



## Original Research Article

### Effect of metal ions and chemical compounds on chitinase produced by a newly isolated thermotolerant *Paenibacillus* sp. BISR-047 and its shelf-life

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#### A B S T R A C T

##### Keywords

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Enzyme assay

In this study, we aimed to find out effects of metal ions and chemical compounds on activity of chitinase produced by *Paenibacillus* sp. BISR-047. We also evaluated shelf-life stabilities of the produced enzyme using various stabilizers at different temperatures. A 10-80% increase in relative activity was observed in presence of most of the divalent ions used such as  $Hg^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{2+}$  and  $Mg^{2+}$ . Maximum enzyme activity (80%) was attained by  $Fe^{2+}$  at a concentration of 1 mM whereas  $Ag^+$  was found to be a potent inhibitor. Analysis revealed that Tween-80 (T-80) and cetyltrimethylammonium bromide (CTAB) had a positive effect on enzyme activity at a concentration of 0.1%. The shelf-life of chitinase at 4°C was 50 days (retained 91% activity) and at room temperature (RT) was 180 days (retained 59% activity).

## Introduction

Chitin is a linear polysaccharide consisting of  $\beta$ -1,4-linked *N*-acetyl-D-glucosamine (NAG) moieties. It is the second most abundant biopolymer on earth and is a constant source of renewable raw materials (Annamalai *et al.*, 2011). Chitinases (EC 3.2.1.14) are enzymes that selectively hydrolyze  $\beta$ -1,4 glycosidic bonds that link NAG residues of chitin (Ahmadi *et al.*, 2008). The bioconversion of chitin in nature is extremely important to convert complex chitin to its simpler carbon and nitrogenous

compounds that may be utilized by other microorganisms (Meena *et al.*, 2013). Microbial chitinase have received increased attention due to their wide range of biotechnological applications, particularly in agriculture for biocontrol of fungal phytopathogens and harmful insects. Moreover, chitinases have been used as a potential additive or alternative to chemical-based insecticides and fungicides (Singh, 2010). Several reports are available on chitinolytic bacterial strains, and their

enzymes have been studied in detail (Bhushan, 2000; Nawani, et al., 2002; Kim *et al.*, 2003; Essghaier *et al.*, 2012) but most of them cannot be applied widely in various industrial and biotechnological applications. It is worth mentioning here, that use of the present enzyme toolbox is limited as supply is insufficient to meet most industrial demands (Essghaier *et al.*, 2012). Therefore, efforts are needed to explore new sources of enzymes, higher-yielding production techniques, broader stabilities and their novel applications in unexplored fields.

The heavy metals (HM) are generally used as a group name for metals and semi-metals that have been associated with contamination and potential toxicity or ecotoxicity (Duffus, 2002). In microorganisms, these compounds cause numerous slighter or stronger toxic effects particularly by decreasing some of the enzyme activities essentially required for metabolic activities of the cell. Therefore, microorganisms are known to adapt to presence of HM in the environment and have developed resistance mechanisms (Bruins *et al.*, 2000). Although, the kind of mechanism may be more or less homologous in all species of bacteria (Ji and Silver, 1995) it is expected to obtain different responses to the same toxic concentrations for several bacterial species (Rial *et al.*, 2011). This could be attributable to the specificity of the bacterial enzymes that are involved in metabolic processes required for maintaining the structural configuration of enzymes (Madigan and Martinko, 2005). Many reports are available showing the effects of metal ions and chemical compounds on chitinases produced by bacteria (Ahmadi *et al.*, 2008; Wang *et al.*, 2002; Saima and Roohi, 2013). However, it has been observed that these reported chitinases have lower stabilities in presence of heavy metal ions. On the other hand, shelf-life of an enzyme is important

for enzyme-based bio-catalytic conversions in the field of biotransformation, environmental monitoring, diagnostics, and pharmaceutical and food industries (Cano-Salazar *et al.*, 2011). Enzyme based strategies are increasingly replacing congenital chemical methods in both laboratories and industries with attributes as higher stability, efficiency, better performance and longer shelf-life. However, commercialization of enzymes is still at a lower pace because of their higher production costs and storage problems (Datta *et al.*, 2013).

In our earlier communications, we have reported production and optimization of chitinase by newly isolated bacterial strains from desert soils and evaluated their thermal stabilities and antifungal potential (Meena *et al.*, 2014a; Meena *et al.*, 2014). The enzymes have been found highly thermostable at higher temperatures and have shown potential in controlling the growth of fungal phytopathogens of economically important seed spice crops of India. In the present investigation, we are reporting effects of metal ions and chemical compounds on activity of chitinase produced by *Paenibacillus* sp. BISR-047. Attempts were also made to evaluate shelf-life stabilities of the produced enzyme using various stabilizers at different temperatures.

## **Material and Methods**

### **Chemicals**

The sodium dodecyl sulphate (SDS), CTAB, ethylenediaminetetraacetic acid (EDTA), urea, glutamic acid, aspartic acid, glycerol, NAG and urea were purchased from Sigma Chemical Co. (St. Louis, USA). All of the other chemicals used were of highest grade commercially available.

### **Microorganism and growth conditions**

A thermotolerant bacterial strain BISR-047 was previously isolated from the Great Indian Desert soils and identified as *Paenibacillus* sp. by various morphological, physiological and molecular approaches (Meena *et al.*, 2014a). It was cultivated in colloidal chitin (CC) agar medium containing the following per liter: yeast extract (1.5 g), CC (2.0 g), agar (20.0 g), media pH 7.0 at 45°C and was stored as a glycerol stock at -80°C (Meena *et al.*, 2014b).

Chitinase production was done at 45°C in 250ml Erlenmeyer flasks containing 50ml CC medium (pH 7.0) at 180 rpm for 48 h. The culture broth was centrifuged in a microfuge (Biofuse Primo-R) at 10,000 g for 10 min and supernatant was used for enzyme assay (Meena *et al.*, 2014a).

### **Enzyme assay**

The chitinase activity was determined colorimetrically by detecting the amount of NAG released from colloidal chitin substrate as previously described (Meena *et al.*, 2014a). Briefly, the reaction mixture (1.0ml) for enzyme assay consisted of enzyme (340 µl) with CC (3 mg) as a substrate in 50 mM sodium acetate buffer (SAB, pH 5.0). This mixture was incubated at 55°C for 1 h and the supernatant was used for enzyme assay by estimating the released sugar (Nelson, 1944). One international unit (IU) was defined as the activity that produced one µmol of the product per h at optimal conditions (Vyas and Deshpande, 1989).

### **Effect of metal ions and chemical compounds**

The extracellularly secreted enzyme was pre-incubated with different metal ions and chemical compounds at three different

concentrations (1, 5 and 10 mM) and incubated at 45°C in a dry bath (Techne DB-3D, Cambridge, UK). After 1 h, samples were centrifuged at 10000 g for 10 min and used for enzyme assay to find out the residual activity. A control without metal ion or chemical compound was also maintained throughout the study. Finally, the relative activity was calculated in comparison with the control and reported.

### **Effect of surfactants**

The effect of surfactants on chitinase activity was determined by pre-incubating enzyme with Tween-20 (T-20), Tween-80 (T-80), sodium dodecyl sulphate (SDS), Triton X-100 or cetyl trimethylammonium bromide (CTAB) at 0.01 and 0.1% (w/v or v/v) concentration using the method as reported earlier (Annamalai *et al.*, 2011).

Briefly, the mixture was pre-incubated at 45°C in a dry bath (Techne DB-3D, Cambridge, UK). After 1 h, samples were centrifuged at 10000 g for 10 min and used for enzyme assay to find out the residual activity. A control without surfactant was also maintained throughout the study and the relative activity was calculated in comparison with control.

### **Shelf-life of enzyme**

The shelf-life of enzyme was determined by incubating it at -20, 4, 15 and 28°C RT with different stabilizers; NaCl (100 mM), glycerol (20%; v/v), T-80 (0.2%; v/v) and EDTA (0.3%; w/v) up to 180 days. A control without stabilizer was also maintained to compare storage stability of enzyme. For each optimization parameter, three sets of independent experiments were carried out and the mean values were reported.

## Results and Discussion

### Effect of metal ions and chemical compounds

Various metal salts/ions and compounds were evaluated for their promotory or inhibitory effects on enzyme activity. The chitinase activity was determined at optimal conditions by incubating the extracellularly secreted enzyme with different metal ions and compounds at various concentrations for 1 h and the result have been shown in Table 1. It was observed that some divalent cations, such as  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Hg^{2+}$  and a trivalent cation ( $Fe^{3+}$ ), enhance the enzyme activity up to 35% at 1 mM concentration of their salt over the control. The maximum relative activity (180%) was determined with divalent cation  $Fe^{2+}$  at 1 mM concentration that sharply decreased with increase in the related salt concentration (10 mM). Whereas,  $Li^+$ ,  $Ag^+$ ,  $Cd^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$  and  $Pb^{2+}$  were found inhibitory at any of the concentration used. The maximum inhibition was detected with  $Cd^{2+}$  at 10 mM concentration (10%).

Reducing agents such as L-glutamic acid, glycine and L-methionine were found to enhance the relative enzyme activity (up to 130%) at all the three concentrations (Table 1). Whereas, L-aspartic acid and L-asparagine have been detected as enzyme inhibitors at higher concentration (10 mM) with relative enzyme activity determined below 90%. In these cases, L-arginine was found to be a potent inhibitor with relative activities as low as 48% at 10 mM concentration. Various other chemical compounds were also examined for their effect on the chitinase activity. At lower concentrations (1 mM), all of them have been found to enhance the enzyme activity (Table 1). EDTA showed maximum increase in enzyme activity (155%) when used at 1

mM concentration, whereas, urea and thio urea showed increased activity with increase in concentration up to 10 mM. Other chemicals, when used at 10 mM concentration, have been found to inhibit the enzyme activity.

### Effect of surfactants

The enzyme was pre-incubated at two different concentrations (0.01 and 0.1%) of surfactant and the results have been depicted in Fig. 1. It showed that T-80 and CTAB had a positive effect on enzyme activity at both the concentrations. Within these two, CTAB showed maximum increase in chitinase activity (127%) whereas; it was 120% in case of T-80 at 0.1% concentration. Maximum enzyme inhibition was observed with SDS at both the concentrations.

### Shelf-life of enzyme

The shelf-life of chitinase was investigated by incubating enzyme with stabilizers at four different temperatures (-20, 4, 15 and 28°C). The activity of enzyme was monitored up to 180 days in respect to control (without any stabilizer). The results of this analysis have been shown in Fig. 2. When the enzyme was incubated with glycerol at 28°C, it served as an effective stabilizer by maintaining approximately 60% enzyme activity up to 180 days. At this temperature, T-80 was found least effective in maintaining the enzyme activity at different storage periods and the enzyme showed minimum activity (21%) at 180 days. In case of incubation at -20°C, glycerol again served as an effective stabilizer by maintaining approximately 80% enzyme activity up to 180 days. At this temperature, T-80 was found least effective in maintaining the enzyme activity at different storage periods and showed minimum activity (59%) at 180 days.

Overall, glycerol was found as an effective stabilizer for enhancing storage stability of chitinase that can maintain more than 50% activity at all the studied temperatures, even up to 180 days. In all of these cases, NaCl and T-80 were found ineffective in maintaining the enzyme activity and the activity values were found less than the control. When temperature was decreased from 28°C to -20°C, activity of enzyme was found to increase up to 180 days.

The activity of chitinase in presence of metallic ions is highly valued property with regard to potential industrial applications (Laribi-Habchi *et al.*, 2012). Our study revealed that  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Hg}^{2+}$  had positive effect on activity of chitinase produced by isolate BISR-047. These results are in support of previous findings where  $\text{Mg}^{2+}$  enhanced activity of chitinase produced by *Streptomyces* sp. DA11 (Han *et al.*, 2009). Similarly, Lee *et al.*, 2007 observed increased activity with  $\text{Mg}^{2+}$  in chitinase produced by *Bacillus* sp. DAU101 while in case of *Bacillus* sp. HU1, enzyme activity was found to be significantly increased with 4 mM of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (Dai *et al.*, 2011). Contrary to this, Saima and Roohi (2013) reported inhibition of chitinase by  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}$ , produced by *Aeromonas hydrophila* and *Aeromonas punctata*. Metal ion  $\text{Fe}^{2+}$  has been found to inhibit chitinase activity of *Vibrio* sp. 98CJ11027 (Park *et al.*, 2000). We observed inhibitory effect of  $\text{Ag}^+$ ,  $\text{Li}^+$  and  $\text{Zn}^{2+}$  on chitinase activity. Kim *et al.*, (2003) have reported similar inhibition, where  $\text{Ag}^+$  strongly inhibited enzyme produced by *Streptomyces* sp. M-20. Singh (Singh, 2010) reported inhibitory effect of  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  on activity of chitinase produced by *Paenibacillus* sp. D1 at lower concentrations (1 mM).

Metal ions such as  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  have been reported as a common inhibitor for

most of the chitinases (Nawani and Nawani *et al.*, 2002; Lee *et al.*, 2007; Sri *et al.*, 2004; Lien *et al.*, 2007). Whereas, chitinase produced by isolate BISR-047 appears to be another different kind of chitinase that showed stimulatory effect with  $\text{Hg}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Mg}^{2+}$ . This stimulation could be due to presence of aspartic and glutamic acid residues that provide active binding sites for certain divalent cations, thereby stimulating activity (Annamalai *et al.*, 2010).

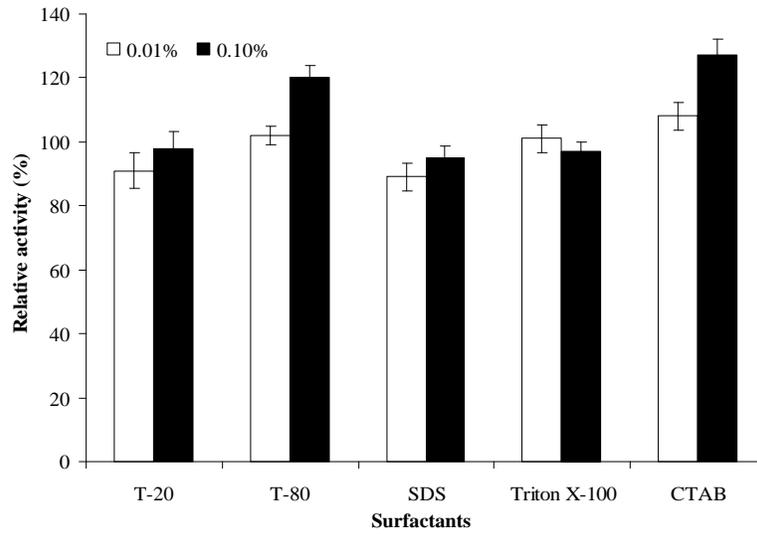
The reducing agents such as glycine, L-glutamic acid, L-methionine, L-aspartic acid and L-asparagine showed stimulatory effect whereas, L-arginine showed inhibitory effect on chitinase activity. Amino acids and their analogs such as tryptophan, tyrosine, glutamine and arginine have shown stimulation of chitinase activity in *Bacillus* sp. BG-11 (Bhushan and Hoondal, 1998). In case of enzyme produced by *Streptomyces* sp. PTK19, valine induced enzyme activity whereas, alanine, asparagine, methionine and histidine suppressed the activity (Thiagarajan *et al.*, 2011).

We studied effects of other chemical agents and found that EDTA (1 mM) had maximum increase in enzyme activity (55%). On the other hand, urea and thio urea (10 mM) stimulated enzyme activity. Nawani *et al.*, (2012) reported 10% increase in activity of chitinase produced by *Microbispora* sp. V2 with 1 mM EDTA. Similarly, addition of urea showed increased activity of enzyme produced by *Paenibacillus* sp. D1 (Singh 2010). Contrary to this, Inglis and Peberdy, 1997 observed reduced activity of chitinase produced by *Ewingella americana* with EDTA. Similarly, Kavitha and Vijaylakshmi, 2011) showed inhibitory effects of urea and SDS in chitinase produced by *Streptomyces tendae* TK-VL\_333.

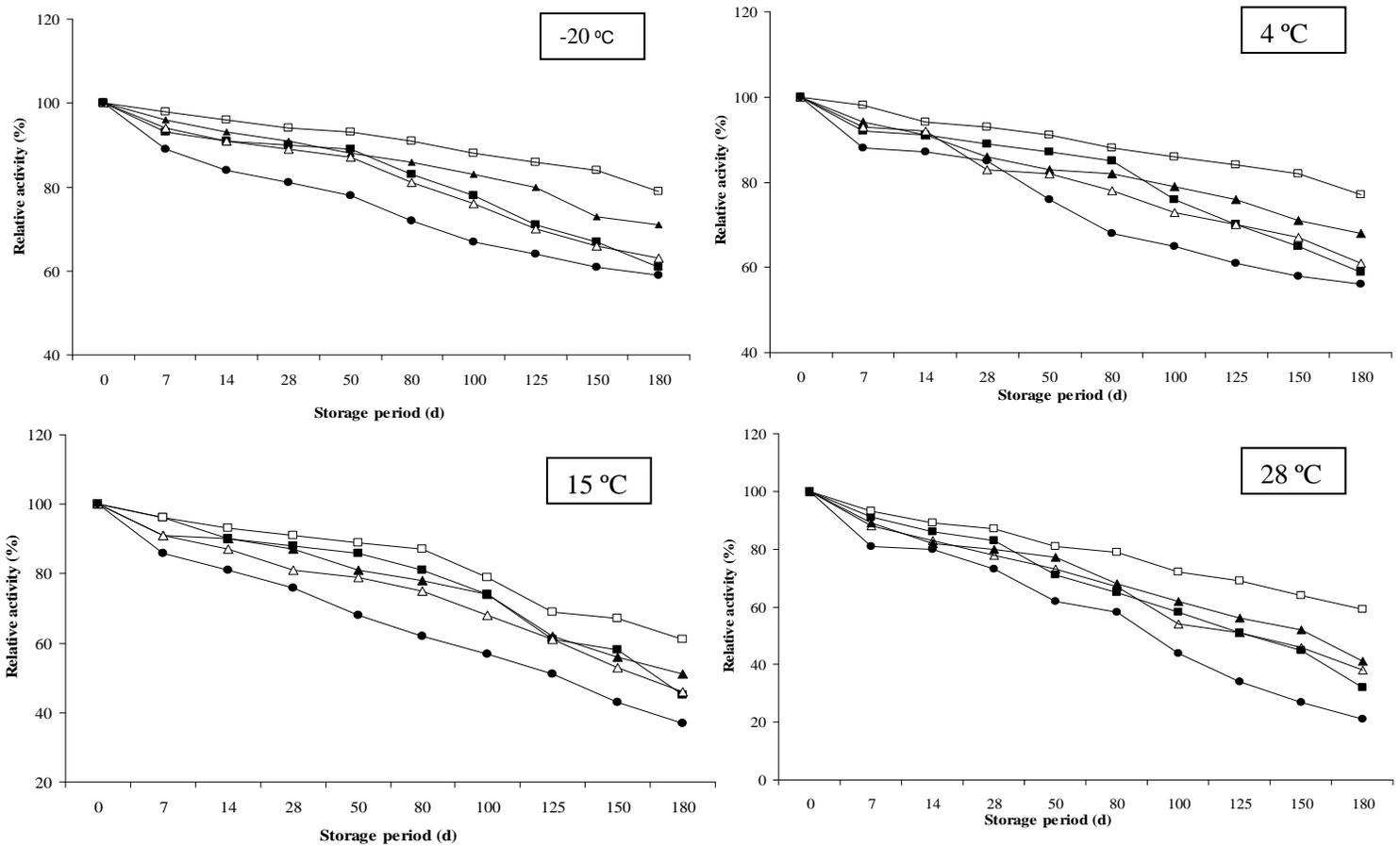
**Table.1** Effect of various metal ions and chemical compounds on chitinase produced by *Paenibacillus* sp. BISR-047

Ions and compounds	Relative activity (%)		
	1 mM	5 mM	10 mM
Control	100 ± 1.1	100 ± 1.7	100 ± 1.3
FeCl <sub>3</sub>	102 ± 1.5	52 ± 1.9	15 ± 2.4
MgSO <sub>4</sub> .7H <sub>2</sub> O	113 ± 2.5	99 ± 1.2	85 ± 1.8
MgCl <sub>2</sub> .6H <sub>2</sub> O	106 ± 3.1	122 ± 1.3	87 ± 1.9
ZnCl <sub>2</sub>	54 ± 1.2	42 ± 1.4	23 ± 1.9
LiCl	67 ± 2.1	69 ± 2.7	87 ± 1.6
CdCl <sub>2</sub> .H <sub>2</sub> O	83 ± 1.9	34 ± 2.0	10 ± 3.1
CuSO <sub>4</sub> .5H <sub>2</sub> O	91 ± 2.9	64 ± 2.4	21 ± 2.3
AgNO <sub>3</sub>	45 ± 1.7	38 ± 1.1	32 ± 2.4
Pb(NO <sub>3</sub> ) <sub>2</sub>	78 ± 3.5	49 ± 2.5	16 ± 2.1
NiCl <sub>2</sub> .6H <sub>2</sub> O	87 ± 1.2	95 ± 1.9	99 ± 2.2
AlCl <sub>3</sub>	78 ± 3.5	65 ± 2.5	32 ± 2.8
CaCl <sub>2</sub> .2H <sub>2</sub> O	104 ± 2.9	109 ± 1.3	111 ± 1.7
FeSO <sub>4</sub> .7H <sub>2</sub> O	180 ± 1.2	121 ± 1.8	71 ± 2.9
MgSO <sub>4</sub> .H <sub>2</sub> O	116 ± 1.5	134 ± 1.5	159 ± 1.5
HgCl <sub>2</sub>	135 ± 1.5	46 ± 1.5	8 ± 1.5
CoCl <sub>2</sub> .6H <sub>2</sub> O	98 ± 1.5	47 ± 1.5	41 ± 1.5
L-Arginine	65 ± 3.9	53 ± 2.1	48 ± 3.9
Glycine	123 ± 4.0	130 ± 1.4	102 ± 4.2
L-Glutamic acid	116 ± 1.9	125 ± 1.4	121 ± 1.3
L-Methionine	103 ± 2.9	112 ± 3.1	119 ± 2.2
L-Aspartic acid	108 ± 2.0	98 ± 1.8	87 ± 1.1
L-Asparagine	124 ± 1.9	129 ± 2.9	89 ± 3.8
Urea	121 ± 1.7	129 ± 1.2	134 ± 1.9
Thio urea	114 ± 2.7	124 ± 3.8	141 ± 1.6
2-Mercaptoethanol	117 ± 2.4	93 ± 1.6	87 ± 1.8
KI	132 ± 3.1	100 ± 2.9	68 ± 1.7
NaCl	118 ± 0.9	69 ± 1.8	62 ± 1.2
EDTA	155 ± 2.2	112 ± 3.1	102 ± 1.3
8-Hydroxy quinoline	109 ± 2.4	103 ± 4.5	98 ± 3.9

**Fig.1** Effect of surfactants on chitinase produced by *Paenibacillus* sp. BISR-047 at two different concentrations. Each point represents the mean of three independent experiments and error bars indicate SD



**Fig.2** Storage stability of chitinase at different incubation temperatures with (Glycerol □, EDTA ▲, NaCl △, Tween 80 ●) and without (Control ■) stabilizers



These results showed that T-80 and CTAB had a positive effect on chitinase activity. Chakraborty, *et al.*, 2012 reported that non-ionic detergents (T-20, T-80 and Triton X-100) significantly enhanced chitinase activity in *Serratia marcescens*. Similarly, addition of T-20, T-60, Triton X-100 and Tergitol N P 35 have been reported earlier to enhance activity of enzyme produced by *Alcaligenes xylosoxydans* (Vaidya *et al.*, 2001). Whereas, Faramarzi, *et al.*, 2009 showed a negative effect of T-20 and T-80 on activity of chitinase produced by *Massilia timonae*.

The effects of stabilizers on shelf-life of chitinase were also evaluated in the present study. Our results showed that enzyme retained 32% of its relative activity up to 180 days at RT without addition of any stabilizer (control) which increased approximately two fold (59%) by addition of glycerol. The higher shelf-life stability at RT (>50% up to 125 days) shows that the enzyme can be utilized effectively for biotransformation studies. A potential drawback of utilizing chitinase in cell-free biotransformation reactions is generally shorter shelf-life. Previous studies have shown that addition of stabilizers enhance shelf-life of chitinase at lower (4°C) storage conditions [Bhushan, 2000; Bhushan and Hoondal, 1998]. In addition, we recently reported extremely high thermal stability ( $t_{1/2}$  of 4 h at 100°C) of this enzyme that shows another potential characteristic in utilizing this enzyme for various biotechnological applications (Meena *et al.*, 2014a).

The present study proposes that the chitinase produced by *Paenibacillus* sp. BISR-047 has a wide tolerance towards metal ions, surfactants and other chemical compounds used in this study. Moreover, it has better shelf-life stability, which is an additive factor for single cell protein production, and

could be utilized for management of chitin waste generated in huge quantities by seafood industries. It may also be used as a bio-additive in formulation of chemical based pesticides and fungicides to develop an effective and environment friendly process route.

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