Comparative Analysis of Chitinase Activity by Four Different Assay from Soil Born *Actinomycetes*

Sneha C. Jha * and Hasmukh A. Modi

Department of Life Sciences, University School of Sciences, Gujarat University, Ahmedabad- 380009, Gujarat, India

Abstract: **Eleven Actinomycetal isolate were selected that showed chitin degradation. Four different methods were tested for measuring chitinase activity by ten different Actinomycetal isolate which included p-DMAB method, Boric acid method, DNSA method and Schales method. Amongst these assay, p-DMAB method was selected as it was most sensitive and reproducible results were obtained. Chitinase activity was found maximum by SP24 isolate by all the four assays.**

Keywords**: Chitinase, plant growth promotion, *Streptomyces rubiginosus* SP24, p-DMAB, potassium tetraborate method, DNS assay and Schales method**

I. INTRODUCTION

Actinomycetes, Gram positive filamentous bacteria have a high G+C content. Most important function of this group is to decompose organic matter such as chitin, cellulose, lignocelluloses and starch with an aid for formation of enzymes corresponding to it. *Streptomyces sp*. is one of the main microbes which have high ability for formation of various lytic enzymes. As reported by Gupta et. al. (1995) amongst *Streptomyces sp*. screened *S. viridificans* was found to be good chitinase producer. Amongst those lytic enzymes, one such enzyme is chitinase, which has ability to degrade cellwall of phytopathogenic fungi, partial degradation of exoskeleton of invertebrates, nematodes and thus making it important in biological research (Felse and Panda 2000, Saadoun I, 2009, Honee & Visser, 1993, Ruiz-Herrera & Martinez-Espinoza, 1999). Chitinase enzyme breaks chitin into oligomers or monomer such as N-acetyl D-glucosamine. Chitinase enzyme is divided into three categories such as exochitinase, endochitinase and N-acetylglucosaminidase demonstrating its activity for formation of tetrameric, trimeric, dimeric monemeric units of N-acetyl glucosamine. Different methods having different substrate or reagents are used for estimating chitinase activity.

Hence, our research focus is first isolating *Actinomycetes* that were able to degrade chitin and simultaneously producing chitinase enzyme in sub-merged condition. Secondly, with different assay determining best suitable method for estimating chitinase activity.

II. METHODOLOGY

Isolation of Actinomycetes

Soil samples were collected in sterile polypropeline bags from the rhizosphere of *Gossypium* sp. (Cotton) grown in Dastan farm, Naroda, Gujarat. For isolation, Bennet's agar medium was used. First, little amount of the soil from different fields were taken in petriplates and on them calcium carbonate was sprinkled, these was kept for drying for a period of 4-5 days to allow *Actinomycetes* to sporulate. Dilutions (10^{-3} to 10^{-6}) of enriched soil samples were plated which were incubated at 30°C. Desired colonies culturally appeared as *Actinomycetes* were picked up and restreaked for obtaining pure colonies. *Actinomycetes* were isolated by the serial dilution and spread plate techniques (Jha S. et. al 2016).

Colloidal chitin preparation

Chitin flakes (10 gm) were suspended in concentrated HCl (60 mL) and incubated on rotary shaker for 1-2 hr at room temperature until chitin flakes were completely dissolved. The resulting solution was then poured into doubly deionized water (400 mL) with rapid stirring to form the precipitates of colloidal chitin, which were subsequently collected by centrifugation at 7000 xg for 10 min at 4°C. The precipitates were washed with sterile distilled water (10 mL each) several times to bring the pH value of the colloidal chitin suspension to 2.0-3.0. After neutralization with sodium hydroxide (1 M), the low-salt colloidal chitin was obtained on repeated centrifugation (7000 xg, 10 min) and washing with sterile water 2-3 times. The colloidal chitin was kept at 4°C as a pellet for further applications (Wu, Cheng, & Li, 2009).

Screening of Chitinase producing Actinomycetes:

Actinomycetal isolates obtained from Bennet's agar media were then screened for chitin hydrolysis by spoting on the centre of 1% CCA (Colloidal Chitin- 1%, $NaNO_3$-0.2 gm, K_2HPO_4-0.1 gm, $MgSO_4$-0.1 gm, $CaCO_3$-0.1 gm, $FeSO_4.7H_2O$-0.001 gm, KCL-0.05 gm) media at neutral pH and were incubated at room temperature. The zone of clearance due to chitin hydrolysis was recorded up to 8 days. The isolates those formed clear zones above 8 mm were selected. Actinomycetal isolate were selected on the basis of zone of degradation i.e. Selection ratio

Chitinase Assay by different assay:

1) p-DMAB assay

The reaction mixture contained 1 ml of 0.1% colloidal chitin in phosphate buffer (0.05 M, pH 7.0) and 1 ml culture filtrate

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was incubated at 37ºC for 1 hr. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by following the spectrophotometric method of Reissig et. al. (1955). The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, (pH 9.2) to 0.5 ml of reaction mixture and then was boiled in a water bath for 3 min. Then 3 ml of diluted p-dimethylaminobenzaldehyde [10 gm of DMAB dissolved in 100 ml of glacial acetic acid containing 12.5% 10 N (v/v) hydrochloric acid] reagent was added and again incubated at 37ºC for 15 min. The released product in the reaction mixture was read at 585 nm in a visible spectrophotometer. Chitinase activity was determined using N-acetylglucosamine (Hi-media) as the standard. One unit of chitinase activity is defined as the amount of enzyme, which produces 1 µ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition (Mathivanan et al., 1998).

2) Boric acid assay

Chitinase activity was assayed by the method of Yanai *et al.* (1992). The reaction mixture consisited of 250µl of 0.5% colloidal chitin, 250 µl of 0.2 M sodium acetate buffer (pH 4), and 500 µl of enzyme (crude enzyme) solution was incubated for 2hr at 37°C. After centrifugation at 3000 rpm for 15 mins, 500 µl supernatant was mixed with 100 µl of 0.8M boric acid, and the pH of this mixed solution was adjusted to 10.2 with KOH. The solution was heated for 3 min in boiling water. After the mixture was cooled, 3ml of *p*-dimethyl aminobenzaldehyde (DMAB) (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% 6N V/V hydrochloric acid) was added and the mixture was incubated for 20 min at 37°C. Absorbance at 585 nm was measured against water as a blank.

3) DNSA assay

The reaction mixture consisted of 2.5 ml buffer, 2.5 ml 1% colloidal chitin followed by 0.5 ml of crude enzyme. Tubes were incubated at 37°C for 1 hr. The reaction was stopped by adding 3.0 ml of 10% DNSA and heating in boiling water bath for 15min. The colored solution was centrifuged at 8000×g for 5 mins at 4°C. The absorption of supernatant was measured at 540nm spectrophotometrically. The reducing sugar was estimated from standard curve of glucose.

4) Modified Schales assay

In this assay, 96-well Microtitre plates were used for estimation of chitinase activity. The reaction mixture contained 1ml of 1% colloidal chitin in phosphate buffer (0.05, pH 7.0) and 1ml culture filtrate was incubated for 1 hr at 30°C. After incubation, centrifuge mixture at 4°C for 15 mins. About 100 µL of supernatant was transferred in a new plate. A volume of 100 µL of Schales' reagent (a solution of 0.5 M sodium carbonate and 0.5 g/L potassium ferricyanide in water) was added. The plate covered with aluminum foil and was incubated at 100°C for 15 minutes and allowed to cool. Read the absorbance at 420 nm spectrophotometrically. As a positive control, 50 mM of N-acetyl-D-glucosamine was used (Ferrari et. al. 2014).

III. STATISTICAL ANALYSIS

Chitinase activity obtained by different methods after 72 hours of incubation period were analyzed for knowing the significant difference within the assay. Experiment was designed in such a way that five replicates were obtained and mean results were analyzed by performing analysis of variance test (ANOVA) followed by tukey post-hoc test using software IBM SPSS 23.0 trial version (IBM Corp. Released 2015)

IV. RESULTS & DISCUSSION

Actinomycetes were screened on the basis of hydrolysis of colloidal chitin (substrate) against creamy background. Selection Ratio was measured on zone of hydrolysis and colony size. Maximum chitin hydrolysis was observed by isolate SP 24 about 2.13 and minimum hydrolysis was observed by SP 12 about 1.5 as described in Fig. 1.

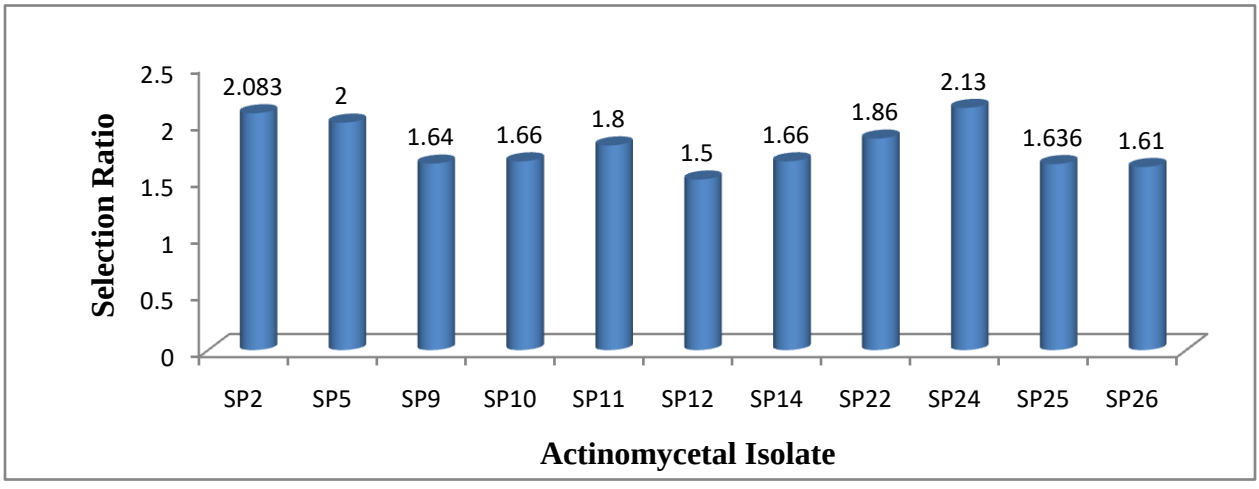

Fig. 1 Selection ratio of Actinomycetal isolate based on chitin degradation

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Eleven *Actinomycetal* isolates were found to give high selection ratio. So, a study was conducted with eleven *Actinomycetal* isolate with different methods to find chitinase production to assess best suitable method. Colloidal chitin as a substrate was most preferred because chitin as such is insoluble in water or buffer or acid/base and hence cannot be digested when incubated with enzyme. It is also cheaper in comparision with other substrates which are commercially available in market. So, for preparation of colloidal chitin, chitin was hydrolysed by two step acid catalyzed process: Depolymerization and De-N-acetylation. Four different reagents were used that produced color based on corresponding method used (Potassium tetraborate method, Schales method and Boric acid method) which measured exact amount of N- Acetyl glucosamine which were formed from colloidal chitin present in medium whereas, By DNS method, all reducing sugars were measured instead of N-acetyl glucosamine. SP24 gave maximum chitinase activity in all four methods. In potassium tetraborate method, SP24 gave maximum chitinase activity after 72 hours of incubation period about 2.79 U/ml (Fig. 2) and in Boric acid method SP24 also gave maximum chitinase activity about 2.06 U/ml (Fig. 3). Comparing selection and reagents of both method, Potassium tetraborate method was selected because it gives more chitinase activity than Boric acid method and it was very reliable method. The compounds which are used in Potassium tetraborate method are also less cheaper. In case of DNS method, maximum chitinase activity was obtained (4.98 U/ml) as shown in fig.4 because it measured all the reducing sugars present in the solution. Hence this method was not specific for measuring N-acetyl glucosamine that was measured by potassium tetraborate method. With Schales method, a modification were made in method of incubation time of substrate and enzyme but still chitinase activity obtained were less about 1.32 U/ml (Fig. 5) in comparision with p-DMAB method, boric acid method and DNS assay. All the four methods used in this research has an advantage of using insoluble colloidal chitin as a substrate in reaction medium which can be separated before addition of acidic/alkaline medium. In potassium tetraborate method, certain chromophoric substances of cyclic intermediate structure of N-acetyl glucosamine was produced on addition of p-Dimethylaminobenzaldehyde under acidic condition (10 N HCL). NAG,Dimers, trimers and tetramers released in medium were further hydrolysed at 100°C. Released product were further treated with DMAB and can be spectrometrically measured (Takahashi T, 2003). Statistical analysis results revealed that values with same letters within the column are not significantly different from each other. Results of chitinase assay for SP24 isolate by statistical software was found to be maximum after 72 hours of incubation period and were analyzed for tukeys test which revealed as most significant among other isolates.

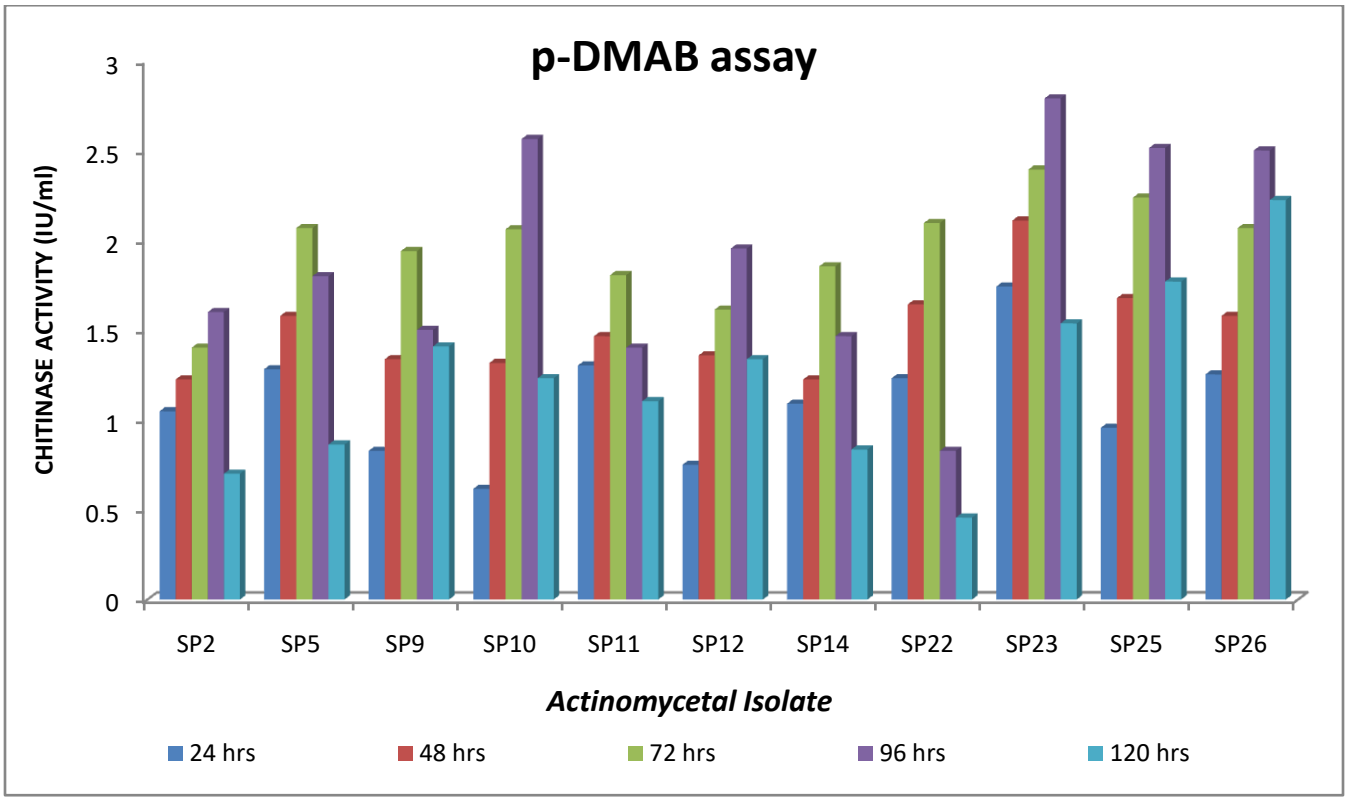

Fig. 2 Chitinase assay by p-DMAB method

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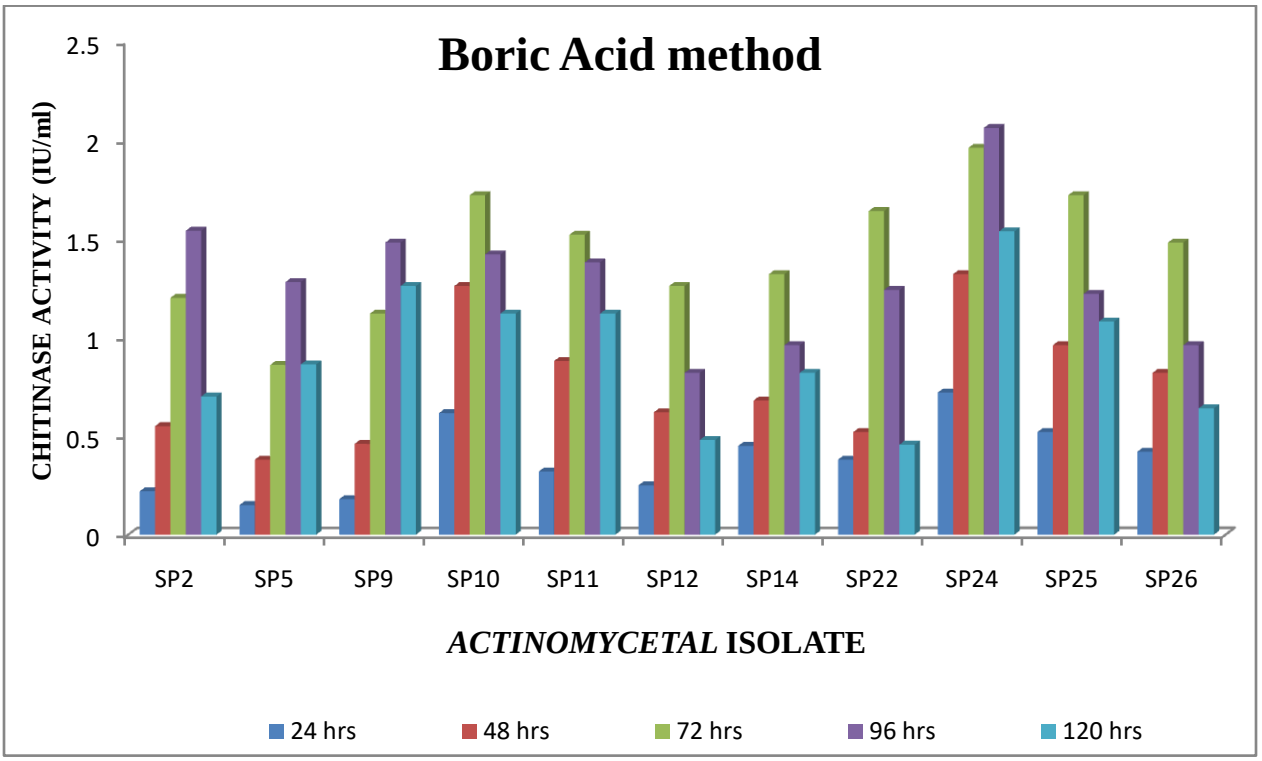

Fig. 3 Chitinase assay by Boric acid method

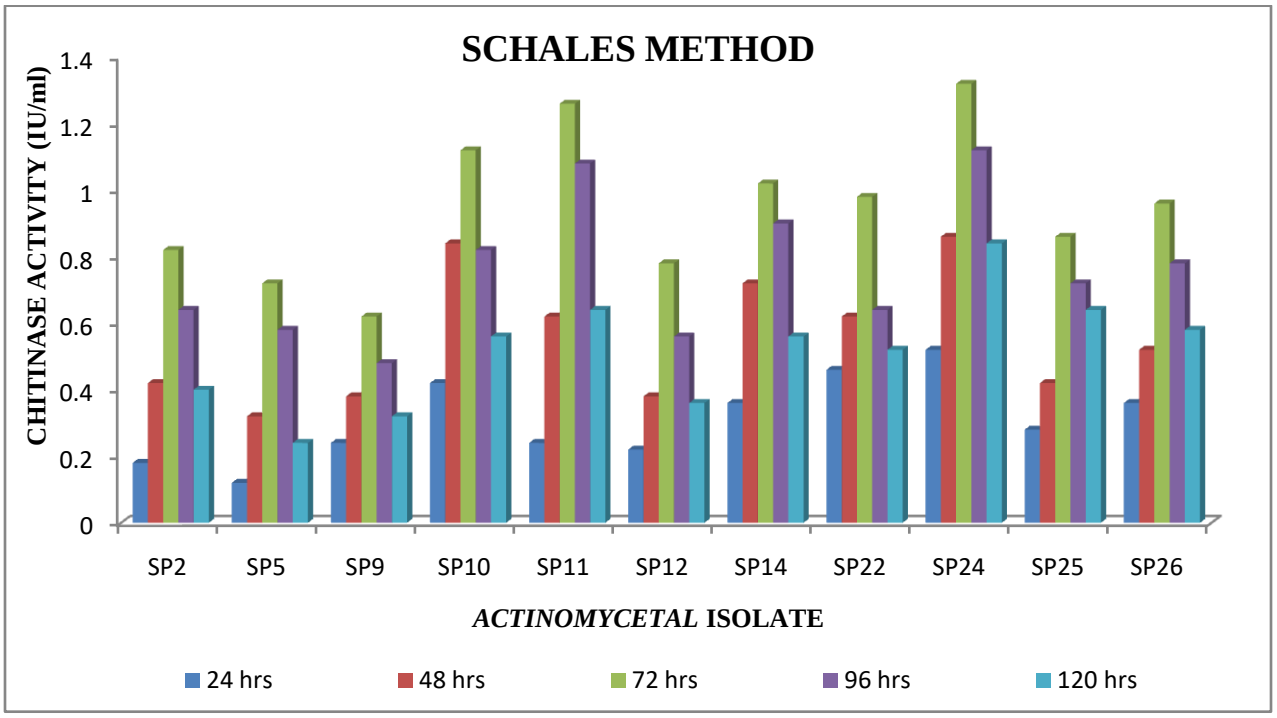

Fig. 4 Chitinase assay by Schales method

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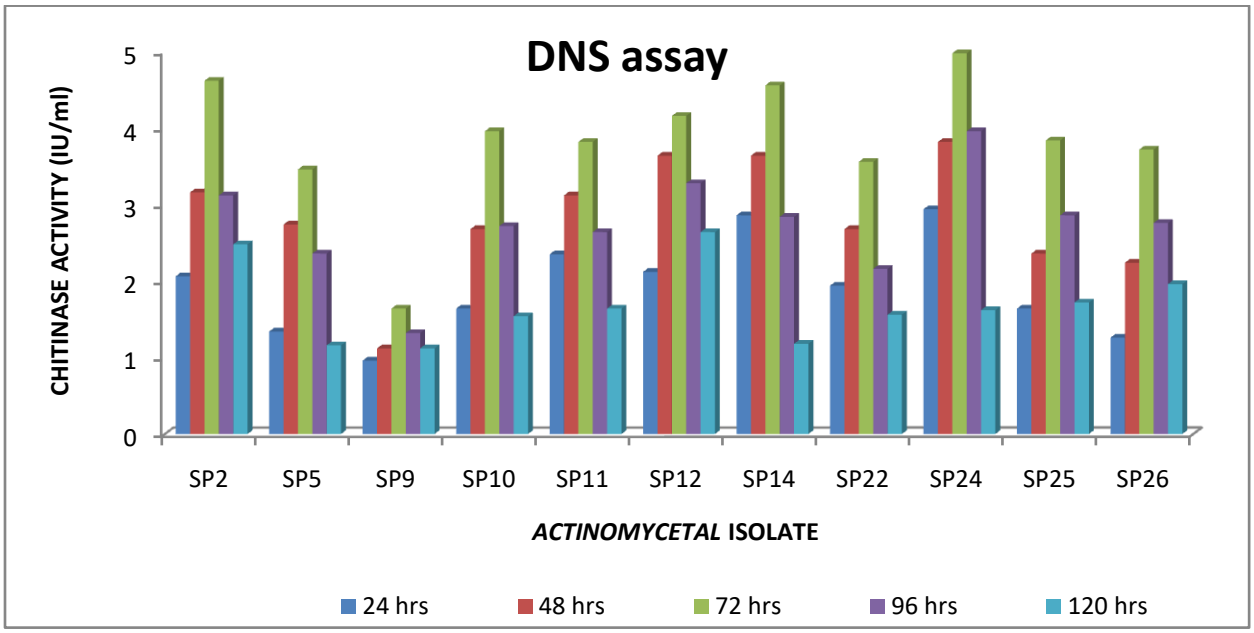

Fig. 5 Chitinase assay by DNS assay

Table 1 Chitinase activity by different assays after 72 hours of incubation period for selected isolates.

Method	P-DMAB	Boric	Modified Schales	DNSA
Isolates	72 hrs	72 hrs	72 hrs	72 hrs
SP2	1.402^{d}	1.2^{cd}	0.82^{cde}	4.62^{b}
SP5	2.068^{ab}	0.86^{e}	0.72^{de}	3.46^{g}
SP9	1.94^{bc}	1.12^{de}	0.62^{e}	1.64^{h}
SP10	2.061^{ab}	1.72^{ab}	1.12^{ab}	3.96^{d}
SP11	1.806^{bcd}	1.52^{bcd}	1.26^{ab}	3.82^{e}
SP12	1.614^{cd}	1.26^{cd}	0.78^{cde}	4.16^{c}
SP14	1.855^{bc}	1.32^{cd}	1.02^{bc}	4.56^{b}
SP22	2.096^{ab}	1.64^{b}	0.98^{bc}	3.56^{fg}
SP24	2.394^{a}	1.96^{a}	1.32^{a}	4.98^{a}
SP25	2.238^{ab}	1.72^{ab}	0.86^{cde}	3.84^{de}
SP26	2.068^{ab}	1.48^{bc}	0.96^{bcd}	3.72^{ef}

Values with the same letters (abcdefgh) within the column are not significantly different and values with different letters within the column are significantly different from each other according to tukey's test .

V. CONCLUSION

In the present investigation, isolate SP 24 was considered to be most potent *Actinomycetes* amongst all tested isolates for giving maximum chitinase activity. Different assays used in present research showed that p-DMAB method was most sensitive, rapid, low cost reagents needed as well as precise for giving maximum chitinase activity. It had added advantage of using colloidal chitin as a substrate which is easily prepared in comparision with other substrate such as glycol chitin and swollen chitin which are too expensive.

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