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INFLUENCE OF CHEMICAL STRESS ON THE STABILITY OF CHITINASE PRODUCED BY *GLUTAMICIBACTER URATOXYDANS* KIBGE-IB41

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ABSTRACT

Chitinase is a glycosyl hydrolase that cleaves chitin into oligomers. Stability of an enzyme during industrial procedures is a challenge because it directly affects the industrial processing of enzyme catalyzed bioproducts. In this study, chitinase was produced by *Glutamicibacter uratoxydans*. Chitinase was subjected to stability studies in the presence of different chemicals that could either acts as enhancers, stabilizers or inhibitors. Current results suggested that chitinase was greatly stimulated by Cu^{+2} ions whereas, the order of potency of inhibition of chitinase by different metal ions was $K^{+} > Fe^{+3} > Hg^{+2} > Ni^{+2} > Ba^{+2} > Zn^{+2} > Co^{+2} > Mn^{+2} > Cs^{+} > Ca^{+2} > Na^{+}$. Two non-ionic (Tween-20 and Tween-80) and one anionic Sodium dodecyl Sulfate (SDS) surfactants inhibited the enzyme activity. Cetyl trimethylammonium bromide (CTAB) exhibited a stabilizing effect. Only 4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol (Triton X-100) was able to enhance the catalytic performance of chitinase in 1.0 mM and 5.0 mM concentrations. Percent relative activity decreased in the presence of different organic solvents and the order of inhibition noticed was isopropanol > formaldehyde > dimethyl sulfoxide (DMSO) > methanol > chloroform > ethanol. The results suggested that chitinase from *G. uratoxydans* KIBGE-IB41 is sensitive against different chemicals except for Triton-X100. Therefore, it is suggested that chitinase will be an ideal candidate along with Triton-X100 to be used in the bioremediation of hydrophobic compound at the contaminated sites for the solubilization of hydrophobic contaminants from the environment.

Keywords: Chitinase, Chitin, Metal Ions, Organic Solvents, Surfactants, Scavengers.

INTRODUCTION

Hydrolases are commercially significant enzymes that are used in several industrial applications. There are several physical and chemical factors that are optimized in the bioprocessing of a bioproduct. Both physical and chemical conditions effect the stability of an enzyme. In some of the cases, if the processing conditions are not favorable then the enzymes are either inhibited or exhibits lower catalytic performance. In both of the cases, a bioprocess needs to be optimized according to the catalytic performance of the enzyme selected for its optimum performance. Stability of an enzyme requires a specific environment in order to perform enzyme substrate catalytic reactions. The presence of various chemical compounds like organic solvents, metal ions or different kinds of surfactants highly effects the enzyme kinetics. These compounds are involved in providing stability or can also inhibit an enzyme by developing covalent and non-covalent interactions within an active site of the enzyme (Robinson, 2015).

Chitinases are enzymes [EC 3.2.14] that belong to a class of glycoside hydrolase family. This hydrolase has a potential to cleave glycosidic linkages present within chitin (Kasprzewska, 2003; Ohno *et al*., 1996). A major portion of chitinolytic enzymes are produced by microbes that belongs to either actinomycetes, bacteria or fungi. Chitinase is capable to cleave chitin in order to produce a variety of metabolites such as deacylated oligomer chitosan $(GlcNAc)_n$, disaccharide chitobiose $(GlcNAc)_2$ and monomers of N-acetyl-β-D-glucosamine (Jung and Park, 2014). These derivatives are reported for their effective use in agriculture, cosmetic and biomedicine industries (Tachu *et al*., 2007; Marcum and Seanor, 2007; Flessner *et al*., 2002).

Chitinase is a commercially important enzyme that is involved in the hydrolysis of chitin and results in the biosynthesis of commercially important product known as glucosamine. Glucosamine is used as a sweetener in food products; is used to treat bowel syndrome; can also act as a growth factor and as an anti-arthritic agent in pharmaceutical products (Agullo *et al*., 2003; Liu *et al*., 2008; Tamai *et al*., 2003). This compound also acts as a substrate in production of salicylic acid and is also used along with hyaluronic acid in cosmetics (Maru *et al*., 1998). Chitinase also has the ability to act as an antifungal and an antinematode agent in pest control management and act

as a potent biocontrol agent (Tachu *et al*., 2007). Current study deals with the catalytic performance and stability of chitinase produced by *Glutamicibacter uratoxydans* KIBGE-IB41 in the presence of various chemical compounds. The effect of different concentrations of organic solvents, metal ions and surfactants were studied on the kinetic behavior of bacterial chitinase.

MATERIALS AND METHODS

Microorganism used

Glutamicibacter uratooxydans KIBGE-IB41 [GenBank Accession: KY938041.1] was isolated from soil sample and was identified based on polyphasic approach. The selected strain was used for the production of chitinase. The bacterium was inoculated in colloidal chitin containing medium of (gL^{-1}) Tryptone, 1.0; Yeast extract, 0.5; NaCl, 1.0; $MgSO_4.7H_2O$, 0.1; KH_2PO_4, 0.5; K_2HPO_4, 1.0 and colloidal chitin, 10.0. The pH was adjusted to 7.0±0.2 before sterilization. The flasks were kept at 25˚C for 72.0 h. The fermented broth was centrifuged at 40,000 × *g* for 10.0 min at 4˚C to obtain a clear supernatant.

Partial Purification

The clear cell free supernatant was partially purified using ammonium sulfate precipitation method (60%). The precipitated protein was collected through centrifugation at 40,000 × *g* for 15.0 min at 4˚C. The precipitates were dissolved in potassium phosphate buffer (pH-7.0) and stored at 4˚C for further experimentation.

Enzyme Assay

The enzyme activity was performed using chitinase (0.5 mL) which was mixed with 1.0% colloidal chitin (1.0 mL). The colloidal chitin was prepared in potassium phosphate buffer (pH-7.0). The reaction was continued for 2.0 h at 35.0°C and reaction mixture was centrifuged at 40,000 × *g* for 10.0 min at 4.0°C. The enzyme activity was measured by a modified method as previously described (Kobashi and Matsuda, 1974) using N-acetyl β-D-glucosamine as standard. Absorbance was measured at 680nm against a reagent blank. One unit of chitinase is defined as "an amount of enzyme required to yield 1.0 µmol of N-acetyl-D-glucosamine under standard assay conditions".

Estimation of Total Protein

Total protein content was analyzed using modified method of Lowry *et al*., (1951) and bovine serum albumin (BSA) used as a standard.

Influence of Metal Ions on Chitinase Activity

The effect of different metal ions on the chitinolytic activity was studied by mixing chitinase with different metal ions in ratio of 1:1 for 1.0 h at 30˚C. The final concentration of metal ions used was 1.0 mM and 5.0 mM. After the exposure of enzyme with a respective metal ions, enzyme activity was performed as described earlier. The metal ions which were used in this study were chloride salts of the following ions: Na^+ (NaCl), K^+ (KCl), Cs^+ (CsCl), Mg^{+2} ($MgCl_2$), Ca^{+2} ($CaCl_2$), Mn^{+2} ($MnCl_2$), Hg^{+2} ($HgCl_2$), Ba^{+2} ($BaCl_2$), Co^{+2} ($CoCl_2$), Zn^{+2} ($ZnCl_2$), Cu^{+2} ($CuCl_2$) and Fe^{+3} ($FeCl_3$). The percent relative activity was calculated with respect to the control. The control was not treated with any metal ion.

Influence of Surfactants on Chitinase Activity

Different surfactants (4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol (Triton X-100), Tween 20, Tween 80, Cetyl trimethylammonium bromide (CTAB) and Sodium dodecyl sulfate (SDS)) were used in this study to observe their impact on chitinase stability. For this purpose, chitinase was mixed with surfactant (1:1) in the concentration of 1.0 mM, 5.0 mM and 10.0 mM. This reaction mixture was placed at 30˚C for 1.0 h. Enzyme substrate reaction was conducted using standard assay procedure. The data was interpreted by calculating percent residual activity (%) with reference to control.

Influence of Organic Solvents on Chitinase Activity

Compatibility of chitinase with reference to its stability in the presence of different organic solvents (methanol, ethanol, isopropanol, dimethyl sulfoxide (DMSO), formaldehyde and chloroform) was also determined. Chitinase was mixed with different organic solvents in 1.0 mM, 5.0 mM and 10.0 mM concentration. This was kept at 30˚C for 1.0 h. Enzyme which was not treated with any of those organic solvent was considered as control (100%).

Influence of Scavenging Element on Chitinase Activity

Stability of chitinase was observed in the presence of an scavenging element (ethylenediamine tetraacetic acid (EDTA)). Chitinase was mixed with 1.0 mM, 5.0 mM and 10.0 mM concentration of EDTA at 30˚C for 1.0 h. Enzyme assay was performed and residual activity (%) was calculated in comparison to control.

RESULTS AND DISCUSSION

There are several applications of industrial enzymes. Most of the enzymes belonging to hydrolase family are exposed to various chemical agents when they are used for different industrial purposes. Hydrolase which are widely used in detergent, textile, paper sizing, waste water management and pharmaceutical industries are exposed to several chemical stresses (surfactants, solvents and metal chelators). All these compounds are responsible for either reducing the enzymatic properties of a biocatalyst or could also completely inhibit the enzymatic activities (Chapman *et al*., 2018).

Chitinases belongs to a group of glycosyl hydrolase family of GH18, GH19 and GH20. Chitinase from bacteria, viruses, fungi and animals are mainly part of glycosyl hydrolase family GH18, while some chitinase from *streptomyces* belongs to glycosyl hydrolase family GH19 (Ohno *et al*., 1996). Family GH19 chitinases have been recognized from plants, nematodes and some bacteria (Kasprzewska, 2003).

The current study aims to focus on the stability of bacterial chitinase in the presence of chemical compounds which are frequently used in different industrial purposes. In this present study, chitinase was produced by *G. uratoxydans* KIBGE-IB41. The enzyme was partially purified and its stability was studied in terms of enzyme kinetics with reference to control. Chitinase exhibited diverse array of activation and inhibition in the presence of various metal ions, surfactants, solvents and an scavenging agent.

Under *in-vivo* or *in-vitro* studies, metal ions either acts as an enhancer or as an inhibitor of an enzyme. In the current study, different metal ions in the form of chloride salts were used (Fig. **1**). In all the metal ions used, it was observed that 5.0 mM concentration of any metal ion relatively inhibited the enzyme activity more than 50% except Cu^{+2} which exhibited similar relative activity as compare to control. It was also observed that all the metal ions in 1.0 mM concentration also inhibit the activity of chitinase except for Cu^{+2} and Mg^{+2} which enhanced the relative activity up to 42% and 17%, respectively. The order of potency of inhibition of chitinase for different metal ions in 1.0 mM concentration was found to be $K^{+} > Fe^{+3} > Hg^{+2} > Ni^{+2} > Ba^{+2} > Zn^{+2} > Co^{+2} > Mn^{+2} > Cs^{+2} > Ca^{+2} > Na^{+}$. Metal ions are commonly known to affect the enzyme complex system where they are responsible for either maintaining or disrupting the 3D structure of a protein complex which ultimately effects the enzyme kinetics (Andreini *et al*., 2008). Several bacterial chitinases are reported to be inhibited strongly by Hg^{+2} ions exclusively by reacting with the −SH group of cysteine residues (Karthik *et al*., 2015). However, Cu^{+2} ions have also been reported to act as an inhibitor or enhancer for chitinase activity. Chitinase from *Microbulbifer thermotolerans* MtCh509 and *Chitinibacter* sp. GC72 were profoundly inhibited by Cu^{+2} ions (Lee *et al*., 2018; Gao *et al*., 2015). Cu^{+2} usually assist the formation of intramolecular S−S bridges by auto-oxidation and also forms sulfenic acid (Gao *et al*., 2015). In some of the cases bacterial chitinase activity was stimulated with Cu^{+2} (Annamalai *et al*., 2010). Similar results were observed in the current study. This stimulated activity is due to the formation of complex by divalent metal ions at the active site of the chitinase with carboxylic group of aspartic acid and glutamic acid (Annamalai *et al*., 2010).

The effect of a variety of surfactants (Triton X-100, Tween-20, Tween-80, SDS and CTAB) in different concentrations was observed on chitinase activity. It was observed those two non-ionic surfactants (Tween-20 and Tween-80) and one anionic (SDS) surfactant inhibited the enzyme activity with reference to control. Tween-20 and SDS strongly inhibited the enzyme activity in all tested concentration of these surfactants. However, CTAB had a stabilizing effect on chitinase activity. Only Triton-X100 was able to enhance the chitinolytic activity in 1.0 mM and 5.0 mM concentration **(**Fig. **2)**. Surfactant increase the contact frequency of substrate and active site of enzyme by disrupting surface tension of surrounding hence enhanced the turnover number of enzymes. SDS is widely reported for its ability to denature protein however, the counter effect of proteins may vary (Chanchi, 2013). Triton-X100 is composed of hydrophilic polyethylene tail and lipophilic aromatic heads which assists this surfactant to interact with protein molecules for its refolding and is also responsible for the removal of inclusion bodies in protein molecules (Lee *et al*., 2006).

The effect of different organic solvents including chloroform, DMSO, ethanol, isopropanol and methanol in the range of 1.0 mM, 5.0 mM and 10.0 mM were studied on chitinase activity **(**Fig. **3)**. It was observed that all of these aforementioned solvents had a very drastic inhibitory effect on chitinase produced by *G. uratoxydans* KIBGE-IB41. More than 50% of inhibition was noticed in all of these solvents under treatment with 1.0 mM concentration. As the concentration was raised, inhibition in enzyme activity was observed. The profile of inhibition by organic solvents

was isopropanol > formaldehyde > DMSO > methanol > chloroform > ethanol. These results suggested that the hydrophobic interactions due to organic solvents may have reduced the enzyme substrate interactions as suggested by Karthik (2015) and Halder *et al*. (2016). However, a recent report suggested that the chitinase from *Microbulbifer thermotolerans* MtCh509 expressed enhanced relative activity in the presence of DMSO, isoamyl alcohol, benzene, toluene and hexane (Lee *et al*., 2018).

The effect of only chelating agent was studied on the chitinolytic activity (Fig. **4**). In this current study, all the tested concentrations (1.0 mM, 5.0 mM and 10.0 mM) of EDTA was found to be inhibiting the enzyme activity. Only 20%, 18% and 10% relative activity of chitinase in comparison to control was detected when 1.0 mM, 5.0 mM and 10.0 mM of EDTA was used, respectively. EDTA usually binds to the metal ions and act as a chelating agent. This compound has been previously reported to reduce the enzymatic activities of different chitinases (Karthik, 2015, Dai *et al*., 2011). Chitinase produced by *Enterobacter* sp. NRG4 was also inhibited in presence of 10 mM EDTA up to 11% while, EDTA also act as inhibitor for chitinase produced by *Enterobacter* sp. G-1 at 10 mM concentration (Park *et al*. 1997).

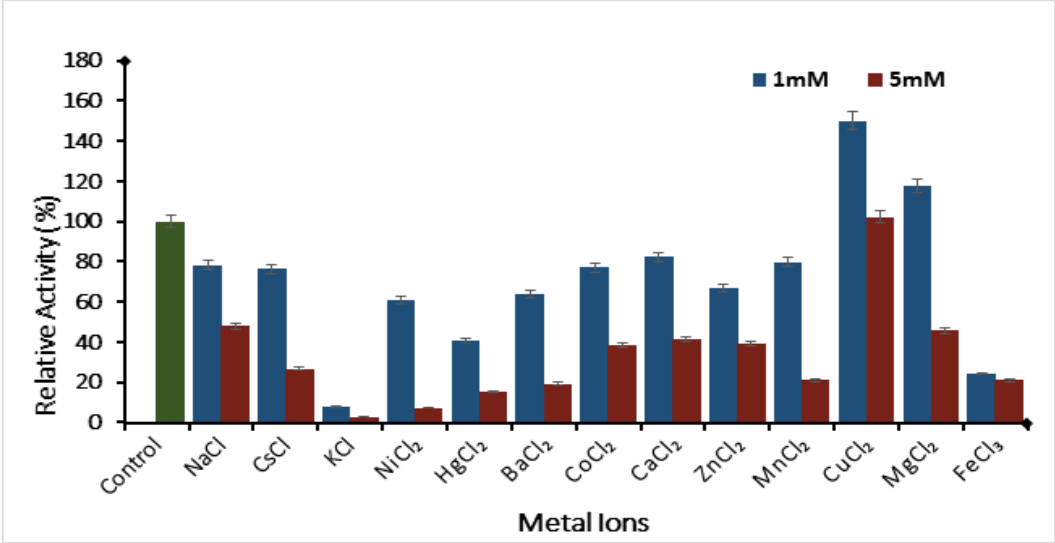

Fig.1. Effect of Metal Ions on Chitinase Activity Produced by *G. uratoxydans* KIBGE-IB41. All the Metal Ions Used were Chloride Salts (n = 3; *p-value* < 0.005).

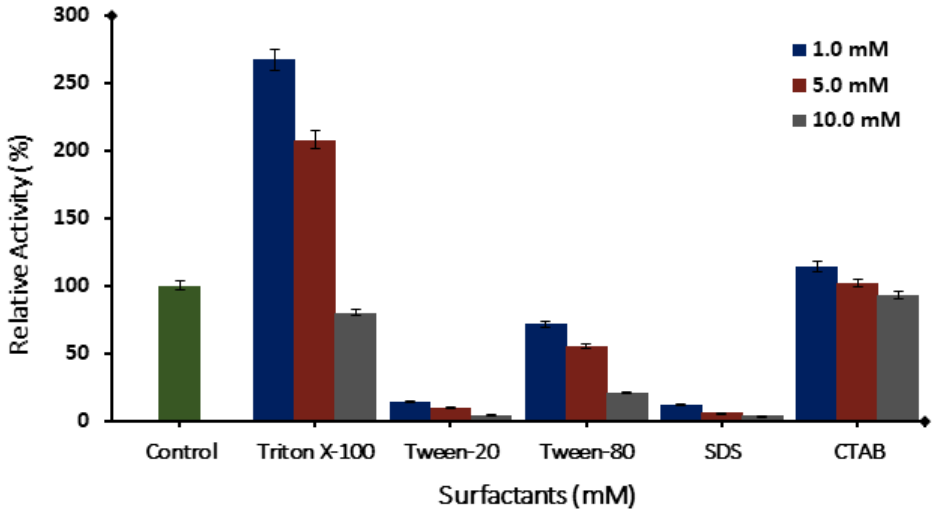

Fig. 2. Effect of Various Surfactants on Chitinase Activity Produced by *G. uratoxydans* KIBGE-IB41 (n = 3; *p-value* < 0.005).

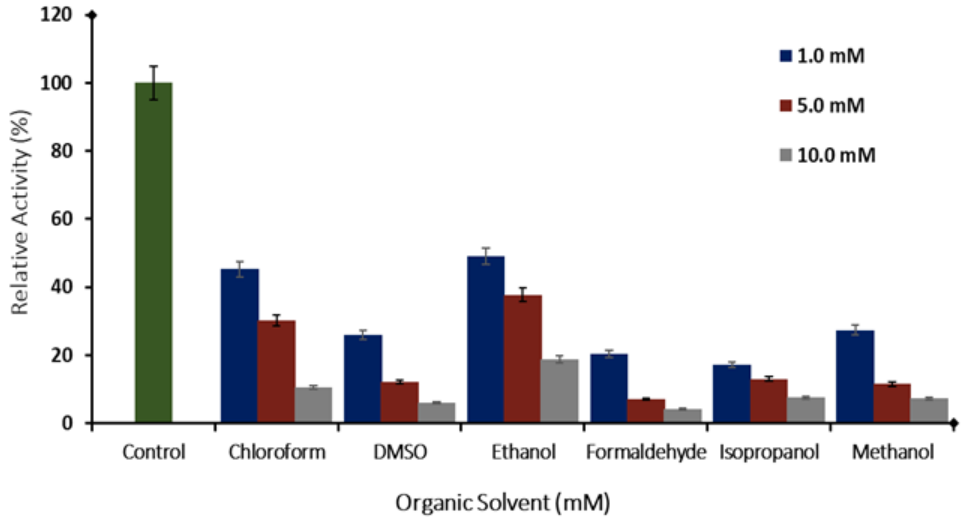

Fig. 3. Effect of Different Organic Solvents on Chitinase Activity Produced by *G. uratoxydans* KIBGE-IB41 (n = 3; *p-value* < 0.005).

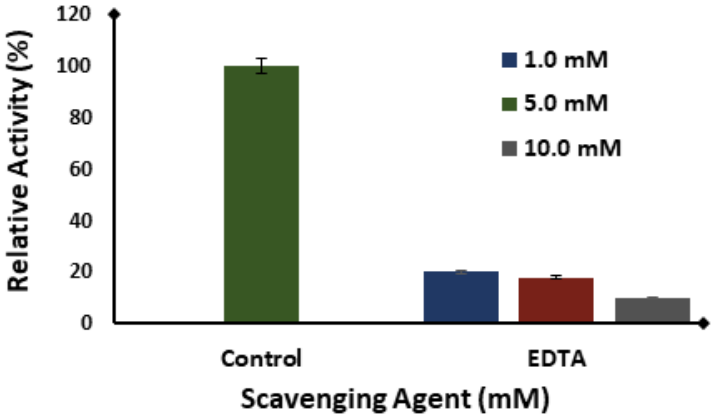

Fig. 4. Effect of an Scavenging Agent on Chitinase Activity Produced by *G. uratoxydans* KIBGE-IB41 (n = 3; *p-value* < 0.005).

Conclusions

It was observed that chitinase is a metal dependent enzyme and specifically requires Cu^{+2} as an activity enhancing agent. It was also observed that Triton X-100 improved the catalytic potential of chitinase. In conclusion, the chitinase produced by *G. uratoxydans* KIBGE-IB41 is highly sensitive to a variety of chemical agents and exhibits a unique catalytic response.

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