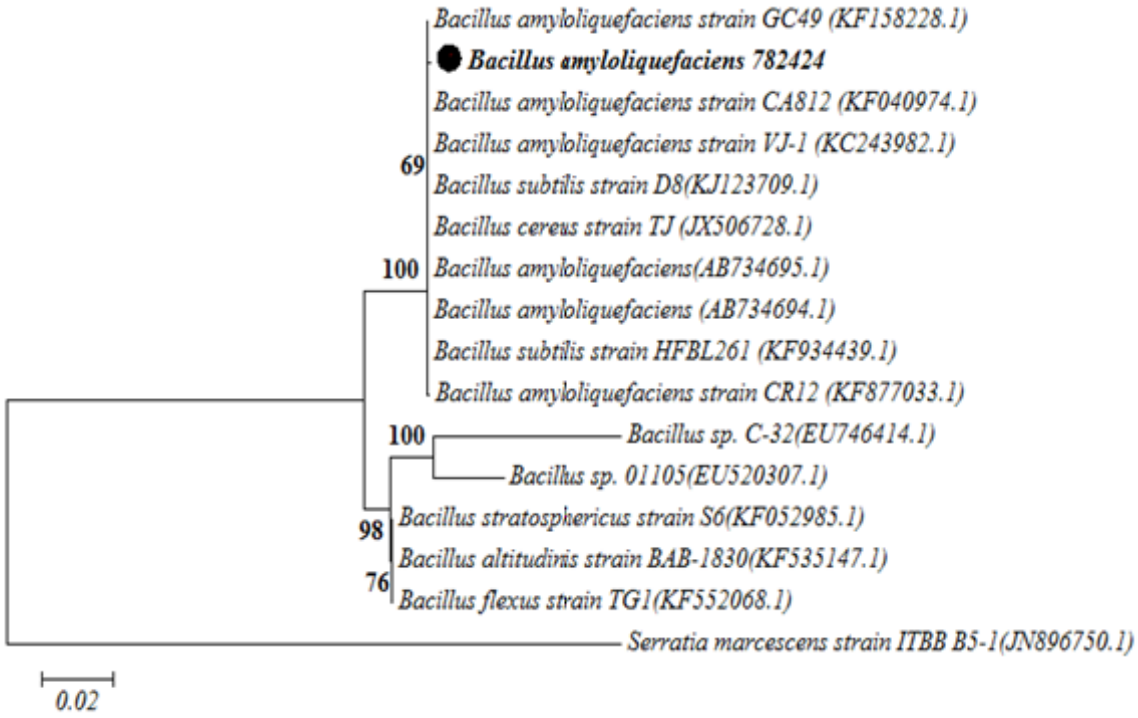

Fig. 1. Phylogenetic tree of CHT-32 isolate. Bootstrap values are indicated at the branch points. The bar indicates a branch length equivalent to 0.02 changes per amino acid. Phylogenetic trees were constructed by the neighbor-joining method[14], using MEGA software, version 5.2.

medium M-6 (Luria Broth) supplemented with 0.5 % (w/v) chitosan was most suitable for the production of chitosanase (Table 1).

Optimization of medium pH for the maximum production of cell mass and chitosanase by *B. amyloliquefaciens*

The variation in medium pH greatly affects the uptake of nutrient by the cells and this phenomenon makes it mandatory to optimize the pH of the medium. The Luria Broth supplemented with 0.5% (w/v) chitosan was used. The maximum growth (4.98 mg/mL) and chitosanase production (4.78 IU) was observed at pH 8.0 (Fig.2). However, with further increase in the initial pH of the production medium, the chitosanase production was found to decrease gradually. It has been reported that the enzyme exhibited greatest activity at an optimum pH of 8.0 [3] and the optimal pH of the recombinant chitosanase obtained from *Bacillus* sp. was observed to be 5.0 [20]. In another study, the chitosanase production from *Penicillium janthinellum* was found to be maximum at pH 7.0-9.0 [12].

Effect of incubation temperature on the production of chitosanase.

The chitosanase production by *B. amyloliquefaciens* was studied by incubating it at different temperature ranging from 25-55°C for

Table1. Selection of medium for maximum production of the chitosanase by *B. amyloliquefaciens*

Medium No.	Composition (%, w/v)	Chitosanase activity (IU)	Cell mass (mg/mL)
M-1	Chitosan 0.5,Peptone 0.5, Yeast extract 0.5, K_2HPO_4 0.2, KCl 0.05,$MgSO_4$ 0.05	3.03	2.9
M-2	Chitosan 0.5, Peptone0.5, Yeast extract 0.5,Glucose 0.1, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.07	2.7	2.9
M-3	Chitosan 0.5, $NaNO_3$ 0.3, K_2HPO_4 0.2, $MgSO_4$ 0.01,KCl 0.5,$FeSO_4$ 0.005	0.96	0.75
M-4	Nutrient broth 1.3,Chitosan 0.5	2.91	2.4
M-5	Chitosan 0.5, K_2HPO_4 0.2, KH_2PO_4 0.2, NaCl 0.5,$(NH_4)_2SO_4$ 0.3, $MgSO_4.7H_2O$ 0.007	0.95	0.74
M-6	Luria broth 2.0, Chitosan 0.5	3.33	3.7
M-7	Chitosan 0.5,Yeast extract 0.5, Peotone0.5, K_2HPO_4, KCl 0.5, $NaNO_2$ 0.2, $MgSO_4.7H_2O$ 0.07, $FeSO_4.7H_2O$ 0.005	2.01	2.5
M-8	Glucose 0.1, Peptone 0.1, KH_2PO_4 0.2 $MgSO_4$ 0.02, Chitosan 0.5	1.05	2.2
M-9	Chitosan 0.5,Yeast extract 0.5, K_2HPO_4 0.2, KH_2PO_4 0.1, $MgSO_4$ $7H_2O$ 0.07, NaCl 0.05, KCl 0.05, $CaCl_2$ 0.01	0.82	0.74
M-10	Na_2HPO_4 0.13, KH_2PO_4 0.3, NaCl 0.05, NH_4Cl 0.1, $MgSO_4$ 0.024, $CaCl_2$ 0.001, Chitosan 0.5	1.58	2.4

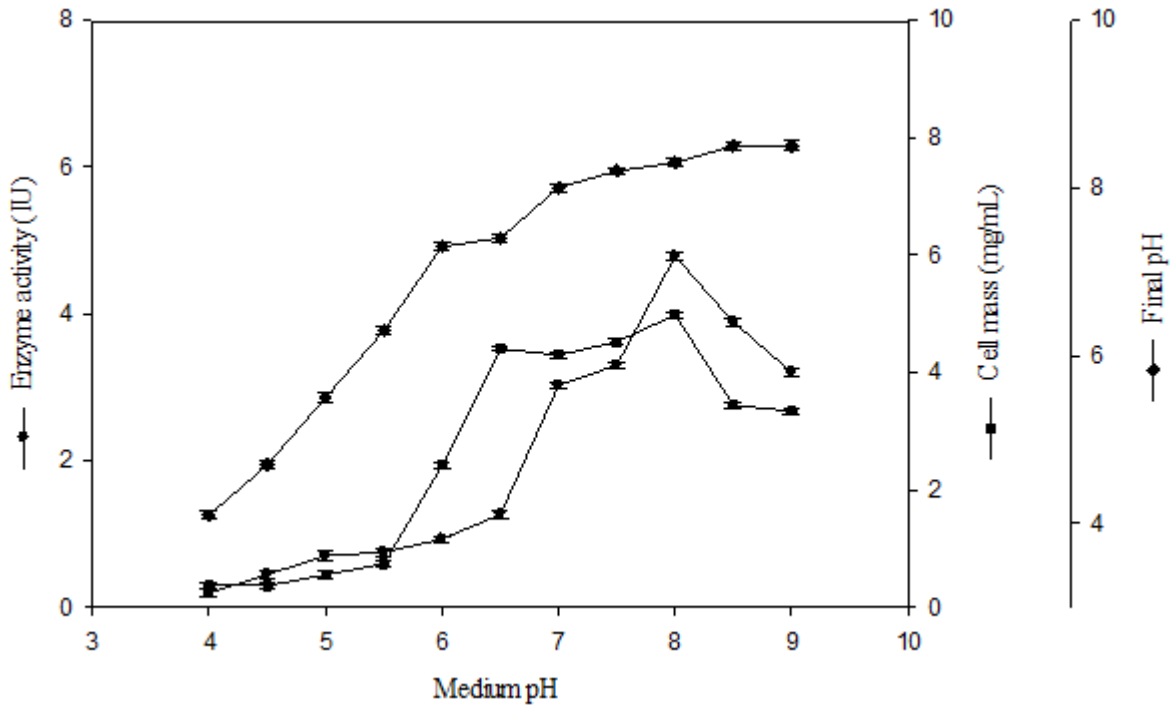

Fig.2. Effect of pH on chitosanase production

24 h at 150 rpm. The bacterium showed narrow range of temperature for growth and enzyme production. Maximum chitosanase production was observed at 30°C (5.80 IU) as shown in Fig.3**.** Above and below this temperature, both the biomass and chitosanase production was decreased. The optimum temperature for production of chitosanase by *Acinetobacter* sp. C-17 was reported to be 30°C [21].

Optimization of incubation time for the maximum production of chitosanase by *B. amyloliquefaciens*

The maximum cell mass and chitosanase production were found to be 7.11 mg/mL and 6.49 IU respectively at 22 h of incubation (Fig.4). In case of chitosanase reported from *Bacillus licheniformis* MB-2 chitosanase activity was detected from 12 h of incubation and increased continuously (1.49 U/mg) up to 60 h of fermentation [6].

Conclusion

Present study establishes the production of an extracellular chitosanase from a novel isolate *B. amyloliquefaciens*. This novel chitosanase hydrolysed the chitosan with high efficiency and provide an alternative to the existing enzyme for production of chitosan oligosaccharides (COS). Since, the bioactive COS has significant applications *viz.* antimicrobial, antioxidant activity, lowering of blood cholesterol and high blood pressure, protective effects against infections, controlling arthritis and enhancing antitumor properties. Moreover, they have growing demand in food and biomedical sectors also. Hence, a bioprocess developed around this novel isolate will prove beneficial with respect to one or another application. This chitosanase can also be used for the control of phyto pathogens.

Acknowledgements

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Conflict of interest

The authors confirm that the contents of this article have no conflicts of interest.

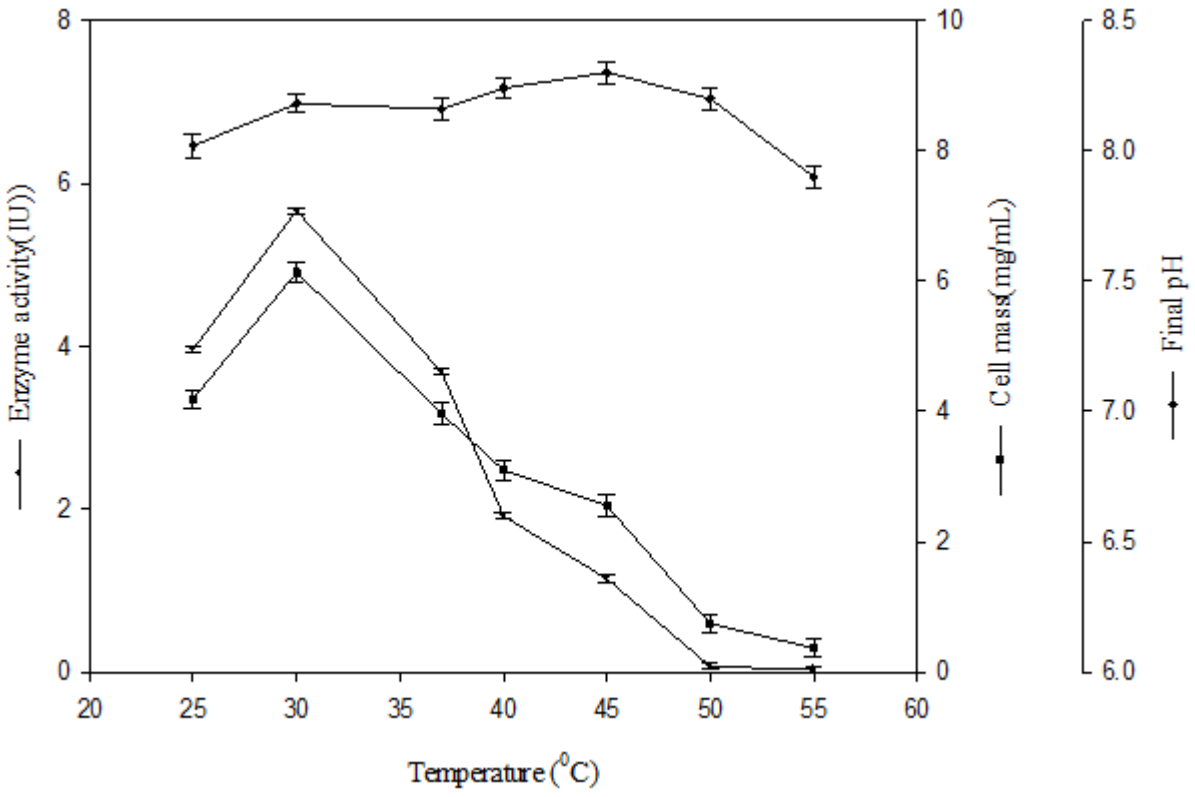

Fig.3. Effect of fermentation temperature on chitosanase production

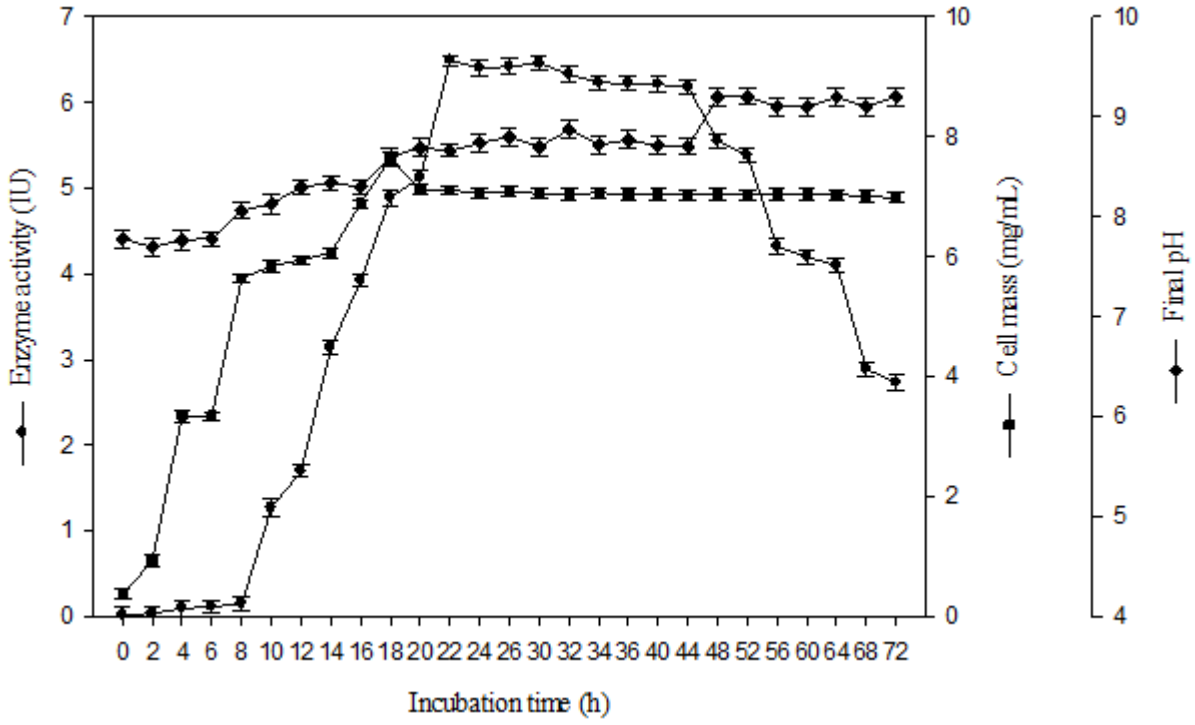

Fig.4. Course of fermentation of *B. amyloliquefaciens*

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