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Comparativestudy of Chitinase Production From Rhizospheric Bacteria Antagonistic To Selected Fungal Plant Pathogens.

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1. Introduction :

Plant sicknesses are a noteworthy issue confronting plant development and are in charge of the loss of 10 % of the aggregate worldwide yield generation **[14**]. Molds, a standout amongst the most forceful plant pathogens, are ordinarily obliterated with substance fungicides. In addition, compound fungicides might be deadly to useful creepy crawlies and microorganisms populating the dirt and may enter the evolved way of life **[3].** Regardless of their high viability and convenience, substance fungicides have numerous impediments. Organic control, which is utilizationof microbethat control plant infections, also provide option, naturally neighborly methodology for controlling phytopathogens. As of late, organic control has been centered around microorganisms creating mycolytic chemicals, particularly chitinases (CHIs), that

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themselves are not chitin, a noteworthy segment of parasitic cell dividers. Chitin degrading microbes are as of now concentrated on appealing distinct option for engineered chemicals as a result of their apparent security and lower natural effect.Chitin, a β-(1,4)- linked polymer of NAG is a standout amongst the inexhaustible normally happening carbohydrates. Naturally, they areobserved in three structures that are crystallinic. The α-structure has chitin microfibrils chains that are not parallel with solid intermolecular hydrogen holding and is the most bounteous polymer in nature, observed in Arthropods. Against it the β-chitin has chitin chains that are parallel to each othetr and happens in squids **[18].**Biopolymer has a wide scope of utilizations in biochemical, nourishment, as well as concoction commercial ventures that shows antimicrobial, anticholesterol, and antitumor movement **[5].**Garbage from the handling of shellfish is considered to be a critical business wellspring of polymer. Significant sums are created in Asia, mostly Thailand, that is a noteworthy exporter of shrimp, and India. Usage of such unused chitin is uncommon, yet in the event that legitimately directed, would take care of the ecological issue of waste transfer and empower the immense monetary estimation of polymer be used **[16].** Important fact is that the generation of polymer in the marine ecosystem is huge. The assessed yearly measure of polymer created by sea zooplankton is more than a few millions of tons **[2],**alongwith aggregate yearly generation of polymer is evaluated to be 1000 to 1050 tons **[8].** The vast majority of this generation is in chitin shells inserted in marine zooplankton and marine shellfish, for example, shrimp, crabs, and lobsters. Whenever shed, the shed exoskeletons may contain up to five times more chitin than the body of the creature. Arthropods have the biggest operational criticalness since they deliver the best measure of chitin. Additionally, the top layer of collections of physical creepy crawlies and 8-legged creature have extensive measures of polymer**[13].** Shockingly, the chitin content in marine residue are very low. That is because of

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conversion procedures completed by marine chitinhydrolysing microscopic organisms **[6],** that change this polymer into natural intensifies which are in this manner utilized by different microbesand wellspring of carbon and nitrogen. Chitin degrading microbes occupy an extensive variety of situations. Scientists**[7]** discovered chitinolytic microorganisms in the dung of herbivores (e.g., buffalo—*Bibosbonasus,vicugnapaca,* and elk—*Elaphurusdavidianus*) and household herbivores (dairy animals). They are likewise found in the rumen liquid of bovines, that can't deliver chemicals for processing chitin and along these lines offers a living domain for chitinolytic microscopic organisms in return for help processing this extreme compound. They hydrolyze the β-1,4-glycosidic bonds between the N-acetyl-d-glucosamine residues that comprise a chitin chain **[7].**

Materials and Methods

Glasswares, glass slides, different chemicals, dyes , certain reagents, different growth medias were provided by the school of Bioengineering and Biosciences. The soil sample was collected from BudhiPind ,Distt. Hoshiarpur, Punjab . The Bacterial strains were isolated from pea plant and grown on nutrient agar at 37℃

2.1 Isolation of bacterial colonies:-

Serial dilution was the first step for bacterial colony isolation. For the process of isolation , serial dilution 10-1 to 10-6 for soil sample was done . The bacterial culture 50 µl was used for spreading on nutrient agar and plates were incubated at 37˚C for 24hrs in incubator . The colonies were observed after 24 hr incubation.

2.2Collection of fungal strains:-

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Aspergillus Niger isolated originally from naturally diseased plants from different agricultural fields .Also , the strains of aspergillus were collected from ground nuts by incubating ground nuts with mild moisture at 22 ℃ .The isolated fungi were grown on Potato Dextrose Broth (200g potato filtered mash , 20g Dextrose per litre).

2.3 Antagonistic activity assay :

Dual culture assay was performed to check biocontrol activity against fungal pathogen . In dual culture assay , the fungal strain and bacterial culture were suspended by spreading on Potato Dextrose Agar . The growth level of both was inspected day by day for the arrangement of inhibition zone and growth reduction.

2.4.Biochemical identification :-

Bacteria was identified by biochemical test like catalase test , oxidase test , motility test

2.4.1 MRVP (Methyl red -VogesProskauer) :-

MR and VP tests were observed from tube inoculated with MR-VP broth. After incubation the MR-VP broth divided into two tubes. One for MR test and other for VP test. This media consist of glucose and peptone. Both the MR and VP tests were depended on end products resulted when the test organism degrades glucose. When the pH indicator methyl red was added to this acidic broth it will be either cherry red (a positive MR test) or yellow color indicating a negative MR test (Aneja, 1993).

2.4.2 Motility test :-

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Motility test is done in order to determine the motility of microorganisms. The motility of microorganism can be seen by variety of mechanism, but the most common is the flagella. It is observed by a diffuse zone of growth spreading from the line of inoculation. The test tubes having motility agar medium is inoculated with the bacterial colony through stabbing and incubated at 37ºc for 24 - 48 hrs for proper growth. A positive result is indicated by diffuse or hazy growth, especially at the top and bottom of the stab and negative result is observed by growth in a zone along the stab (Cappuccino and Sherman, 1992**).**

2.4.3 Citrate test: - Simmon's citrate agar medium was prepared, poured into the tubes and autoclaved at 15 lbs for 15 minutes and slants were prepared. Inoculate the slant with the isolated bacterial strain with the help of inoculating needle. The slants incubated at 37° C for 24 to 48 hrs. Slants were observed after incubation. Bromthymol blue was used as pH indicator. When citric acid hydrolysed, CO_2 producedreacts with Na+ and H2O to produceNaCO3 that leads to change the color of indicator from green to blue and this result into the confirmation of positive citrate test.

2.5 Production of Chitinase :-

Hydrolysis of chitin :- Hydrolysis of chitin was carried out by mixing 200g of chitin in 500 ml of concentrated HCL for 2 hours on magnetic stirrer . After 2 hours , the acid was removed by several washing of chitin with distilled water in order to obtain neutral pH . The dried powder is then used up as colloidal chitin in media for chitin production.

Enzyme Production :-

The enzyme chitinase was produced by inoculating strain in fresh medium of colloidal chitin (5% chitin liquid medium; 5g of colloidal chitin in 100 ml of distilled water). The growth was

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checked by taking OD at 530 nm taking blank without inoculated strain in fresh medium. The standard curve was made of different OD values based on different concentration of NAG (n-acetyl glucosamine).

Chitinase profiling :-

The biochemical estimation of enzyme carried out by chintaseprofiling . In chitinaseprofiling , reaction mixture had 1ml (5% acid swollen chitin) , 1ml (50 mM Acetate buffer) , 1ml enzyme solution and was kept for incubation at 50°C for 1 hour and boiled it for 15 minutes to stop the reaction. The mixture was centrifuged at 5000 rpm for 20 min and the grouping of discharged GlcNac created was examined at 530nm spectrophotometrically with colloidal chitin as substrate (Monreal and Reese, 1969) from the aliquots taking after DNS sugar estimation test utilizing GlcNac as standard.

2.6. Optimization for growth of selected bacterial isolates at different pH, and temperature.

Selected bacterial isolates were grown in their respective growth media (nutrient broth) adjusted with different pH using NaOH and HCl. After proper incubation, absorbance was observed at 600 nm (visible range); the turbidity of the medium causes the increase in absorbance which corresponds to slow, moderate or fast growth of bacteria with respect to different pH range (interval of 0.5 units ranging from 5-8).Similarly, effect of different temperatures was observed by inoculating the respective growth medium followed by incubation at different temperature.

3. Results and Discussions

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3.1. Sample collection: The soil sample was collected aseptically in plastic bags from fields of Budhi village, Tanda , Punjab. The physical parameters of soil sample Budhi field were also checked during sample collection. The temperature of the field was 15˚C and the respective pH was basic i.e 8.0.

Fig. 1.Sample collection from fields of Budhi village , Tanda , Punjab

3.2. Isolation of Bacterial colonies :- Isolation of bacterial colonies was carried out by serial dilution method . Number of colonies were seen but in dillution10^{-7} isolated colonies were observed . The isolates were subjected to different morphological and biochemical tests for further their identification.

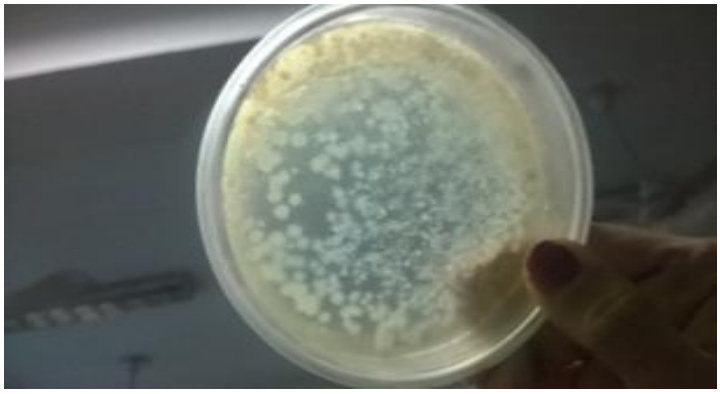

Fig.2.Various colonies were seen during spreading on nutrient agar media.

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3.3. Collection of fungal strains : Fungal strains of *Aspergillus niger* was obtained on moist ground nuts at 22 ℃ after 4 to 7 days of incubation. Also *Aspergillus niger* strains were grown in potato dextrose broth by taking inocculum from lyophilized culture that was made available by our school of biosciences . The culture of fungal strain was then incubated for preservation at 22 ℃ .

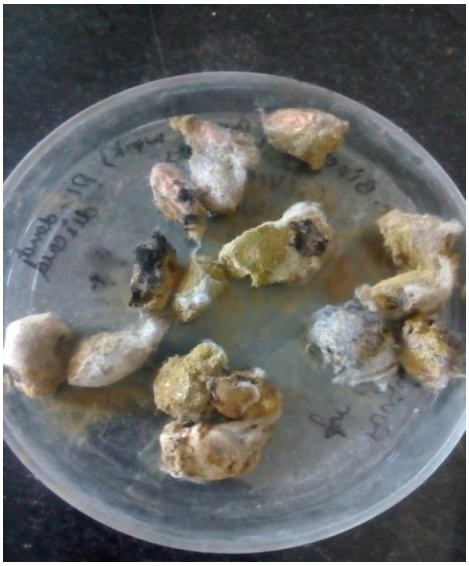

Fig.3.Fungal strain grow after 5 – 6 days of incubation.

3.4. Antagonistic activity assay :-The Antagnositic assay was performed by dual culture . fungal and Bacterial culture was inoculted and spreadedapseptically in laminar air flow . the culture was spreaded by dividing the plate in two halves equally . growth was checked day to day at 22 ℃ . The zone of inhibition was reflecting antifungal and antibacterial activity of both the cultures At this stage , the isolate shows its property of inhibiting the growth of fungi.

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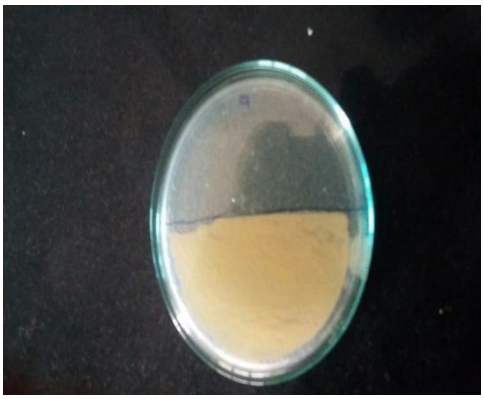

Fig.4. Plates conforming antagonistic activity

3.5.Biochemical identification:

I. MRVP TEST (methyl red-Voges-Proskauer) : Red colored rings were appeared in the culture that indicates positive MRVP test .After the incubation of culture when methyl red reagent was added to the tubes containing culture , culture does not show any red color rings. Similarly, when Barrett's A and B reagent was added to check VP test, isolate showed formation of ring that is positive result.

II. Motility test: After the 24 hour incubation to culture , the motility test was performed , the observation was reflect that isolate was showing cloudy and diffuse growth throughout the medium indicating the isolate is motile in nature.

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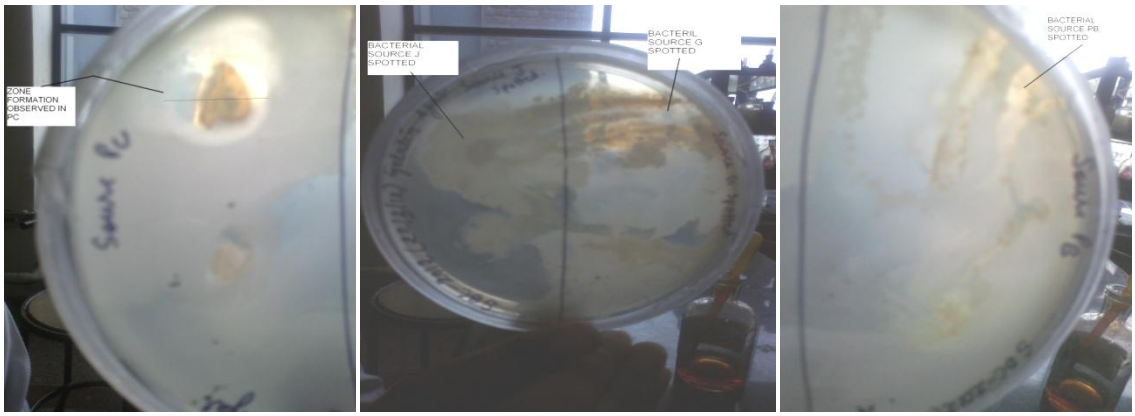

(a)- Isolate PC(Positive) **(b) isolate G(Negative)** **(c) Isolate PB(Negative)**

Fig.5. Zone formation results of gelatin hydrolysis.

III. Starch hydrolysis : Different size of diameter of rings were observed

BACTERIAL SOURCE	ZONE FORMATION	DIAMETER
G	NO ZONE FORMED	NA
J	ZONE FORMED	1.7cm
PB	ZONE FORMED	1.2 cm
PC	ZONE FORMED	1.5 cm

Table 1: Starch hydrolysis reflects only G strain as negative in test

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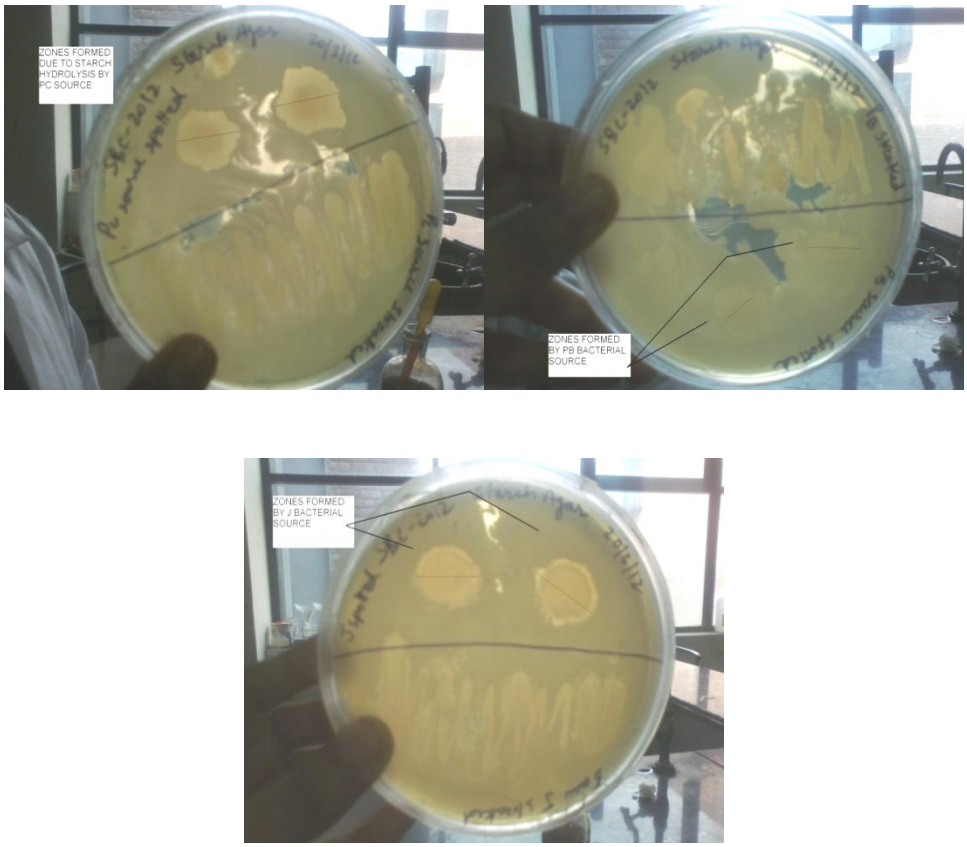

Fig.6. Halo zone formation indicating hydrolysis of Starch by bacterial isolate PB,PC and J.

IV. MILK REACTIONS: LITMUS TEST

BACTERIAL SOURCE	COLOR CHANGE	INTERPRETATION
G	Clear white curdy appearance	Only acid curd formation

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J	White curdy appearance with slight pink color on top	Slight fermentation of glucose and lactose; acid curd formation
PB	Pinkish white curdy appearance	Slight fermentation of glucose and lactose; acid curd formation
PC	Clear white curdy appearance	Acid curd formation because of lactic acid producing curd.

Table 2 : 6.3 Milk Litmus Test interpretation

V. Production of Chitinase :-

The estimation of chintase quantity was determined by OD values taking standard of NAG (N – Acetyl Glucosamine) . The concentration of enzyme was gradually increased after every hour incubation provided to culture at 37℃.

VI. Hydrolysis of Chitin: - The hydrolysis of colloidal chitin was observed by growing the bacterial strain 37℃ on colloidal chitinagar (5% chitin). The differential zones on plates were observed to be hydrolised that was reflecting hydrolisation of chitin that act as substrate for enzyme to act upon it.

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Fig.7.Colloidal Chitin (Chitinhydrolysed in concentrated Hcl)

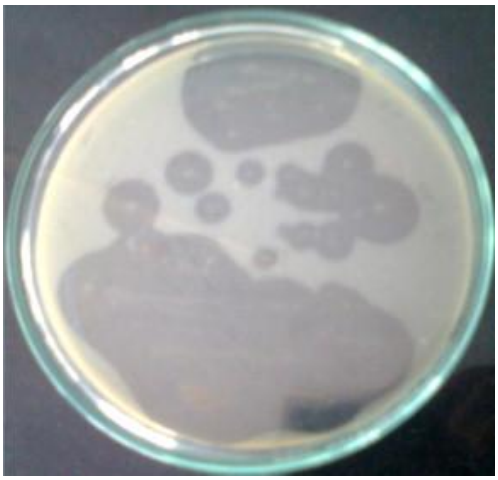

Fig.8. Hydrolysis of Chitin by Chitinase

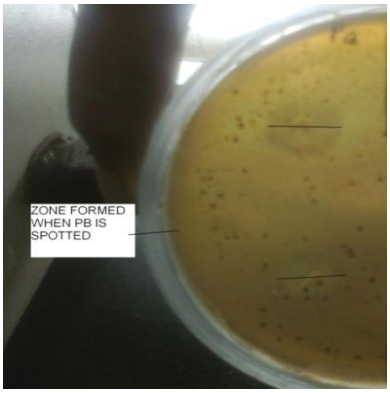

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Fig.9. Hydrolysis of chitin by chitinase by bacterial isolate

VI. Characterization of bacteria secreting chitinase on different temperature and pH

Effect of temperature on Enzyme Activity: Thetemperature was studied in the range of 20 ºC to 47 ºC. The optimum temperature was found to be around 37ºC for the isolate. This is the temperature where the isolates showed maximum growth. The streaking was performed on nutrient agar.

S.No.	Bacterial isolate	Temperature range (0C) 28 37 42
1	SJ 602	+++ +++ +
2	SJ 603	+ + +++ +
3	SJ 604	+++ +++ ++
4	SJ 605	++ +++ +

Where ++ indicates moderate growth while +++ indicates best growth

Table 3:Optimization at different pH.

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VII. Effect of pH on Enzyme Activity: After studying the residual activity of isolates, it has been observed that the isolate shows maximum growth at 7 pH whereas, isolate shows least growth at pH basic approximately 10 . Also isolate shows no growth at very acidic pH i.e.:2.

S.No.	SAMPLE	pH-RANGE 5 5.5 6 6.5 7 7.5 8
1	J	++ +++ ++ ++ +++ ++ ++
2	PB	++ ++ ++ +++ +++ ++ ++
3	PC	++ +++ ++ ++ +++ ++ ++
4	C	++ +++ +++ ++ ++ +++ ++

Where ++ indicates moderate growth while +++ indicates best growth

Table 4: Optimization at different pH.

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