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SCREENING AND IDENTIFICATION OF CHITIN DEGRADING BACTERIA FROM SHRIMPSHELL WASTE DUMPING SOIL ENVIRONMENT AND ITS MEDIA OPTIMIZATION FOR CHITINASE ENZYME PRODUCTION

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ABSTRACT

Chitin is a versatile, environmental friendly and modern material. It is found in our biosphere in abundance and its recycling is essential for an ecological balance. In this work, organisms which produce large clear zone on the colloidal chitin agar were selected as potent chitinolytic organisms. Among the chitinolytic organisms, in the present work soil isolate *Bacillus licheniformis* SSCL10 was found to produce highest amount of chitinase on colloidal chitin agar. The three media tested, colloidal chitin amended with minimal media supported highest chitinase production than Nutrient broth and Luria Bertani broth. Among five different concentrations tested, colloidal chitin at 1% considerably enhanced the chitinase activity (2.4 Units /ml), followed by colloidal chitin at 0.5% (2.1units/ml), 0.3% (0.8 units/ml). Beyond 0.3%, the substrate concentrations decreased the enzyme activity. According to our study, maximum chitinase production was observed at neutral pH and *Bacillus licheniformis* produced enormous chitinase activity (2.5 unit/ml) at 40^0C.

KEYWORDS: Chitinase enzyme, shrimpshell waste, chitinolytic microorganisms etc.

INTRODUCTION

At present aquaculture is in focus as an answer to the growing demand for food. Shrimp industry is a rapidly growing industry in India and all over the world. But major concern of the seafood industry is the large amount of waste materials. They are highly perishable in nature as they are quickly colonised by spoilge microorganisms and can rapidly transform

into a public health hazard (Sini *et al.*, 2005). The annual worldwide commercial production of crustaceans was 10,795,000 ton in 2008 (FAO, 2010). In India, Shrimp waste constitutes more than one lakh tonnes per year (Philip and Nair, 2006). Environmental implication of traditional disposal methods of shell fish waste, coupled with the strengthening of environmental regulations in many countries has created an interest in alternative methods of disposal/utilization of this waste (Wang *et al.*, 2010). This waste can be utilized as an economic source of chitin and its derivative chitosn which are considerably versatile and promising biomaterials (Domard, 2011; Tarafdar and Biswas, 2013).

The biological conversion of chitin by means of microbial chitinases is mainly responsible for the replenishment of carbon and nitrogen to the atmosphere, thereby maintaining the ecological balance (Nawani and Kapadin, 2003). The enzymes themselves have been found to be antifungal and nematicidal agents for agricultural biocontrol methods (Gohel *et al.*, 2004). The disposal of the seafood wastes, particularly shrimp waste without proper treatment in the environment causes biofouling. Hence the identification of the microbes with efficient chitin degradation and it can be used for soil reclamation. The present study was aimed to isolate efficient chitin degrading bacteria from the seafood processing industrial waste polluted soil. For this purpose, soil samples were collected from shrimp shell wastes dumping areas located in and around Thoothukudi which is one of the major sea food processing and exporting city in Tamil Nadu. This study primarily gives an idea of the ecological effects on chitin degradation attribute of bacteria.

MATERIALS AND METHODS

Powder chitin was obtained from Hi-media, India. N-acetyl D-glucosamine was obtained from sigma Co, St. Louis, USA. All other reagents used were analytical grade.

Methods

Collection of sample

A total number of 10 different soil samples were collected from 6 sites of shrimp shell waste dumping area in sea food industry of Thoothukudi District, Tamil Nadu, India. 10g of soil sample was collected from each selected site carefully in an aseptic condition and the samples were placed in a sterile container and kept on ice until returned to the laboratory.

Preparation of colloidal Chitin

Colloidal Chitin was prepared from the Chitin powder (Hi-media, India) by the method of Mathivanan (1995). 5 grams of chitin powder was added slowly to 60 ml of concentrated HCl (10N HCl) and kept overnight at 4^0C with vigorous stirring. The mixture was added to 500ml of ice-cold 50% ethanol with vigorous stirring at 25^0C and kept in the rotary shaker at 200 rpm overnight. The precipitate was collected by centrifugation at 10,000 rpm for 20 minutes and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze dried to powder and stored at 4^0C until further use.

Preparation of colloidal chitin agar medium

Medium for colloidal chitin agar plate preparation was prepared by mixing 5 g of colloidal chitin with the following mineral salts:

KH_2PO_4 - 0.7 g

K_2HPO_4 - 0.3 g

$MgSO_4.5H_2O$ - 0.5 g

$FeSO_4.7H_2O$ - 0.001 g

$ZnSO_4$ - 0.001 g

$MnCl_2$ - 0.001 g

Agar - 20 g

Distilled water -1000 ml

pH

Methods

Screening and isolation of chitinolytic microorganisms on colloidal chitin agar plates

A total number of 10 different soil samples were collected from several sites of shrimp shell dumping area in sea food industry of Thoothukudi. Chitin degraders were isolated by serial dilutions of soil samples and plated on 0.5% Colloidal chitin agar (CCA) medium. After 5 days of incubation at room temperature, the isolates capable of degrading chitin with distinct zone of clearance on CCA were selected and sub-cultured in Nutrient Agar slants and maintained (Shanmugaiah *et al.*, 2008).

Primary screening of chitin degrading bacteria

Primary screening was performed by spot inoculating all the chitin degrading bacterial isolates on CCA using toothpick heads of 2mm diameter and incubated at room temperature. The zone of clearance due to chitin hydrolysis was recorded up to 5 days. The bacterial

isolates producing clear zone over 5mm alone were selected and subjected to secondary screening.

Secondary screening of chitin degrading bacteria

Secondary screening was performed with the culture filtrates of the 16 selected bacterial isolates for chitinase enzyme using well diffusion method. All the16 isolates were grown in nutrient broth containing 1% colloidal chitin. One ml of each test bacterial inoculums with 0.5 OD was inoculated to 100 ml of medium and incubated at 100 rpm in a rotary shaker at room temperature. After 2 days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 15 minutes and the supernatant was collected. Colloidal chitin of 0.5% agar plates were prepared and wells were made using 6 mm sterile cork borer. 100 µL culture filtrate of each isolate was placed in each well and incubated at 37^0C. After 24 hrs, the development of clear zone around the well was observed.

Partial purification of Chitinase

Colloidal Chitin broth (100ml) in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml culture of 0.5OD and incubated at 37^0C for three days. The culture broths were centrifuged at 8000 x g for 20 minutes and cell free supernatant were collected. The clear culture filtrates were saturated with ammonium sulphate to 60 to 70% levels and kept at 4^0C overnight to extract the enzymes. The precipitates were collected by centrifugation at 10,000 rpm at 4^0 C and dissolved in 50 mM Phosphate buffer, pH 7.0 (Bansode and Bajekal, 2006).

Measurement of enzyme activity

Chitinase activity was measured with colloidal chitin as the substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained a 0.5% suspension of the colloidal chitin prepared in a phosphate buffer (50mM, pH 7.0) and the mixture was incubated at 37^0C for 15 minutes. After centrifugation, the amount of reducing sugars produced in the supernatant was determined by the Dinitrosalicylic acid method for estimation of reducing sugars using N-acetyl glucosamine as a standard (Imoto and Yagishita, 1971). One unit of chitinase activity was defined as the amount of the enzyme that produced 1 µmol of reducing sugar per minute (Mathur *et al*., 2011).

Determination of shrimp shell degradation using chitinolytic microorganisms

Determination of shrimp shell degradation was performed by preparing minimal media containing 1g of shrimp shell. The selected isolates SSCL10 and SSCL14 were grown in

nutrient broth containing 0.5% colloidal chitin. One ml of selected bacterial inoculum with 0.5 OD was inoculated to 100 ml of shrimp shell containing minimal media and incubated at 100 rpm in a rotary shaker at room temperature for 4-15 days. After incubation, efficient shrimp shell degrading isolate was identified and selected by completely degrade the shrimp shell within short time. The selected isolates were maintained on nutrient agar slants and used for further studies.

Identification of chitinolytic microorganisms

The identification of microorganism was done by the methods suggested in the Bergey's Manual of Systematic Bacteriology (William *et al*., 1989).

Optimization of cultural conditions of chitinase production

Effect of different culture media on chitinase enzyme production

Three different media namely nutrient broth, Luria Bertani broth and minimal medium amended with 0.5% colloidal chitin were used to determine the growth and chitinase production of the selected bacterial isolates. One ml of strain SSCL10 inoculum with 0.5 OD was inoculated with 100ml of different media and incubated at room temperature in a rotary shaker of 100 rpm. After three days of incubation, the culture medium was centrifuged at 1000 rpm for 15 minutes the supernatant was used for chitinase assay.

Effect of different concentrations of colloidal chitin on chitinase production

Strain SSCL10 was grown on different concentrations (0.1, 0.3, 0.5, 0.8 and 1%) of colloidal chitin amended with minimal medium to determine the optimum concentration of substrate (colloidal chitin) for chitinase production.

Effect of different pH on chitinase production

The effect of pH on chitinase production was determined by strain SSCL10 was grown at different pH range of 3-10. Acetate buffer (50mM) was used for pH 3-6; phosphate buffer (50mM) was used for pH 7; glycine-NaOH (50mM) for pH 10-11 in minimal medium containing 0.5% colloidal chitin to determine the optimum pH for chitinase production.

Effect of Temperature on chitinase enzyme production

The effect of temperature on chitinase production was determined by incubating strain SSCL10 on different temperature of 25, 30, 35, 40 and 45^{0}C in minimal medium containing

0.5% colloidal chitin. The method was used to determine the optimum temperature of chitinase production.

Effect of Incubation period

Strain SSCL10 was grown in minimal medium with optimized growth conditions (1% colloidal chitin, pH 7 and temperature 40^0C up to 6 days). Every day, growth and the production of chitinase was assayed in the culture filtrate.

Activity of chitinase enzyme on crab shell, insect shell and mushroom

The 5ml of partially purified chitinase enzyme (2.5U/ml) was incubated separately into 50ml minimal medium containing 1g of crab shell, insect shell, mushroom with optimized growth conditions (pH 7 and temperature 40^0C upto 15 days).

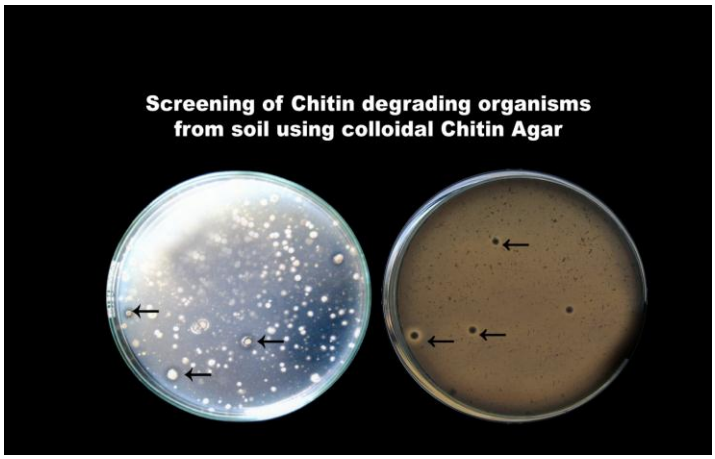
RESULT AND DISCUSSION

Thoothukudi, one of the major port city involved in the processing and export of seafood products next to Cochin in southern India is located in the southern Tamil Nadu with latitude of $8^0$47'10"N, $78^0$7'36" E. The selected study areas where the shrimp waste polluted soil collected were located (Korampallam, Korampallam, SIPCOT, Pudur Pandiapuram, Therespuram and Krishnarajapuram) near seafood industries in and around Thoothukudi city. 10 different soil samples of shrimp shell wastes dumping areas were identified and from there a total of 30 different bacterial types were isolated with chitinolytic activity (Table:1).

TABLE – 1: Isolation of Chitinolytic Bacteria from Soil Sample of Shrimp Shell Dumping Area in Thoothukudi District of Tamilnadu, India

Sl. No.	Sampling Location	No. of Soil Samples	No. of Chitinolytic Bacteria isolated
1	Korampallam	2	7
2	Korampallam	1	3
3	SIPCOT	2	5
4	Pudur Pandiapuram	3	4
5	Therespuram	1	5
6	Krishnarajapuram	1	6
	TOTAL	10	30

In the primary screening, all the 10 soil samples collected show the presence of 30 chitinolytic bacterial types as measured by the zone of clearance in the colloidal chitin agar (fig: 1).



Among them, only 16 bacterial strains produced zone of clearance over 5mm in Colloidal Chitin Agar medium (Table: 2).

TABLE-2: Secondary screening of Chitinolytic Bacteria on Colloidal Chitin Agar

Sl. No.	Bacterial Isolate	Zone of Clearance (mm)
1.	SSCL1	7
2	SSCL2	7.5
3	SSCL3	7
4	SSCL4	8.1
5	SSCL5	9
6	SSCL6	8.5
7	SSCL7	8.3
8	SSCL8	7
9	SSCL9	7.8
10	SSCL10	14
11	SSCL11	8.2
12	SSCL12	7.1
13	SSCL13	9
14	SSCL14	11
15	SSCL15	8.3
16	SSCL16	8.4

The isolates producing clear zones over 5mm alone were selected and subjected to secondary screening. Hsu and Lockwood, (1975) reported that the preparation of colloidal chitin is very important for the screening of chitinolytic microorganisms. In this work, organisms which produce large clear zone on the colloidal chitin agar were selected as potent chitinolytic organisms.

In the secondary screening, the culture filtrates of 2 days old chitinolytic bacterial types were tested for the presence of chitinase enzyme by well diffusion method. The clear zones on Colloidal Chitin Agar medium by the chitinase activity is shown in (Table:2). Interestingly,

only two isolates (SSCL10 and SSCL 14) produced prominent and maximum clear zone of 14 mm and 11 mm respectively and the remaining 14 isolates ranged between 7 mm and 9 mm zone of clearance in Colloidal chitin Agar medium. Table 2 shows the CCA plates with clear zones by the bacterial isolates SSCL10 and SSCL14. These two isolates were selected and preserved for further investigation.

Based on the physiological characterization of Bergy's manual, isolate SSCL10 was identified as *Bacillus licheniformis* and isolate SSCL14 was as *Bacillus subtilis*. To check the chitin degradation ability, the two bacterial isolates were grown on the minimal medium with shrimp shell as substrate for the extracellular chitinase enzyme. Among the two, *Bacillus licheniformis* SSCL10 rapidly degrade the shrimp shell completely under laboratory conditions within12 days while the other isolate *Bacillus subtilis* SSCL 14 took 15 days for the same activity (fig:2).

Since the bacterial isolate *B. licheniformis* SSCL 10 was efficient in natural chitin degradation in the minimal media, it was selected for further studies. Among the chitinolytic organisms, soil isolate *Bacillus licheniformis* SSCL10 was found to produce highest amount of chitinase on colloidal chitin agar. The same work was supported by Waldeck *et al.*, 2006.

Studies on medium optimization for chitinases production are the worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimumzation of the factor. On the other hand, medium optimization is very important not only to maximize the yield and productivity, but also to minimize the production cost (Park *et al*., 2005). Bacterial culture medium plays a vital role in the growth of the bacteria hence each type of medium has different inorganic, organic components and some other

supplements. The growth of *Bacillus licheniformis* SSCL10 reached maximum on fourth day of incubation as measured its density by OD at 660 nm. The cell growth reached log phase at 8 hrs days of incubation was 1.2 OD (Fig:3).

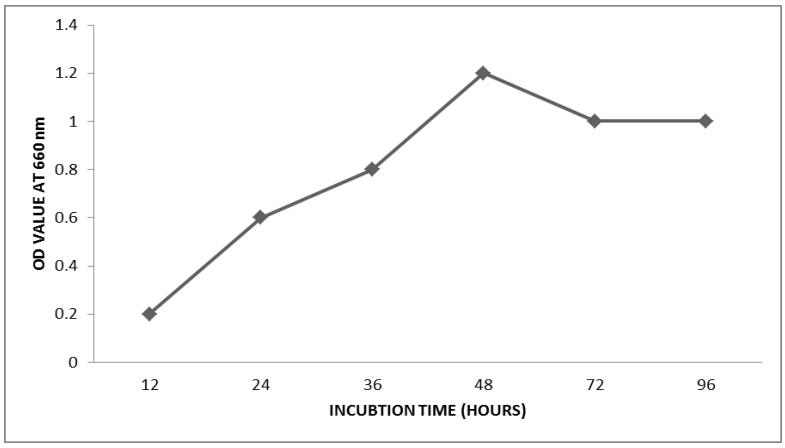


Figure3, Growth curve of *B.licheniformis*

Among the three media tested for maximal production of extracellular chitinase, minimal media with 0.5% colloidal chitin supported high chitinase production (1.8 Units/ml) as compared to Luria Bertani (0.8 Units/ml) and nutrient broth medium (0.2 units/ml) (Fig:4).

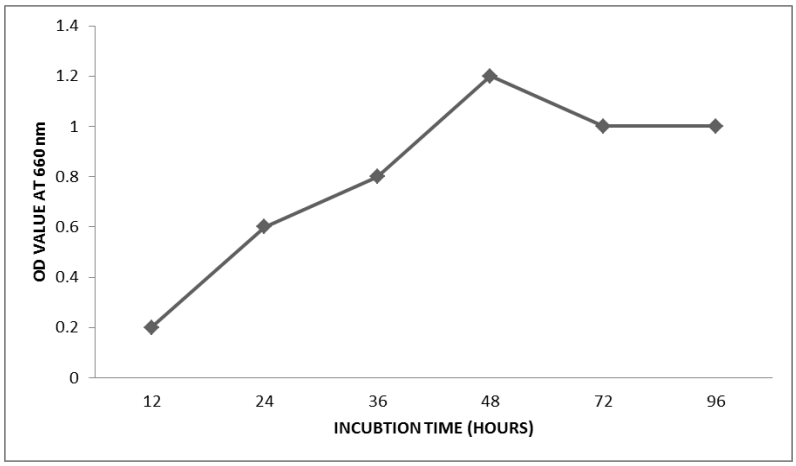


Figure-4, Growth curve of *B.licheniformis* Production of chitinase by *B.licheniformis* SSCL10 in different media (Nutrient Broth, Luria Bertani Broth, Minimal Media), Values are mean of three replicates

Among the three media tested, colloidal chitin amended minimal media supported highest chitinase production than other media because, Akhir *et al*., 2009 reported medium supplemented with low level of yeast extract and peptone and high level of $NaNO_3$ and K_2HPO_4 produced the highest chitinase activity. The chitinase activity increased along with

the cell growth. According to Nampoothiro *et al.*, (2004), the activity of chitinases produced by *Trichoderma harzianum* was most significantly induced by colloidal chitin. In most cases, chitin (colloidal chitin, chitin flakes or chitin powder) was utilized as a carbon source in the production of chitinase (Vaidya et al., 2003; Nawani and Kapadnis, 2005; Gohel et al., 2006).

Colloidal chitin concentration also plays vital factor in inducing high chitinase production (Mathivanan *et al.*, 1997). Among five different colloidal chitin concentrations tested, colloidal chitin at 1% considerably enhanced the chitinase activity (2.4 Units /ml), followed by colloidal chitin at 0.5% (2.1units/ml), 0.3% (0.8 units/ml). Beyond 0.3%, the substrate concentrations decreased the enzyme activity(fig:5). But above (0.5%) of colloidal chitin concentration, chitinase production was significantly increased than the 0.3% and 0.1%. The same work is supported by addition of colloidal chitin 0.5% and 1% induced the maximum chitinase production in *Bacillus sp.* NCTV2 (Wen *et al.*, 2002), *Alternaria alternata* (Sharaf, 2005) and *Trichoderma harzianum* (Sandhya *et al.*, 2005). Similarly, Nakaew's (2000) study demonstrated that specific strains of fungi and bacteria showed high chitinolytic activity in media containing a 1% solution of colloidal chitin.

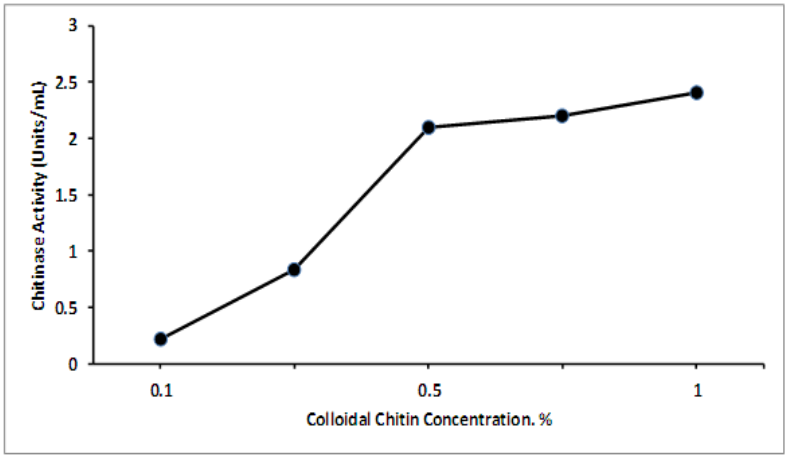


Figure-5 – Production of chitinase by *B.licheniformis* SSCL10 in different colloidal chitin concentration, Values are mean of three replicates

The optimal pH for chitinase production was examined when kept at 40^0C. The chitinase production was highest on pH 7(1.3 units/ml). In pH 6, the chitinase activity was 0.8unit/ml, followed by pH 5 was 0.2 units/ml. In pH 8, chitinase activity was 0.5unit/ml, followed by pH 9 was 0.1 units/ml. chitinase activity was relatively stable at pH between 6.0 and 8.0. However, beyond these pH ranges and above, it rapidly lost its enzyme activity (Fig:6).

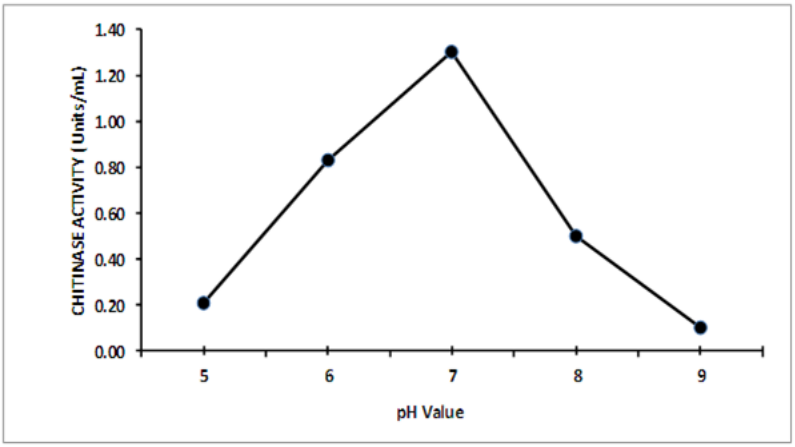


Figure-6, Production of chitinase by *B.licheniformis* SSCL10 in Minimal Media (MM) amended with 0.5% of colloidal chitin in different pH. Values are mean of three replicates

The pH of the culture medium is playing important role in chitinase production. Majority of the bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH (Sharaf, 2005). According to our study, maximum chitinase production was observed at neutral pH. Mubarik *et al.,*(2010) reported *Bacillus* sp. I.5 and *Bacillus cereus* I.21 isolates produced chitinase optimum at pH 7.0. In contrast, Shanmugaiah *et al.*, (2008) reported that *Bacillus laterosporus* MML 2270 produced highest chitinase at pH 8.0.

Among different temperature tested, *Bacillus licheniformis* SSCL10 produced maximum chitinase activity of 2.33 units /ml at 40^0C. It has been observed that in both lower and higher temperature (25, 30, 35, 40and 45^0C) the chitinase activity sharply decreased (Fig:7).

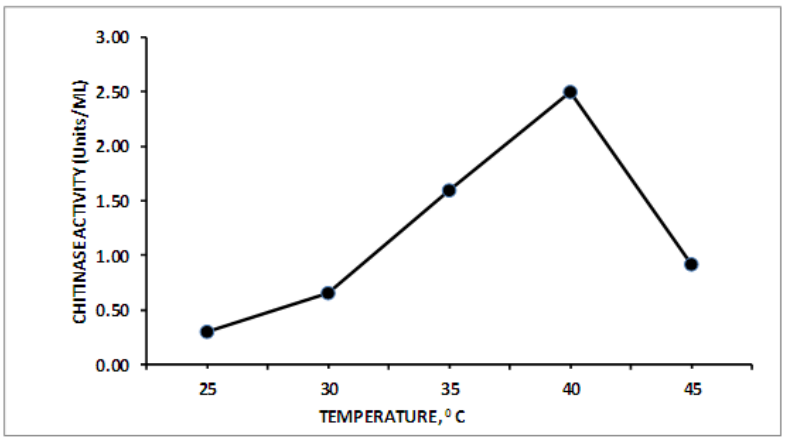


Figure:5 – Effect of different temperature on chitinase Production by *B.licheniformis* SSCL10 in Minimal Media (MM) amended with 0.5% of colloidal chitin at pH 7. Values are mean of three replicates

At 45^{0}C, chitinase activity was 0.92 units /ml, 40 C 35C was 1.6 units /ml and 25^{0}C was 0.30 units /ml. Among the temperatures tested, *Bacillus licheniformis SSCL10* produced enormous chitinase activity (2.35 unit/ml) at 40^{0}C. The same report was supported by Wang *et al.*, 2006. Shanmugaiah *et al.*,*(*2008) reported that *B. laterosporus* produced high chitinase activity at 35^{o}C, in which good bacterial growth has also been recorded.

When the bacterium was grown in all the other standardized parameters, it was observed that *Bacillus lichenformis* SSCL10 produced maximum chitinase production of 3.4 Units/ml on the 4th day of incubation. The chitinase activity gradually declined in subsequent days (fig:8).

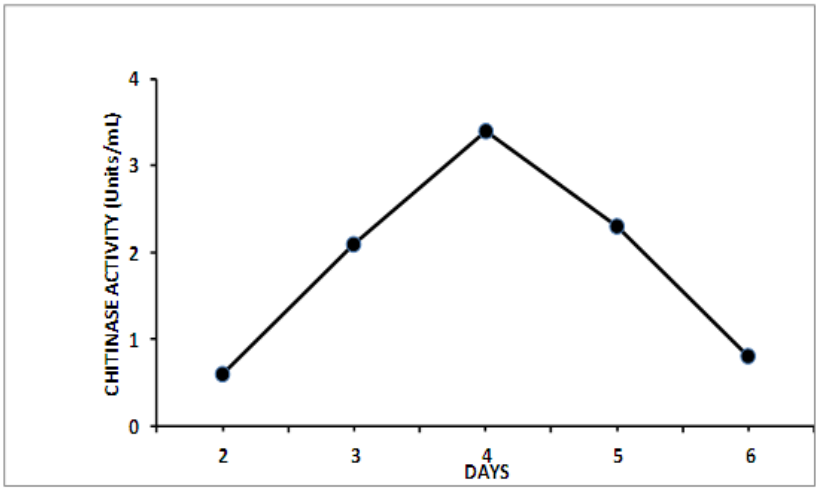
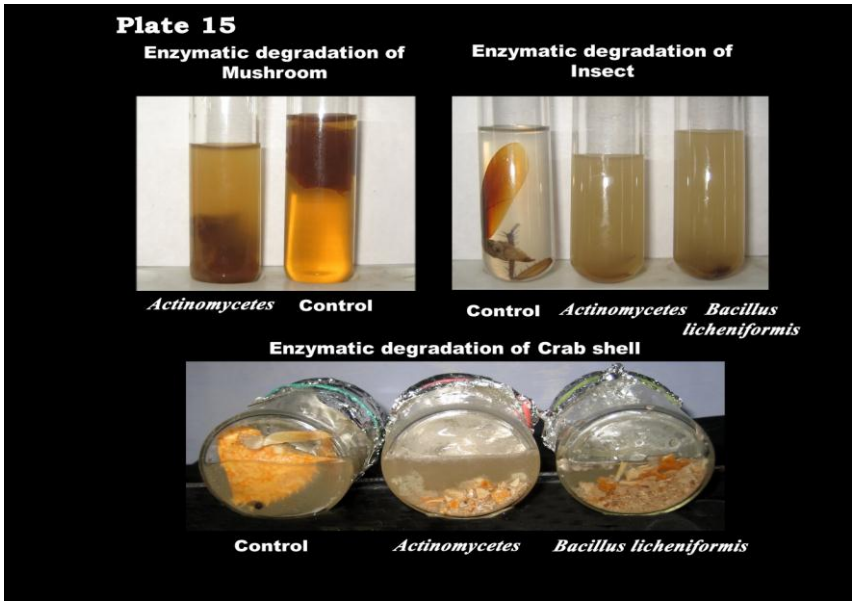


Figure:8 – Production of chitinase by *B.licheniformis* SSCL10 with standardized cultural conditions at different days. Values are mean of three replicates

The chitinase activity was 2.8 Units/ml, 0.8 units/ml on the 5 and 6th day of incubation respectively. The chitinase activity was 2.1 Units/ml on 3rd day of incubation and chitinase activity was 0.6 Units/ml on 2nd day of incubation. The minimum OD(0.013) was recorded after 24 hours of incubation which tended to increase till 96 hours (3.4unot/ml).

To check the specific activity of the extra cellular Chitinase activity on naturally available chitin substrates, partially purified chitinase enzyme from the culture filtrate *B. licheniformis* SSCL10 was incubated separately with different natural chitin substrates from crab shell, cockroach shell, *Plerotus* mushroom using standard assay methods (Fig:9).



Following incubation, the degradation of different substrates was observed visually. Among the substrates, cockroach shell degraded very quickly (after 4 days of incubation) followed by crab shell (8 days of incubation), while the plant chitin from *Plerotus* mushroom take degradation more than 10 days. Chitinase enzyme is very important in the biological control of insects (Reguera and Leschine, 2001). This research also observed the ability of chitinases of *Bacillus licheniformis* SSCL10 was degrading exoskeleton of cockroach effectively. Chitinase produced by *Bacillus licheniformis* SSCL10 was a potential bacterium to be improved and used as a biocontrol agent for insects such as cockroach. Application of chitinase also can be done by spraying it directly to the household things. Similarly, Leaves and fruits of chitinase sprayed strawberry showed absense of any insects bite nor pathogenic fungi (Koga, 2005). *Bacillus thuringiensis* proved to be active against Lepidopteran insects (Moustafa *et al.*,1999). Moreover, chitinase may be applied as insecticides and fungicides to control pests and fungal pathogens of plants respectively (Merzendorfer and Zimoch, 2003).

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