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Full Length Research Paper

Optimization of culture conditions for thermostable chitinase production by *Paenibacillus* sp. D1

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A hyper-chitinase producing isolate *Paenibacillus* sp. D1 was obtained from common effluent treatment plant of seafood industries at Veraval (Gujarat, India). The isolate exhibited chitinase production over a wide temperature (25 - 45°C) and pH (6 - 9) range with maxima at 30°C in medium with initial pH 7.0. The crude chitinase had activity in broad pH (4 - 10) and temperature (30 - 60°C) range with optima at pH 5.0 and 50°C, respectively. The enzyme was highly thermostable with $t_{1/2}$ of 36 to 60 h at 40 and 45°C. Crab shell chitin, urea and K_2HPO_4 were identified as best carbon, nitrogen and phosphorous sources influencing chitinase production by *Paenibacillus* sp. D1. Addition of tween 80 and $FeCl_3$ enhanced the chitinase production by 1.44 and 1.33 fold, respectively. Identification of essential nutrients affecting chitinase production by *Paenibacillus* sp. D1 would help to formulate a suitable medium for its production. Moreover, low cost chitin from crab shells can be used as carbon source for thermostable chitinase production by the isolate for industrial and agricultural applications.

Key words: *Paenibacillus*, optimization, thermostable, chitinase.

INTRODUCTION

Chitin is the second most abundant biopolymer on the earth, with an annual recovery of 1 - 100 billion metric tonnes mainly as chitinous waste by processing of marine invertebrates (Rattanakit et al., 2002). Although, a potential renewable resource most of the chitinous wastes are disposed through ocean dumping, incineration and land filling, owing to lack of cheap and commercially feasible methods for its processing leading to wastage of natural resource, economic loss and problem of environmental pollution (Carroad and Tom, 1978). Since, in India alone 60,000 - 80,000 tonnes of chitinous wastes are generated it is necessary to design alternative method for disposal and utilization of this waste (Suresh and Chandrasekaran, 1998). Disposal by microbial degradation of chitin offers best solution to the problem leading to recycling of nutrients in the environment along with generation of useful products viz. chitinases, chitooligosaccharides, N-acetyl glucosamine and single cell proteins (Gohel et al., 2007; Pichyangkura et al., 2002; Vyas and Deshpande, 1991).

Chitinases are extracellular inducible enzymes that catalyse the first step in chitin digestion, hydrolysis of β-1, 4 linkages between the NAG molecules. Recently chitinases have received increased attention due to their wide range of biotechnological applications, especially in agriculture for biocontrol of fungal phytopathogens (Mathivanan et al., 1998; Maisuria et al., 2008) and harmful insects as fugal cell wall and cuticle of insects contain chitin as an essential component (Mendonsa et al., 1996; Pinto et al., 1997). Moreover, they offer a potential additive or alternative to use of chemical fungicides (Bhushan and Hoondal, 1999; Chao-Ying and Chien-Jui, 2008). However, such applications require chitinase to be produced in large quantities which in turn require optimization of nutritive and physical parameter like pH and temperature for its production by selected isolate. Chitinase are found in a variety of organisms (Bhushan, 2000). *Paenibacillus* spp. is widely distributed in nature and has been reported for its biocontrol activities mainly attributed to production of cell wall degrading enzymes (β-1,3-glucanases, cellulases, chitinases and proteases (Budi et al., 2000). Although, chitinase production has been reported in *Paenibacillus* species, expect for a few reports (Patel et al., 2007),

there is no much information on optimization of culture conditions for its production. Thus an extensive search for factors effecting chitinase production by *Paenibacillus* species is required.

Several statistical and non statistical methods are available for optimization of medium constituents (Montgomery, 2002). Plackett-Burman and response surface methodology are the most widely used statistical approaches. Although optimizing the parameters by statistical method reduces the time and expense, selection of media components for use in Plackett Burman design is either decided by borrowing or by random selection. Borrowing involves literature survey to select components used by other workers for growth and production of desired product from same genus being analysed. The problem with this method is that there are not too many options if the organism under analysis has not been previously studied for production of desired product (Panda et al., 2007).

Thus, before statistical optimization of medium for production of desired product from a new source bacterium it is essential to screen large number of possible medium constituents. Component replacing is the most commonly used method for screening number of carbon, nitrogen and phosphorous sources (Jatinder et al., 2006) while the effect of surfactants, metal ion, antibiotics etc is checked by one factor at time approach (Patidar et al., 2005). This approach can generate information on medium constituents for desired product form organism under study and can also identify new components affecting its production.

In this paper we report effect of various physical and nutritive parameters on chitinase production by *Paenibacillus* sp. D1.

MATERIALS AND METHODS

Isolation and screening of chitinase producing bacteria

Chitinase producing bacteria were isolated from various intertidal zones, fish drying yards and common effluent treatment plants of sea food processing industries along the coast of Gujarat including Bhavnagar (Gogah, Talaja, Alang, etc.), Veraval, Diu and Porbandar. Isolates were screened on the bases of clearance zone: colony size (CZ/CS) ration on chitin agar plates containing fluorescent dye calcofluor white M2R (Vaidya et al., 2003). Isolates with significant zone of clearance were further selected for chitinase production in medium containing (g/l): chitin, 5.0; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.3; and KH_2PO_4, 1.36, pH 7.2 at 30°C under shaking conditions (180 rpm) for 72 h (Monreal and Resse, 1969).

Enzyme production and assay

The culture was grown in above mentioned minimal medium at 30°C for 72 h under shaking conditions (180 rpm). Chitinolytic activity was estimated as described by Vyas and Deshpande (1989). One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 µmole of *N*-acetyl-D-glucosamine equivalent at 50°C per hour.

Effect of pH and temperature on chitinase production by *Paenibacillus* sp. D1

Effect of initial pH on chitinase production by *Paenibacillus* sp. D1 was studied by varying the initial pH of medium from 6.0 - 9.0. Similarly, effect of temperature on chitinase production was studied by growing the culture at different temperatures varying from 30 - 45°C. Samples were collected at interval of 12 h to determine the chitinase activity.

Effect of pH and temperature on chitinase activity

Chitinase activity was measured at different pH values by the standard assay method using acid swollen chitin as the substrate. The pH of reaction mixtures was varied using the 50 mM buffers (pH 3.0, sodium citrate and citrate phosphate; pH 4.0, sodium citrate and sodium acetate; pH 5.0, sodium acetate and sodium succinate; pH 6.0, sodium phosphate and maleate; pH 7.0 sodium phosphate, Tris-maleate and Tris-Cl; pH 8.0, sodium phosphate and Tris-Cl; pH 9.0 Tris-Cl and glycine NaOH; pH 10.0, glycine NaOH and carbonate-biocarbonate). The optimum temperature for enzyme activity was determined by incubating the reaction mixture at different temperatures like 25, 30, 35, 40, 45, 50, 55 and 60°C and assaying the enzyme activity.

The crude chitinase was incubated with 50 mM sodium acetate buffer pH 5.0 at different temperatures 30, 40 and 45°C for 168 hr and chitinolytic activity was determined at 50°C with intervals of 12 h. The residual activity was expressed as percentage of the initial activity.

Screening of essential medium components

Sixty different possible media constituent including 13 carbon sources, 13 nitrogen sources, 8 phosphate sources, 10 surfactants and 16 different metal ions were screened for their effect on chitinase production by *Paenibacillus* sp. D1. Effect of different carbon sources was checked by replacing chitin in the basal media with different chitin sources (equivalent weight) or 5 g/l of different test sugars or 10mM of test sugars (with chitin). Nitrogen and phosphorus sources were screened by replacing the corresponding source in the basal medium in equimolar concentrations (1.0 g/l for yeast extract and peptone) with respect to N or PO^{-}_{4}. Surfactants and metal ions were screened by supplementing the media with the test component. The concentration of surfactants used while screening was 0.1% while the concentration of metal ions used were 5mM for macronutrients and 1mM for micronutrients.

RESULTS AND DISCUSSION

Isolation and screening of chitinase producing bacteria

Total 140 chitinolytic bacteria isolated from various habitats were screened on the bases of CZ/CS ratio. These isolates were further analysed for chitinase production in liquid medium. Isolate D1 was found to be the best chitinase producer and was selected for rest of the studies. The isolate was identified as *Paenibacillus* sp. D1 by 16S rDNA sequence analysis the NCBI GeneBank accession no. for which was given as DQ908925.

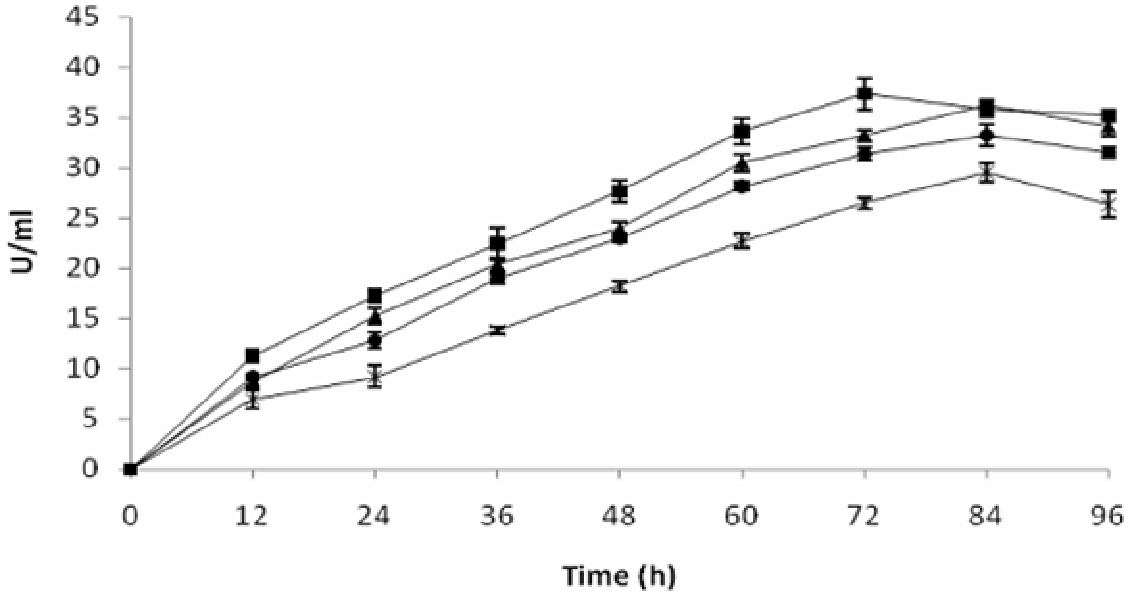

Figure 1. Effect of initial pH of the medium on chitinase production by *Paenibacillus* sp. D1. Symbols circle, square, triangle and cross represent chitinase activity in the medium with initial pH 6.0, 7.0, 8.0 and 9.0, respectively.

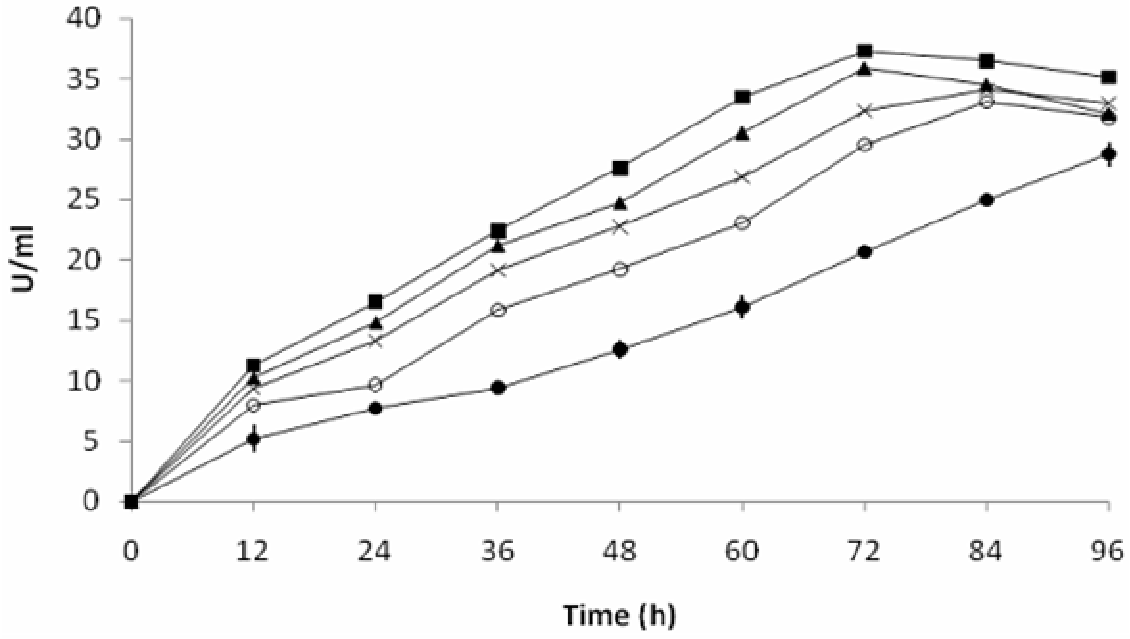

Figure 2. Effect of temperature on chitinase production by *Paenibacillus* sp. D1. Symbols solid circle, square, triangle, cross and empty circle represent chitinase activity at temperatures 25° C, 30° C , 37° C , 40° C and 45° C, respectively

Effect of pH and temperature on chitinase production and activity

Maximum chitinase production by *Paenibacillus* sp. D1 was observed when initial pH of the medium was set at 7.0 (Figure 1). Significant amount of chitinase was produced by the isolate irrespective of the initial pH of production medium. 89% and 79% of chitinase production was retained at pH 6.0 and 9.0, respectively. Maximum chitinase production by *Paenibacillus* sp. D1 was achieved at 30℃ after 72 h (Figure 2). No considerable decrease in chitinase production was

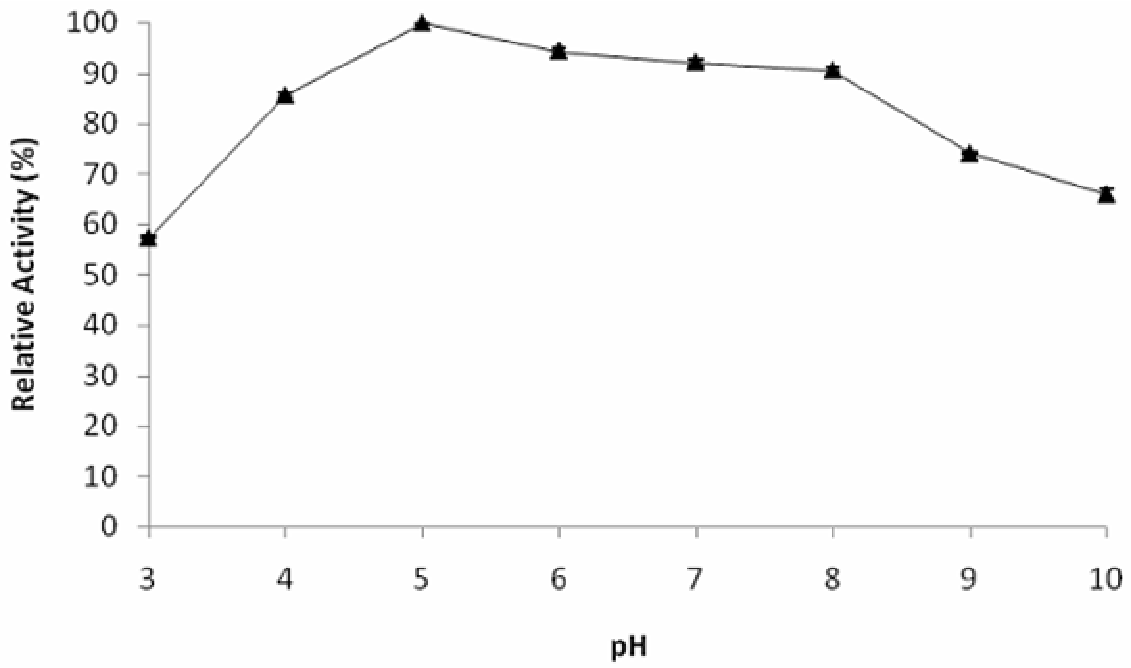

Figure 3. Effect of assay pH on chitinase form *Paenibacillus* sp. D1.

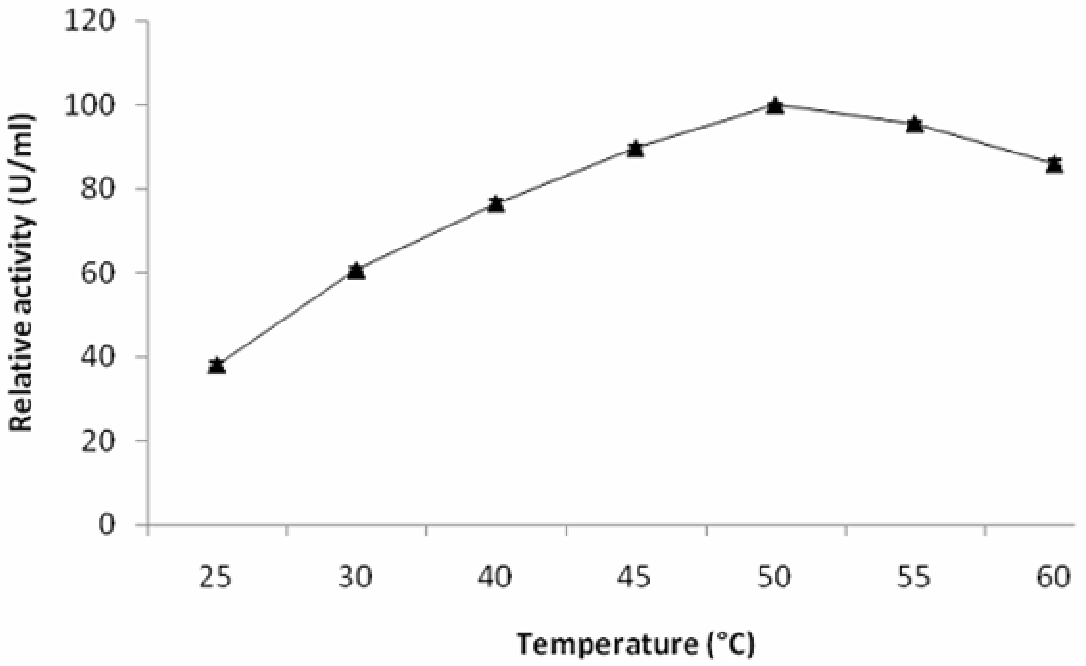

Figure 4. Effect of assay temperature on chitinase form *Paenibacillus* sp. D1.

observed at elevated temperatures up to 45°C. 91.5% of chitinase production was retained at 40°C and 88.7% at 45°C. The initial pH of medium and temperature for optimal chitinase production by *Bacillus pabuli* was reported to be 8.0 and 30°C (Frandberg and Schnurer, 1994). Shanmugaiah et al. (2008) had reported optimum pH and temperature for chitinase production by *Bacillus laterosporous* to be 8.0 and 35°C, respectively.

Chitinase of *Paenibacillus* sp. D1 was active at broad pH and temperature values. Optimum chitinase activity was observed at pH 5.0 (Figure 3) and 50°C (Figure 4). 85.6 and 66% chitinase activity was retained at pH 4 and

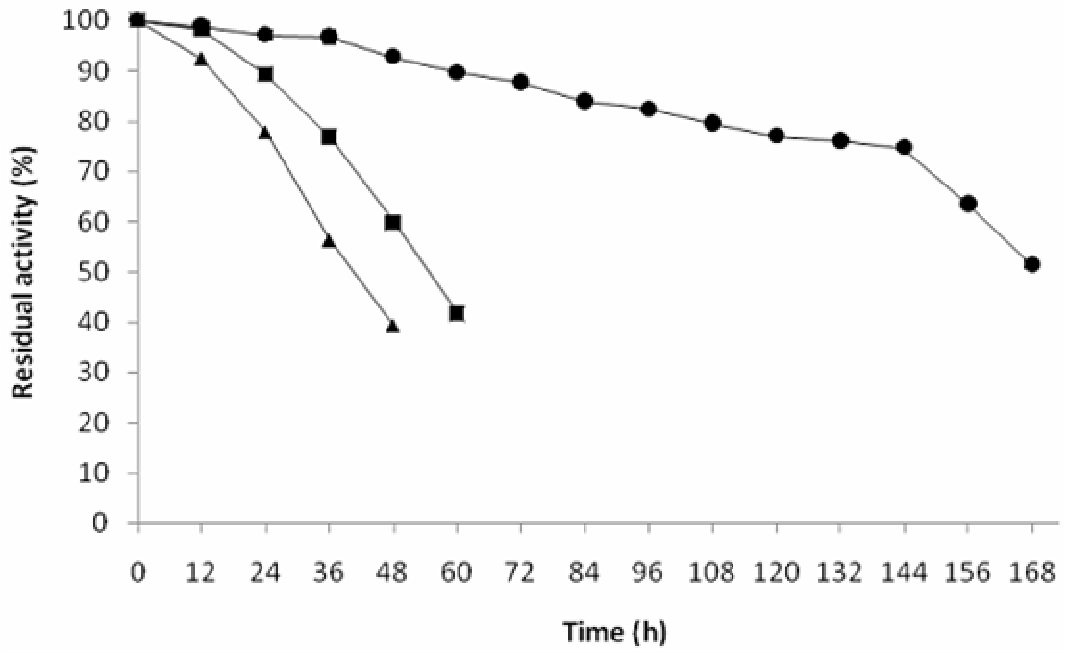

Figure 5. Thermostability of chitinase form *Paenibacillus* sp. D1 at different temperatures. Symbols circle, square and triangle represent thermostability of chitinase at 30, 40 and 45°C, respectively.

10, respectively, whereas, 95.5 and 86% activity was retained at 55 and 60°C suggesting that the enzyme was highly active at high temperatures. The enzyme was highly stable at 30°C with $t_{1/2}$ of 168 h. The $t_{1/2}$ of enzyme activity at 40 and 45°C was attained between 36 to 48 h and 48 to 60 h, respectively (Figure 5). These results revealed that the chitinase produced by *Paenibacillus* sp. D1 was highly thermostable.

Several workers have reported broad range of pH and temperature optima for chitinase, from 4.5 to 7.5 for *Bacillus cereus* chitinase (Pleban et al., 1997), pH 5.0 to 8.0 for *Aeromonas hydrophila* H-2330 (Hiraga et al., 1997), pH 7.5 to 9.0 for *Bacillus* sp. BG-11 (Bhushan and Hoondal, 1998). The temperature activity and stability profile of *Enterobacter* sp. NRG4 chitinase revealed that the enzyme was optimally active at 45°C and was stable at 40°C for more than 3 h. Chitinase from *Vibrio alginolyticus* TK-22 was stable at 40°C for 30 min (Ohishi et al., 1996) and purified chitinase of *Vibrio* sp. P-6-1 was stable at 40°C but completely inactivated at 55°C in 30 min (Takahshi et al., 1993).

The broad pH and temperature range for chitinase production and its activity along with stability at high temperatures is especially suitable for field application as the pH of soil in Gujarat, India ranges from near neutral to alkaline while the temperature in fields during the cultivation of most of the crops in India varies form 25 - 40°C (Gujarat: 20.6 and 24.42° north latitude and 68.10 and 74.28° east longitude).

The laboratory scale studies and field trials with *Paenibacillus* sp. D1 have revealed potential of its chitinase in controlling *Fusarium* wilt of *Cajanus cajan* and plant growth promotion (data not shown). Moreover, we have recently discovered that the chitinase of *Paenibacillus* sp. D1 is also tolerant to a range of pesticides commonly used in fields (Singh et al., 2009). Chitinase of *Paenibacillus* sp. D1 can thus prove as a nontoxic and thermostable additive to commercially used formulation of fungicides, reducing the dose of toxic chemical fungicides in fields. However, such agricultural applications require large quantities of enzyme that can be obtained by optimizing the medium constituents for enhanced enzyme production by the potential organism. Effect of various nutrients on chitinase production by *Paenibacillus* sp. D1 was, therefore, investigated.

Effect of carbon source in chitinase production

Various chitin sources were tested for chitinase production. Crab shell chitin was identified as the best carbon source among all the carbon sources checked (Table 1). Andronopoulou and Vorgias (2004) previously reported colloidal chitin as best chitin source for chitinase production by *Thermococcus chitonophagus*. Addition of simple sugars like glucose, lactose, maltose, arabinose and sucrose in the medium as sole carbon source suppressed the chitinase activity drastically. Similar observation has been reported for *Thermococcus chitonophagus.* Incorporation of these sugars along with chitin showed chitinase production similar to control. The results contradicted the report for enhanced chitinase

Table 1. Effect of carbon on chitinase production.

Source	Chitinase production (units/ml)
Crab shell chitin	37.65 ± 0.3
Collodial chitin	37.13 ± 0.4
Shrimp waste chitin	25.24 ± 0.54
Matsyafed chitin	21.32 ± 0.34
Glucose	1.6 ± 0.37
Lactose	0.29 ± 0.13
Maltose	0.177 ± 0.35
Arabinose	2.6 ± 0.29
Sucrose	2.5 ± 0.25
Chitin + glucose	31.15 ± 3.16
Chitin + lactose	28.46 ± 2.29
Chitin + maltose	32.08 ± 0.82
Chitin + arabinose	32.98 ± 2.03
Chitin + sucrose	32.83 ± 5.42

Table 2. Effect of nitrogen on chitinase production.

Source	Chitinase production (units/ml)
Control	37.65 ± 3.3
Chitin	26.42 ± 3.14
$(NH_4)_2SO_4$	35.24 ± 1.4
$(NH_4)H_2PO_4$	13.84 ± 4.94
$(NH_4)_2HPO_4$	39.55 ± 1.86
NH_4Cl	30.90 ± 4.3
NH_4NO_3	46.08 ± 1.91
KNO_3	42.36 ± 0.53
$NaNO_3$	48.06 ± 2.3
Urea	56.43 ± 2.92
Glutamate	47.05 ± 1.72
Aspartate	29.56 ± 2.2
Peptone	21.63 ± 1.6
Yeast extract	52.03 ± 1.9

production in presence of arabinose by Vaidya et al. (2001) in *Alcaligenes xylosoxydans* and Gupta et al. (1995) for *Streptomyces viridificans.* This suggested that sugars were not required for chitinase production by *Paenibacillus* sp. D1 and chitin served as an essential inducer for the enzyme.

Effect of nitrogen and phosphorus sources on chitinase production

Among various oraginc and inorganic sources tested urea and yeast extract were identified as the best nitrogen source (Table 2). Supplementation of urea and yeast extract (1.0 g/l) in the media increased chitinase production by 1.6 and 1.5 folds, respectively. Addition of yeast extract has been reported to increase chitinase activity in *Alcaligenes xylosoxydans and Paenibacillus sabina* strain JD2 (Vaidya et al., 2001; Patel et al., 2007). Gohel et al. (2006) had reported significant influence of urea, peptone and yeast extract on chitinase production by *Pantoea dispersa*. Chitinase production by *Paenibacillus* sp. D1 was reduced in presence of peptone. Similar observation has been reported for chitinase production by *Streptomyces* sp. Da11 (Han et al., 2008). The results in present study indicated that organic nitrogen sources were better for chitinase production than inorganic nitrogen sources by *Paenibacillus* sp. D1.

Table 3. Effect of phosphorous on chitinase production.

Source	Chitinase production (units/ml)
Control	37.65 ± 3.3
$(NH_4)_2HPO_4$	40.0 ± 2.2
$(NH_4)H_2PO_4$	19.65 ± 1.01
$CaHPO_4$	39.15 ± 2.6
K_2HPO_4	53.2 ± 0.95
KH_2PO_4	34.34 ± 2.56
Na_2HPO_4	43.5 ± 3.42
NaH_2PO_4	36.26 ± 2.59
Phytate	19.55 ± 2.27

K_2HPO_4 was identified as the best phosphorus source for chitinase production by *Paenibacillus* sp. D1. Addition of organic phosphorus source such as phytate drastically reduced chitinase production (Table 3).

Effect of surfactants and metal ions on chitinase production

Effect of various surfactants and metal ions tested is summarised in Table 4 and 5. Detergents tween 80, tween 20, triton X 100, span 80 and Na-taurocholate had positive effect on chitinase production while CTAB, SDS and dicotylsulfosuccinate decreased the chitinase production by *Paenibacillus* sp. D1. Tween 80 was identified as best surfactant supplement with an increase in chitinase production by 1.44 folds. Effect of surfactants on chitinase production has not been much studied. Vaidya *et al.* had reported positive effect of non ionic detergents on chitinase production by *Alcaligenes xylosoxydans* (Vaidya et al., 2001). Triton X 100 was identified as best surfactant supplement for chitinase production by *Aeromonas* sp. (Ahmadi et al., 2008). Surfactants are known to alter the porosity of cell membrane resulting in leakage of enzyme into the external milieu. Addition of surfactant in the medium can, therefore, improve the enzyme production.

Effect of metal ions on chitinase production by bacteria has not been studied in detail. Chitinase production by

Table 4. Effect of surfactants on chitinase production.

Source	Chitinase production (units/ml)
Control	37.65 ± 3.3
CTAB	13.14 ± 4.38
Dicotylsulfosuccinate	14.2 ± 1.1
Hexamine hydrate	32.59 ± 1.2
Na-taurocholate	48.23 ± 0.96
SDS	13.92 ± 1.35
Span 80	48.93 ± 1.67
Tergitol	24.34 ± 0.54
Triton X-100	51.57 ± 1.14
Tween 20	46.53 ± 1.95
Tween 80	54.4 ± 0.64

Table 5. Effect of metal ions on chitinase production.

Source	Chitinase production (units/ml)
Control	37.65 ± 3.3
$BaCO_3$	30.84 ± 0.56
$CaCl_2$	34.68 ± 1.64
$CoSO_4$	26.6 ± 1.63
$CuSO_4$	25.087 ± 2.8
$FeCl_3$	48.38 ± 0.97
$FeSO_4$	30.89 ± 4.27
H_3BO_3	7.1 ± 4.64
KCl	31.85 ± 0.56
$MgCl_2$	25.99 ± 0.53
$MgSO_4$	36.37 ± 3.95
$MnSO_4$	35.99 ± 0.33
Na_2CO_3	33.8 ± 1.76
Na_2SO_4	29.3 ± 1.57
NaCl	33.76 ± 1.3
$ZnSO_4$	18.99 ± 1.2

Paenibacillus sp. D1 was investigated in presence of various metal ions. None of the metal ions except $FeCl_3$ had any significant effect on chitinase production by *Paenibacillus* sp. D1. Supplementing $FeCl_3$ in medium increased chitinase production by 1.33 folds compared to control. $FeCl_3$ has not been previously reported to influence chitinase production in bacillus strains. $CaCl_2$ had no effect on chitinase production by *Paenibacillus* sp. D1. This is in contrast with the report by Patel et al. (2007) on *Paenibacillus sabina* in which $CaCl_2$ significantly affected chitinase production. Gohel et al. (2006) has also reported $CaCl_2$ as important media constituent for chitinase production by *Pantoea dispersa.*

Conclusion

Fungal plant diseases contribute significantly to yield losses in agriculture. Conventionally, fungicides are used to control damage caused by fungal phytopathogens. However, their excessive use has lead to several problems related to environmental degradation, pollution, development of resistant strains and lethal effect on beneficial rhizobacteria. Thus, there is need for alternate fungal disease control strategies. Chitinolytic bacteria producing thermotolerant chitinase offer potential alternate or additive to toxic chemical fungicides to reduce their dosage in agriculture. Present investigation involved isolation of a hyperchitinase producing isolate, characterization of its crude chitinase and optimization of culture conditions for maximal chitinase production. The isolate exhibited chitinase production over a wide temperature (25 - 45°C) and pH (6 - 9) range. The crude chitinase from *Paenibacillus* sp. D1 had activity in broad pH (4 - 10) and temperature (30 - 60°C) range and was highly thermostable at 45°C. Broad temperature and pH range for production, activity and thermostability at maximum temperature (45°C) that exists in fields across India of chitinase is of special importance as it ensures stability on the culture and chitinase under different field condition. Identification of essential nutrients influencing chitinase production by the *Paenibacillus* sp. D1 can help in formulating a cheap medium for high chitinase production need for field applications.

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