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Optimization of Cultural Conditions for Production of Chitinase by *Bacillus Sp.* Isolated from Marine Water using Substrate as Marine Crab Shell Waste

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Abstract Chitinases are group of enzymes which play a major role in degrading chitin. Chitinase is involved in the process of producing mono and oligosaccharide starting chitin. The present study chitinase production with crustacean waste powder at different concentrations of substrate (0.5, 1.0 and 1.5%) was increasing. Among the two forms of substrate with three altered concentrations, the combined substrate (crustacean waste powder and colloidal chitin) of 1% was found to be the best substrate for best possible chitinase production by Bacillus sp. Subsequently, the optimal production with such substrate was achieved at pH8. For further work, it is necessary to over express the enzyme in the suitable production, which will be followed by the purification and characterization of the chitinase in order to know its properties.

Keywords *Chitinases, Chitin, Water, Marine, Sheel*

1. INTRODUCTION

Chitin is nitrogen containing polysaccharide consisting of β-1,4-linked N-acetyl-D-glucosamine which is chemicallyanalogous to the cellulose, except that one of the hydroxyl groups of each glucoside residue is replaced by an acetylated or deacetylate amino group.(Saima *et al.*,2013). It is the second most abundant organic polysaccharide in nature after cellulose. In nature this homopolysaccharide is present in the shells of crustaceans, exoskeleton of insects, fungal and algal cell wall, zooplankton and several phytoplankton species the second most abundant biopolymeron Earth and a constant source of renewable raw materials(Hamid et al.,2013).Approximately 75% of the total weight of shellfish, such as shrimp, crabs and krill are considered as waste, and chitin comprises 20–58% of the dry weight of the said waste.

Organic degradation of chitin is accompany by endo- and exo-enzymes known as chitinases and β-N-acetylhexaminidases. Enzymes participate in chitin degradation are formed not only by organisms contain chitin in their body but by bacteria, upper plants and mammals where chitin is not present on a regular basis (Felse and Panda, 2000).

Chitin is often tightly bound with other compounds like protein, lipids, pigments and calcium carbonate. Conversion of these chitinous wastes to useful chitin and related oligomers involves processes like demineralization, deproteinization or hydrolysis, which was earlier carried out with strong acid and bases that involves high cost, low yields and corrosion problem. The probable alternative to solve this problem is utilization of chitinolytic enzyme, chitinase and β-N-acetyl hexosaminidase (Sourav Bhattacharya *et al., 2012*). In our present study to optimization of cultural conditions for production of chitinase by *Bacillus Sp* isolated from marina water using substrate as marine crab shell waste.

1.1Applications of chitin: Major application includes photographic products, cements,

chelating agents of heavy metals, cosmetics (Haki and Rakshit 2003) in most cases, chitinases from bacterial source acts as a fungicidal compound. Chitinolytic enzymes can be used as supplements for chemical fungicides to increase their effectiveness against pathogenic melds and reduce the required concentrations of these harmful chemicals (Brzezinska et al., 2014). The biodegradable and antifungal properties of chitinase are also useful for environmental and agricultural uses, food technology and cosmetics (Manivasagan et al., 2014).

Chitosan

1.2 Medical applications: Chitinases are used along with antifungal drugs to increase the efficiency in treating the fungal infections. Chitinases are also used as additives in skin lotions and antifungal creams .The antifungal activity and highly biocompatible quality make chitinase and its derivatives particularly useful for biomedical applications, such as wound healings drug delivery, cartilage tissue engineering and nerve generation .Chitin and chitosan are used as membrane for drug delivery and also in tissue engineering. This in turn increases the need of highly purified enzymes. Chitinases are also used in anti-cancer therapy (Dahiya *et al*., 2006)

1.3 Roles of chitinases: Chitinases are present widely in various organisms like viruses, plants, animals, fungi, bacteria, insects and it plays a diverse role in these organisms. Chitinases are involved in many physiological and bioconversion processes. It plays a major role in nutrition and parasitism in bacteria. In case of plants and vertebrates, it is involved in the defence mechanisms. Chitotriosodase enzyme was also used as marker of lysosomal storage disorder. Chitinase enzyme also has activity in human serum too and Chitinases participate a major structural role in some fungi and arthropods than source of energy or defence part (Stoykov et al., 2015).

Chitinases (E.C. 3.2.1.14) are a group of enzymes which are responsible for degradation of chitin. They play a pivotal role in recycling chitin in the nature. Chitinases are known to perform many biological functions and they occur in organisms such as bacteria, fungi, actinomycetes, insects and higher plants. Microorganisms produce chitinase in order to utilize chitin as energy source whereas fungi and insect produce chitinases as they are involved in morphogenesis (Shanmugaiah et al., 2008). Thus presence of chitin degrading organisms in nature presents an economical and environmental friendly alternative in order to obtain chitinases. Over the decade, the exploration of microbes as the source of chitinases has increased due to its various applications. Chitinases obtained from microbial sources have been employed in various fields of medicine, biotechnology, food, wastewater and agricultural industries (Riddhi *et al*., 2013). High production costs of chitinases necessitate the need for Scientists to explore and understand the properties of microbial chitinases in order to formulate inexpensive and reliable chitinases mixture. This will serve two purposes: reducing the environmental hazard and generating various value added compounds of industrial Interest .In our present study to optimization of cultural conditions for production of chitinase by *Bacillus Sp* isolated from marine water using substrate as marine crab shell waste. (Kitamura and Kamei 2003)

2. MATERIALS AND METHODS

The marina water sample were collected from the kannyakumari and isolate the bacterial strains from the samples by serial dilution method (Dubey and Maheshwari.,2002) using the plates from the above experiment, observe the colony draw and name the colony draw and identified the Microorganism by staining method.

2.1 Biochemical characterization: (IMVIC Test): Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. The purified bacterial culture was subjected to a range of biochemical tests for identification.

2.2 Preparation of chitin substrates for enzyme assay: In our experiment, chitinase production was observed towards the substrate, marine crab shell waste powder as well as its combination with colloidal chitin. Each substrate was prepared at three following concentrations: 0.5%, 1.0% and 1.5%.

2.3 Preparation of chitin powder from marine crab shell waste: The crustacean waste powder used in this experiment was prepared from crabs waste. Preparation of the crustacean waste powder was carried out according to the method modified by (Oktavia *et al.,* 2005). Colloidal chitin was made from commercially available chitin powder (Sigma) based on the method as described by (Arnold and Solomon 1986).

2.4 Optimization of chitinase production: Production of chitinase from the bacterium was performed by growing it at 55°C in the 150-ml broth Minimal Synthetic Medium (MSM) containing 0.1% 2HPO4, 0.01% MgSO4.7H2O, 0.1% NaCl, 0.7% (NH4)2SO4, 0.05% yeast extract. Then the bacterial cultivation was carried out with crustacean waste powder at different concentrations (0.5, 1.0, and 1.5) and then culture sampling was conducted regularly everyday for 6 days. In addition, chitinase production was also observed towards the mixture of colloidal chitin and crustacean waste powder.

2.5 Extraction of chitinase: Isolate extracellular chitinase, 10-ml cell culture was centrifuged at 10.000 rpm, 4°C for 25 minutes. The resulting cell-free supernatant was used further for activity assay. Chitinase activity was determined by a dinitrosalicylic acid (DNS) method (Miller, 1959). This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymic action.

2.6 Influence of temperature on crude extract: The minimal synthetic medium was prepared for the optimization condition under different temperature (17°C, 27°C, 37°C, 47°C and 57°C). The minimal synthetic medium was prepared and the marine bacillus *sp* (inoculum) were inoculated and incubated at different temperature of (17°C, 27°C, 37°C, 47°C and 57°C) after incubation the crude were extracted and tested for antimicrobial activity (National Committee for Clinical Laboratory Standards, 1997).

2.7 Influence of other factors on crude extract: The minimal synthetic medium was prepared for the optimization condition under different pH (5, 6, 7, 8 and 9) Carbon and nitrogen sources. The *bacillus sp* (inoculum) was inoculated and incubated at optimized temperature of 47°C. After incubation the crude were extracted and tested for antimicrobial activity.

2.8 Partial purification of enzyme: Purification steps were carried out with the help of two different methods of ammonium sulphate precipitation and ion-exchange chromatography. The eluted samples were assayed for enzyme activity and separate of protein and estimate molecular weight by SDS- PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) and preserved enzyme by immobilization method (Meena and Raja, 2004).

3. RESULTS AND DISCUSSION

3.1 Isolation of bacteria (*Bacillus spp.*): The marina water sample was serially diluted and spread into the nutrient agar medium. After incubation period the colonies were counted and the dominated colonies were picked out and their morphological characterization was studied. The dominated colonies were streaked and the pure culture was stored for further studies. Based on morphological, microscopical and biochemical characterization, the bacterium was identified as *Bacillus subtilis* and *Bacillus cereus*

3.2 Preparation of chitin substrates for enzyme assay: In our experiment, chitinase production was observed towards the substrate, marine crab shell waste powder as well as its combination with colloidal chitin. Each substrate was prepared at three following concentrations: 0.5%, 1.0%, and 1.5%. The chitinase production was well good in the percentage of 1.5% concentrations was shown in (Fig.1).

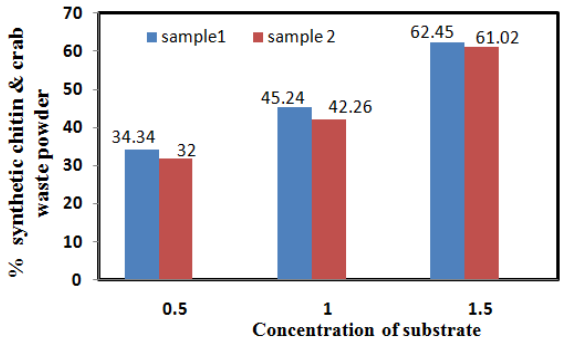

Fig.1: Chitinase assay crude (different substrate in different concentration)

3.3 Effect of pH: The effect of initial pH of the medium on chitinase production was studied by adjusting the initial pH from 6 to 10 by using 0.1N HCl- NaOH. The optimization of cultivation condition for the production of chitinase with different pH was carried out. The best pH for the production of chitinase was shown in (Fig. 2).

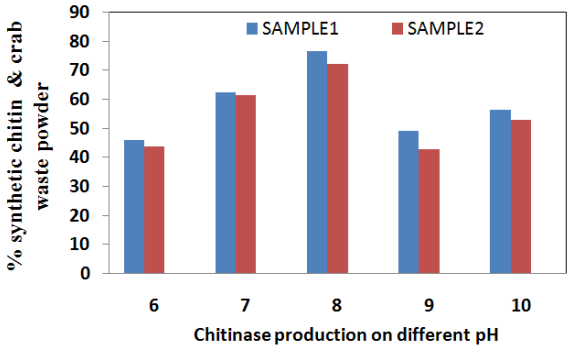

Fig.2: Chitinase assay on different pH

3.4 Effect of temperature: The effect of temperature of the medium on chitinase production was studied by altering the incubation temperature. The temperature was adjusted in the range from 17°C to 57°C. The best temperature for the production of chitinase was shown in (Fig 3).

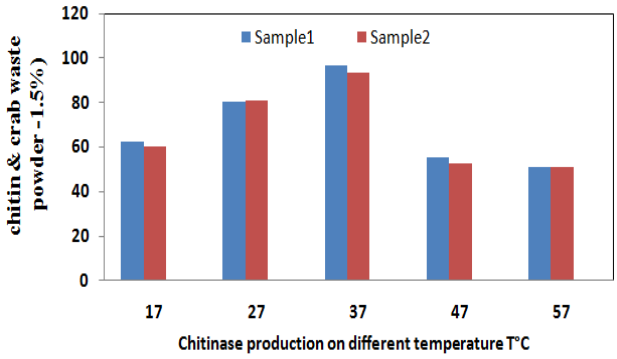

Fig.3: Chitinase assay on different temperature

3.5 Effect of carbon source in different concentration: Carbohydrate utilization by bacteria for the production of chitinase enzyme in aerobic fermentation was studied. Different carbon sources were used in 50ml level in the cultivation medium (Glucose, sucrose, lactose, maltose and fructose). The optimization of its cultivation conditions for the chitinase producing with different carbon source. The best carbon source for the production of chitinase, the activity was shown in (Fig. 4).

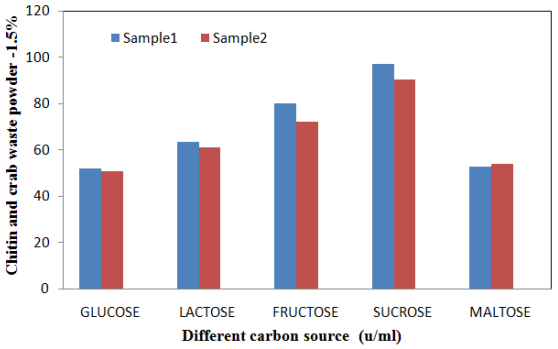

Fig.4: Chitinase assay on different carbon source

3.6 Effect of nitrogen source in different concentration: Nitrogen utilization by bacteria for the production of chitinase enzyme in aerobic fermentation was studied. Different nitrogen sources in 50ml level in the cultivation medium. The optimization of its cultivation conditions for the chitinase producing with Nitrogen source in different concentration. The best nitrogen source in different concentration for the production of chitinase activity was shown in (Fig.5).

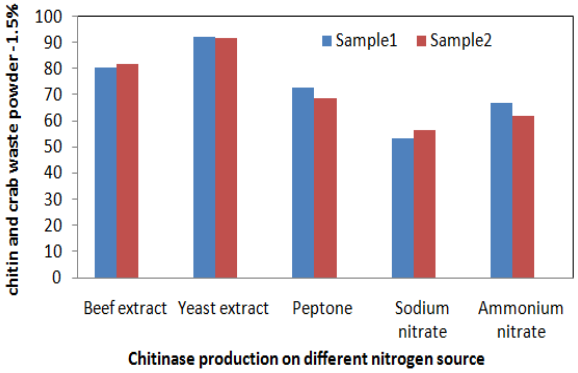

Fig.5: Chitinase assay on different nitrogen source

3.7 Optimization of culture conditions: Chitinase produced by *Bacillus subtilis and Bacillus cereus* were incubated under all optimal nutritional and environmental fermentation conditions. These properties include: effect of pH, effect of temperature, effect of carbon source in different concentration, effect of nitrogen source in different concentration table 1.Among the two species Bacillus species, the *bacillus*

subtilis possess the high chitinase production 62.45 which was shown in fig 2. The optimization of the medium with the pH of the 8, temperature 37°C, carbon source-sucrose, nitrogen source –yeast extract. Similarly, the chitinase production in our experiment was probably induced by the chitin added into the growth medium. Optimization of chitinase production was carried out by cultivating *Bacillus* sp. in the media containing crustacean waste powder at three different concentrations (0.5, 1.0, and 1.5%).

In addition to that, chitinase production was also observed towards the mixture between crustacean waste powders and colloidal chitin at such three different concentrations. It was assumed that the continuous production of chitinase was due to the enough availability of chitin in the growth medium, as the result of the slow rate of chitin biodegradation during the cultivation. Subsequently, the slow rate is probably due to the low solubility of crustacean waste powder in the medium.

3.8 Purification: The extracted sample was determined activity of enzyme initial treatment of Ammonium salt with stirring. The dialyzed sample was purified with DEAE (Di Ethyl Amino Ethyl cellulose) column. The eluted samples were collected and assayed chitinase enzyme activity, protein assay (Table 2). This molecular mass was confirmed by single band in SDS-PAGE. The protein is a monomeric had a molecular mass of *Bacillus subtilis* and *Bacillus cereus,* The produced enzymes were entrapped with Sodium alginate and Calcium chloride.

Table.1 Optimized production medium for chitinase assay

Organisms	PH	Temperature (°c)	Carbon source in different concentration (sucrose) (g/ 50ml)	Nitrogen source in different concentration (yeast extract) (g/ 50ml)	Enzyme concentration (u/ml)
Sample1	8	37°C	1.50	0.25	**192.64**
Sample2	8	37°C	1.50	0.25	**187.00**

Table.2 Chitinase assay for purification

Content	Enzyme concentration (u/ml) by salt cut	Enzyme concentration (u/ml) by ion-exchange
Sample1	197.11	196.56
Sample32	190.50	194.00

4. DISCUSSION

Chitinases are group of enzymes which play a significant role in degrading chitin. Chitinase is involved in the process of producing mono and oligosaccharide from chitin. The present study chitinase production with crustacean waste powder at different concentrations of substrate (0.5, 1.0 and 1.5%) was increasing. Among the two forms of substrate with three different concentrations, the combined substrate (crustacean waste powder and colloidal chitin) of 1% was found to be the best substrate for optimal chitinase production by Bacillus sp. Subsequently, the optimal production with such substrate was achieved at pH8.

For further work, it is necessary to over express the enzyme in the suitable production, which will be followed by the purification and characterization of the chitinase in order to know its properties.

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