Screening for Chitinase-Producing Bacteria

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Abstract

Six accessions of bacteria, belonging to four genera from the Microbial Culture Collection and Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH), UP, at Los Baños were selected as chitinase-producing based on their ability to show moderate to heavy growth and form moderate to large zone of clearing around colonies in chitin agar. The chitinolytic bacteria selected were: ***Bacillus circulans*** *BIOTECH 1037 (5Bc),* ***Arthrobacter luteus*** *BIOTECH 1077 (6A1),* ***B. circulans*** *BIOTECH 1045 (7Bc),****Streptomyces griseus*** *BIOTECH 1562 (9Sg), and* ***Serratia marcescens*** *LPC19 BIOTECH 1748 (11Sm) and S.* ***marcescens*** *LPM42 BIOTECH 1749 (14SSm).*

The bacteria, when grown in chitin broth, excreted chitinase enzymes into the culture medium as assayed using turbidimetric and cylinder cup methods.

Introduction

In recent years, the search for alternative methods to curb some pathogens without excessive use of chemical pesticides has become the subject of extensive research. The use of microorganism in the control of certain pathogens drew significant interest among researchers as it holds prospects for an ecologically sound system for pest management. As such, understanding the interactions among microorganisms takes center stage as some of the interactions may be operational in the biological control process.

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Parasitism, a form of microbial antagonism, relies on lytic enzymes for the degradation of cell walls of pathogenic fungi (Chet et al., 1990). Fundamental to the lytic phenomenon is the liberation of various hydrolytic enzymes by some species of bacteria to degrade fungal cell walls. One group of the hydrolytic enzymes is chitinases.

Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin. In plants, the enzymes make-up part of the pathogenesis-related proteins (PR proteins) involved in several biochemical defense mechanisms against fungal pathogens (Flach et al., 1992). Some fungal chitinases may be involved in the growth of the fungus itself (Pedraza-Reyes and Lopez-Romero, 1989), required in cell separation as in *Saccharomyces cerevisiae* (Kuranda and Robins, 1991) or may perform tropic functions. On the other hand, the chitinases produced by certain species of bacteria have been implicated in the biological control process via degradation of the chitinous cell walls of pathogenic fungi, and play a large role in mineralization in marine waters and sediments (Herwig et al., 1988 cited by Flach et al., 1992).

The chitinolytic activity of bacterial chitinases plays an important role in the decomposition of chitin and, potentially, in the utilization of chitin as a renewable resource (Tsujibo et al., 1993). Chitin, a ß-1,4 polymer of N-acetylglucosamine (GlcNAc), is a major structural component of many agronomically important pests including insects, nematodes and fungi. The enzymatic degradation or deformation of chitin in these organisms could present an effective method for their control (Fuchs et al., 1986).

This study aimed to (1) screen for bacteria available from the culture collection of the National Institute of Molecular Biology and Biotechnology (UPLB) that have the capacity to produce higher quantity of chitinases, and (2) test the chitinolytic activity of the crude chitinase extract in medium containing colloidal chitin.

This study provides information on the available accessions of chitinolytic bacteria that could be used as biocontrol agent against certain fungi and as possible sources of chitinase genes to be introduced to plants in order to boost their resistance to fungal attacks.

Review of Literature

In bacteria, chitinases were shown to be extracellular enzymes (Flach et al., 1992) involved in nutrition as many chitinolytic bacteria can grow on chitin as sole carbon and nitrogen source and in pathogenicity in *S.*

marcescens acting as insect pathogen (Gooday, 1991).

Chitin, an insoluble linear ß-1,4-linked polymer of N-acetylglucosamine (GlcNAc), occurs ubiquitous in nature as common constituent in some plants, in exoskeleton of arthropods and in fungal cell walls. As such, certain bacteria play a large role in chitin mineralization. A number of bacteria have been reported to produce chitinases. Campbell and Williams (1951) reported twenty chitinoclastic strains of bacteria composed of several species of *Acrhromobacter, Pseudomonas, Flavobacterium* and *Micrococcus* that were isolated from marine mud by enrichment cultures. Some soil-and water-borne species of bacteria belonging to *Chromobacterium, Pseudomonas* and *Klebsiella* were found to be chitinase-producing (Clarke and Tracey, 1956).

Chitinolytic complexes had also been isolated from culture filtrates of many actinomycetes including *Streptomyces* sp. (Reynolds, 1954; Skujins et al., 1970), *S. griseus* (Berger and Reynolds, 1958), *S. orientalis* (Tominaga and Tsujisaka, 1976), *S. erythraeus* (Kamei et al., 1989), *Streptomyces* sp. S-84 (Ueno et al., 1990) and *S. thermoviolaceus* (Tsujibo et al., 1993).

Monreal and Reese (1969), in testing about 100 bacterial stocks, found *Serratia marcescens* trailing closely. *Serratia liquefaciens* was reported to be lytic to *Fusarium* sp. hyphae (Sneh, 1981) while a certain species of *Enterobacter* (probably *E. cloacae* was found to be chitinase-producing (Yamasaki et al., 1992). Other species of bacteria reported to be chitinoclastic include *Bacillus* sp. which was found to be lytic on *Rhizopus* (Tsujisaka et al., 1973), *Bacillus cereus* (Mitchell and Alexander, 1963), *Bacillus circulans* WL-12 (Watanabe et al., 1990; 1990; 1992), *Bacillus licheniformis* (Takayanagi et al., 1991), *Aeromonas* sp. (Yabuki et al., 1986; Ueda and Arai, 1992), *Nocardia orientalis* (Nanjo et al., 1989), *Arthrobacter* sp. (Morrissey et al., 1976; Sneh, 1981) and probably a lot more.

Materials and Methods

Several species of bacteria have been reported to produce chitinases. All of the reported chitinolytic bacterial species available at the National Institute of Molecular Biology and Biotechnology (BIOTECH), UPLB, were screened for their ability to produce chitinase.

The bacteria were streaked on chitin agar A (Ueda and Arai, 1992) plates, afterwhich they were incubated at room temperature (25-27°C) for one week. The bacteria that formed clear zones were selected and maintained in chitin agar B slants, chitin broth plus 15% agar (Ueda and Arai, 1992).

Production of crude chitinase followed the procedure of Ueda and Arai (1992). The chitinase-producing bacteria selected were grown in chitin broth. One ml of the seed culture (16-18 hour culture) was transferred to 100 ml of the same broth and was incubated for 48h at room temperature for the production of chitinase. The culture broths were then centrifuged for 20 min at 12,000 rpm and the supernatants were assayed for chitinase activity. The cell-free supernatant (crude enzyme extract) was stored in the refrigerator for future use.

Two methods were used to assay chitinase activity in the culture filtrates, namely: turbidimetric (Imoto and Yagashita, 1971) and cylinder cup assay methods (modified from Frandberg and Schnurer, 1994).

A. Turbidimetric method

Preparation of Standard Curve. A volume of 1.5 ml of the different concentrations of N-acetylglucosamine (GlcNAc) (0.02 - 0.20m g) were prepared. Two ml of the color reagent was mixed with the GlcNAc solutions, afterwhich, the mixtures were incubated in boiling water bath for 15 minutes in aluminum-foil covered tests tubes. After cooling, the absorbance (OD) of the mixtures was read at 420 nm against water. The change in OD (ΔOD) was plotted against mg GlcNAc.

Assay Procedure. A reaction mixture containing 1.0 ml of 0.3% colloidal chitin, 2.0 ml of phosphate-citrate buffer (pH 4.0) and 1.0 ml of the crude enzyme extract was incubated for 1 hr at 37oC. Distilled water served as control. After the incubation period, the mixture was spinned to remove the residual chitin. A volume of 1.5 ml of the mixture was added with 2.0 ml of the color reagent, and incubated in boiling water bath for 15 minutes. After cooling, the change in OD (ΔOD) was determined. The amount of GlcNAc was determined based on the standard curve previously prepared. One unit of enzyme activity is defined as the amount which liberateds ! umole of GlcNAc/min.

B. Cylinder cup assay method

Preparation of Standard Curve. Pure chitinase enzyme from *Serratia marcescens* (Sigma) (0.03U/mg) was used. A volume of 0.1 ml of the different concentrations (0.1200U - 0.0012) of this enzyme were prepared. One unit of activity is defined as the amount of enzyme which liberates 1 umole of GlcNAc/min.

Assay Procedures. The same procedures above were followed except

that 0.1 ml of the crude enzyme extract was pipetted into cylinder cups instead of the pure enzyme solutions. Distilled water served as control.

The unit of activity of the supernates was determined based on the standard curve prepared above.

Results

Screening For Chitinase-Producing Bacteria

Fourteen different accessions of bacteria (Table 1) belonging to 8 genera obtained from the Microbial Culture Collection and Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH), UPLB, were screened for chitinolytic activity. Based on their growth and capability to form clear zones in chitin agar (Table 2), six accessions belonging to 4 genera were selected, namely; *Bacillus circulans* BIOTECH 1037 (5Bc), *Arthrobacter luteus* BIOTECH 1072 (6A1), *B. circulans* BIOTECH 1045 (7Bc), *Streptomyces griseus* BIOTECH 1562 (9Sg); and *Serratia marcescens* LPC19 BIOTECH 1948 (11Sm) and *S. marcescens* LPM 42 BIOTECH 1749 (14Sm). A representative chitinolytic bacterium is shown in Figure 1.

As shown in Table 2, screening of accessions of bacteria that grew and formed colonies in chitin agar was carried out for seven days. Among the accessions selected, *Serratia marcescens* (11Sm and 14Sm) and Streptomyces

Table 1. List of bateria tested for chitinolytic activity.

BACTERIA	ACCESSION NO.	DESIGNATION
Micrococcus luteus	BIOTECH 1061	1Ml
Bacillus cereus	BIOTECH 1635	2Bc
B. licheniformis	BIOTECH 1005	3Bl
B. cereus	BIOTECH 1509	4Bc
B. circulans	BIOTECH 1037	5Bc
Arthrobacter luteus	BIOTECH 1077	6Al
B. circulans	BIOTECH 1045	7Bc
Flavobacterium aurantiacum	BIOTECH 1492	8Fa
Streptomyces griseus	BIOTECH 1562	9Sg
B. licheniformis	BIOTECH 1331	10Bl
Serratia marcescens LPC19	BIOTECH 1748	11Sm
Pseudomonas aeruginosa NRRL B-23	BIOTECH 1335	12Pa
Klebsiella pneumonia NRRL B-14232	BIOTECH 1754	13Kp
S. marcescens LPM 42	BIOTECH 1749	14Sm

Figure 1. A chitinolytic bacterium (*Serratia marcescens* LPC19 BIOTECH 1748) showing zone of clearing around colonies in chitin agar A.

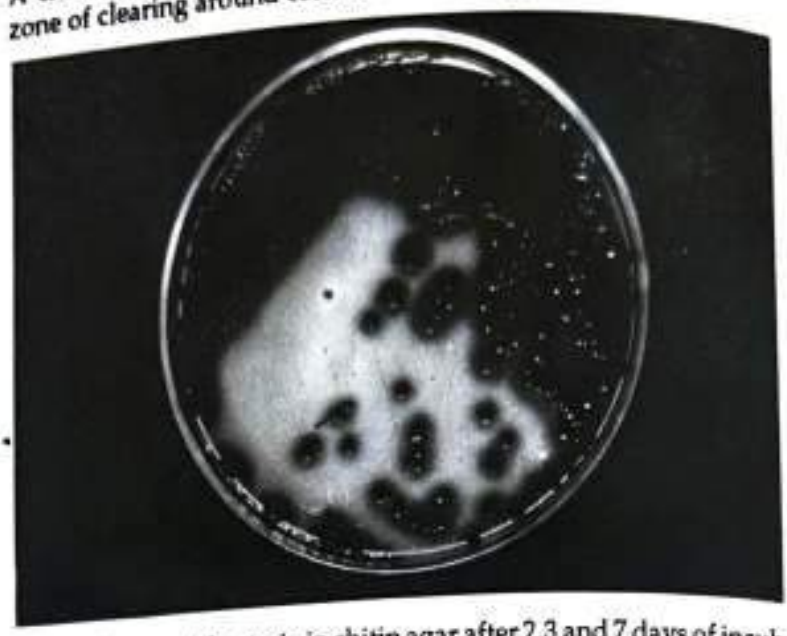

Table 2. Growth of suspect bacteria in chitin agar after 2,3 and 7 days of incubation at room temperature (25-27°C).

BACTERIA	DAY 2		DAY 3		DAY 7	
	Growth	Cleaning	Growth	Clearing	Growth	Clearing
1Ml	-	-	-	-	-	-
2Bc	-	-	-	-	-	-
3Bl	-	-	-	-	+	-
4Bc	-	-	+	-	++	-
5Bc	-	-	+	-	++	+
6Al	-	-	+	+	++	++
7Bc	-	-	+	+	++	++
8Fa	-	-	+	-	+	-
9Sg	+	+	++	++	+++	+++
10Bl	-	-	-	-	+	-
11Sm	+	+	++	++	+++	+++
12Pa	-	-	-	-	-	-
13Kp	-	-	+	-	++	-
14Sm	+	+	++	++	+++	+++

Legend:

Growth

- = no growth
+ = small
++ = moderate
+++ = heavy

Clearing

- = no zone of clearing
+ = minimal
++ = moderate
+++ = large

griseus (9Sg) showed heavy growth and formed large zones of clearing around the colonies of bacteria. *Bacillus circulans* (5Bc and 7Bc) and *Arthrobacter luteus* (6Al) showed moderate growth and formed moderate zones of clearing. All other accessions did not grow or some showed minimal to moderate growth but formed no zone of clearing on chitin agar.

Assay for Chitinase Activity of the Crude Enzyme Extracts

A. Turbidimetric Method

A standard curve (Figure 2) was constructed on the basis of the changes in optical density in the reaction mixtures containing various concentrations of N-acetylglucosamine (GlcNAc) (Table 3). Based on the standard curve, the amount of GlcNAc released per minute in the different reaction mixtures containing 0.3% colloidal chitin and the crude enzyme extract was estimated. Table 4 shows the estimated amount of GlcNAc released in each of the reaction mixtures and the equivalent unit of enzyme activity. A unit of chitinase is the amount of enzyme which catalyzes the release of one micromole GlcNAc per minute. Apparently, all the enzyme extracts showed almost similar enzymatic activity with values ranging from 0.0112 - 0.0117. *S. griseus* had the least activity while the two *S. marcescens* showed the highest activity.

B. Cylinder Cup Assay Method

Pure chitinase enzyme isolated from *Serratia marcescens* (Sigma) was used in the preparation of standard curve (Figure 3) based on the diameter of zones of clearing around the cylinder cups (Table 5) on chitin agar A measured up to three days after plating.

As presented in Table 6, two of the crude enzyme extracts; 6Al, and 9Sg, did not show zone of clearing around the cylinder cup one day after plating. Fourty-eight hours after, 9Sg still did not show zone of clearing. On the third day, all the crude enzyme extracts showed zone of clearing with *S. marcescens* extracts (11Sm an 14Sm) producing the largest measurement followed by the *B. circulans* extracts (5Bc and 7Bc). The *S. griseus* extract (9Sg) produced the smallest zone of clearing. Formation of zone of clearing on chitin agar around the cylinder cup by a crude chitinase extract is shown in Figure 4. The corresponding chitinase activity estimated based on the

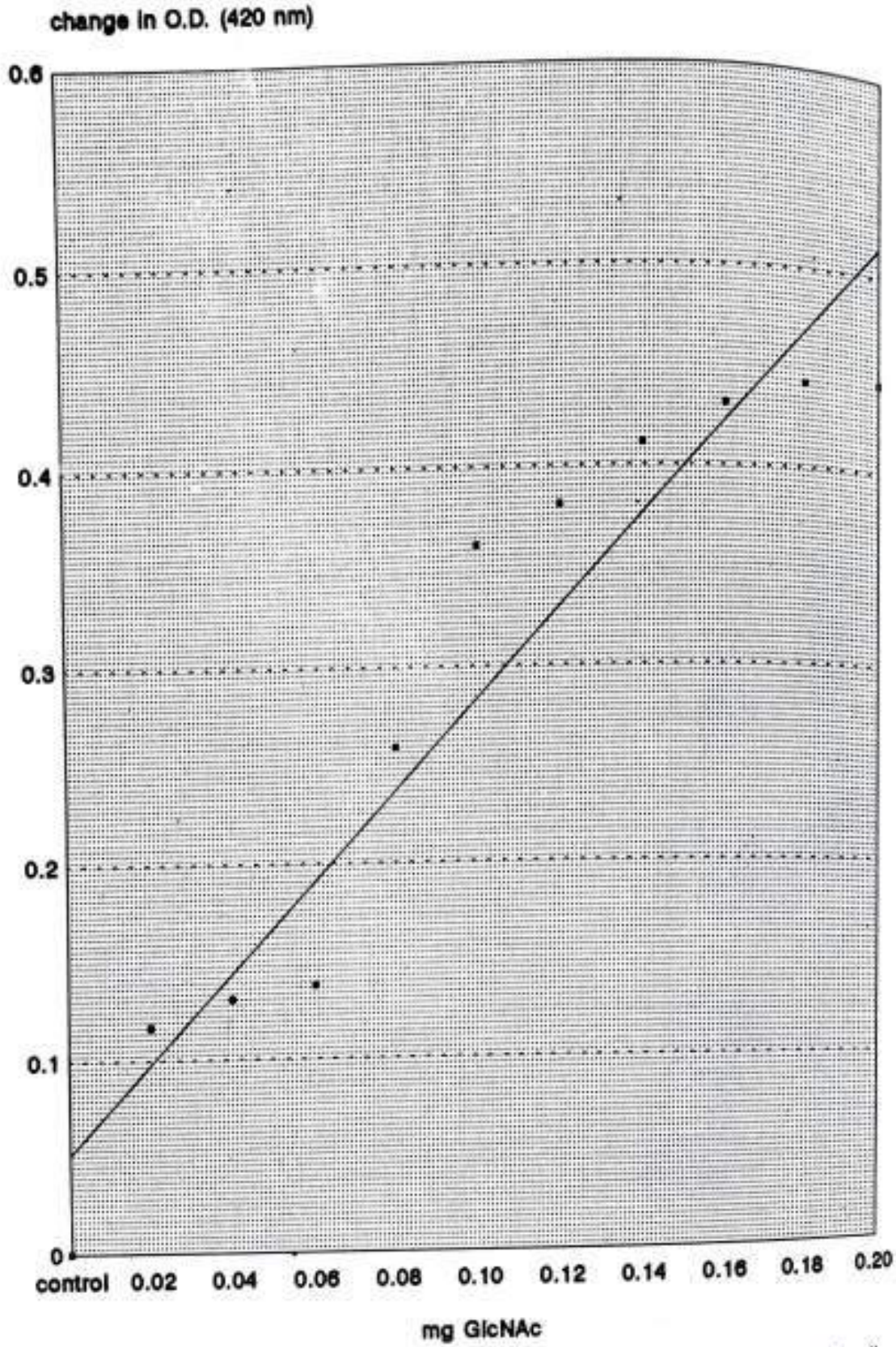

Figure 2. Standard curve for chitinase activity (turbidimetric method)

Table 3. Standard curve for N-Acetylglucosamine (Turbidimetic method).

mg N-acetylglucosamine:	CHANGE IN ABSORBANCE (420nm) (ΔOD 420nm)*
control (-)	0
0.02	0.117
0.04	0.131
0.06	0.138
0.08	0.259
0.10	0.361
0.12	0.381
0.14	0.411
0.16	0.431
0.18	0.443
0.20	0.446

* average of three trials with two replications per trial

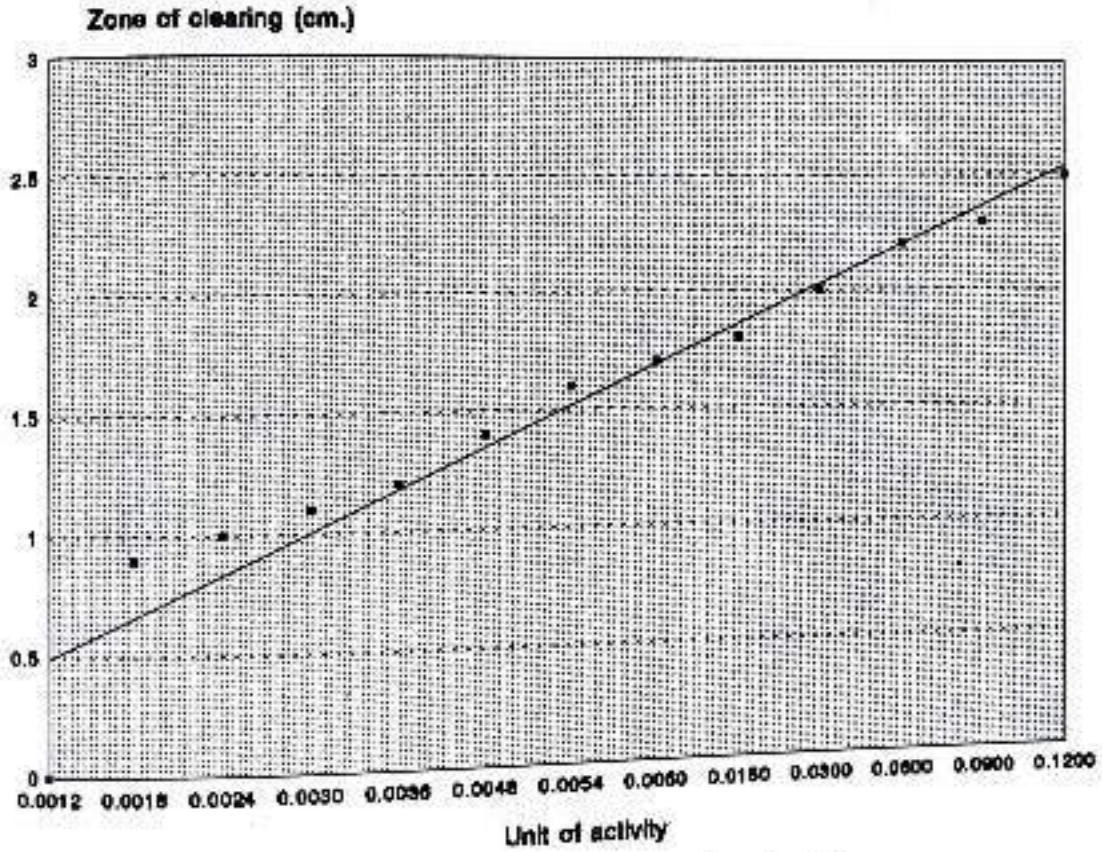

Figure 3. Standard curve for chitinase activity (cup cylinder method)

Table 4. Chitinase acitivity of the crude enzyme extract using turbidimetric method*

EXTRACT	mg GlcNAc	*u*MOLE GlcNAc	UNIT OF ACTIVITY**
Control (-)	0	0	0
5Bc	0.1525	0.0115	0.0115
6Al	0.1492	0.0113	0.0113
7Bc	0.1497	0.0113	0.0113
9Sg	0.1488	0.0112	0.0112
11Sm	0.1553	0.0117	0.0117
14Sm	0.1553	0.0117	0.0117

* average of three trials with two replications per trial

** One unit of chitinase activity is defined as the amount of enzyme which produced one *u*mole of GlcNAc per minute.

Legend:

Control	-	distilled water	9Sg	-	*Streptomyces griseus*
5Bc	-	*Bacillus circulans*	11Sm	-	*Serratia marcescens*
6Al	-	*Arthrobacter luteus*	14Sm	-	*S. marcescens*
7Bc	-	*B. circulans*			

Table 5. Standard curve for pure chitinase from *Serratia marcescens* (Cylinder cup method)*

UNIT (U)	DIAMETER OF ZONE OF CLEARING (cm)		
	Day 1	Day 2	Day 3
0.1200	1.8	2.2	2.5
0.0900	1.7	2.1	2.3
0.0600	1.6	2.0	2.2
0.0300	1.4	1.6	2.0
0.0150	1.2	1.5	1.8
0.0060	1.3	1.45	1.7
0.0054	1.1	1.4	1.6
0.0048	1.0	1.3	1.4
0.0036	0	1.0	1.2
0.0030	0	0.07	1.1
0.0024	0	0	1.0
0.0018	0	0	0.9
0.0012	0	0	0

* average of three trials with two replications per trial.

Table 6. Chitinase activity of the crude enzyme extracts using the cylinder cup method.*

EXTRACT	DIAMER OF ZONE OF CLEARING (cm)					
	Day 1 Mean+/-S.E.		Day 2 Mean +/- S.E.		Day 3 Mean +/- S.E.	
Control (-)	0a		0a		0a	
	1.0b	0	1.70c	0.188	2.28d	0.112
5Bc	0a	0	1.10b	0.1 70	1.68c	0.112
6AI	1.0b	0	1.65c	0.208	2.28d	0.112
7Bc	0a	0	0a		0.70b	0
9Sg	1.30d	0.188	1.800d	0.240	2.50d	.269
11Sm	1.25c	0.193	1.700c	0.30	2.50e	0
14Sm						

Mean values with dissimilar letters show significant differences from each other based on Duncan's Multiple Range Test (DMRT)

* average of three trials with two replications per trial

Legend:

Control - distilled water
5Bc - *Bacillus circulans*
6AI - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

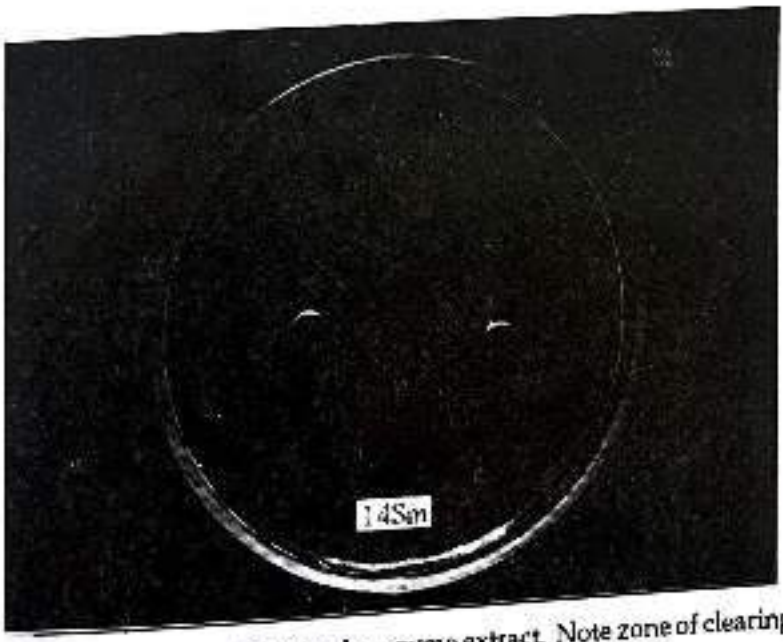

Figure 4. Cup cylinder assay of the crude enzyme extract. Note zone of clearing around the cup cylinders.

Table 7. Estimate of chitinase activity (in U) of the crude extracts using the cylinder cup assay.

EXTRACT	ZONE OF CLEARING (AFTER DAY 3)* (in cm)	UNIT OF ACTIVITY**
Control (-)	0	0
5Bc	2.3	0.0680
6Al	1.7	0.0060
7Bc	2.3	0.0680
9Sg	0.7	0.0019
11Sm	2.5	0.1200
14Sm	2.5	0.1200

* average of three trials with two replications per trial

** One unit of chitinase activity is defined as the amount of enzyme which produced one umole GlcNAc per minute.

Legend:
Control - distilled water
5Bc - *Bacillus circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces grisues*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

standard curve is presented in Table 7. Highest enzymatic activity was observed in the *S. marcescens* extracts while least enzymatic acitivity was observed in *S. griseus*.

Discussion

Bacterial chitinases such as those of *Arthrobacter* P-35 (Morrissey et al., 1976), *Bacillus circulans* WL-12 (Watanabe et al., 1990), *Serratia marcescens* (Monreal and Reese, 1969 and Cabib, 1988) and *Streptomyces griseus* (Ohtakara et al., 1988 and Ohtakara et al., 1990) are known to be extracellular and hence

are excreted into the medium containing chitin. The enzymes once present in a chitin-containing solid medium, hydrolyze the substrate leading to the formation of zone of clearing. Ueno and Arai in 1992, isolated chitinase-producing microorganism from the soil by their ability to form clear zones in medium containing chitin as sole source of carbon. Also in search for agents to prevent post-harvest mould growth in foods and feeds, a number of chitinolytic bacterial strains were selected according to their ability to form clearing zone in chitin agar (Framberg and Schnurer, 1994).

Chitinase activity of the crude extracts was assayed by turbidimetry and cylinder cup method. Turbidimetric measures the amount of GlcNAc monomers and soluble GlcNAc oligomers released into the reaction mixtures (Domard and Vasseur, 1991). The released sugars are then measured by the decrease in optical density (O.D.) of the mixture due to the reduction of ferricyanide, a component of the color reagent, to ferrocyanide by the reducing end groups of GlcNAc (Linker, 1996 and Rajagopalan and Handler, 1966). The said method is extensively used for the estimation of chitinolytic activity of several bacteria (Horikoshi and Iida, 1959; Mitchell an Alexander, 1963; Morrissey et al., 1976, Tominaga and Tsujisaka, 1976; Ueda and Arai, 1992; and Watanabe et al., 1992) while the cylinder cup measures the zone cleared of chitin by the enzymes.

The bacteria that were screened for chitinolysis were previously reported to be chitinolytic. The failure of some to grow and form clear zones in chitin agar A is attributed to strain differences while the absence of clearing in the colonies that showed minimal growth is probably due to low production of chitinase.

Observations made in this study revealed that 6Al and 9Sg extracts possess low enzyme activity when assayed using the cylinder cup method but more or less comparable with the other extracts when measured through turbidimetry. This is not surprising because Franberg and Schnurer (1994) found that there is no agreement between the release of reducing sugars (measured by turbidimetry) and the formation of clearing zones on chitin agar. When the activity of crude chitinases from different bacterial isolates were compared, Franberg and Schnurer (1994) observed that the clearing zone assay was simple but may take several days and sensitivity was low.

In general, the activity of the crude chitinases produced by the bacterial isolates in this study was low (0.0112 - 0.0117 obtained from turbidimetry and 0.0019 - 0.1200 obtained from cylinder cup assay) compared with some reports. Unit of activity of chitinases of *Serratia marcescens* concentrated filtrate was 6.73U (Cabib, 1988), of a certain *Streptomyces* sp.

0.469U (Ueno et al., 1990) and of *Streptomyces antibioticus* 0.034U (Jeuniaux, 1966). However, the activity of chitinase (0.0113 and 0.0060 from turbidimetry and cylinder cup assay respectively) from *Arthrobacter luteus* which was obtained in this study compared well with the value reported by Morrissey et al., (1976) which was 0.006 exhibited by *Arthrobacter* P 35. The differences in activity of the crude chitinases in this study with the previous reports are due to several factors like strain differences, differences in growth conditions (pH and temperature), media composition, and nature of chitin and assay method (viscosimetry, turbidimetry, and radiometry).

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