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RESEARCH ARTICLE

CHITINASE PRODUCTION BY ASPERGILLUS TERREUS FROM MARINE WASTES AND ITS EFFICACY IN ANTIFUNGAL ACTIVITY

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Abstract

The present investigation aims to isolate and identify chitinase producing mycoflora and the chitinase production by efficient *Aspergillus terreus* on different shell powder wastes substrates such as prawn, crab and molluscs at an extracellular and intracellular level and its efficacy in assessing the antifungal activity. Shell fish wastes disposed soil samples were collected and screened for chitinolytic fungi (hydrolyzing zone). The chitinase production on different substrates was assessed and the enzyme extracts were screened for antifungal activity by agar well and disc diffusion methods. Among the number of mycoflora isolated only 4 fungal strains *Aspergillus terreus, A. fumigatus, A. flavus,* and *A. clavatus* showed maximum hydrolyzing zones. A significantly highest hydrolyzing zone (clearance zone) of 20 mm (out of colony diameter of 65 mm) was obtained by *Aspergillus terreus*. Among the different substrates (prawn, crab and molluscs shell powders at 1% concentration), highest chitinase activity was recorded by *Aspergillus terreus* on prawn shell powder waste followed by crab shell powder waste at an intra and extra cellular level. The chitinase produced from prawn shell powder waste as substrates by *A. terreus* showed maximum zone of inhibition in both agar well (28.0 mm) and disc diffusion (24.0 mm) methods against the pathogenic fungi, *A. flavus* compared to positive control (Fluconazole). The chitinase produced from *A. terreus* might have synthesized some antifungal compounds which may be responsible for killing pathogenic fungi.

Introduction:-

Chitin is a polysaccharide, composed of β-1, 4 *N-acetyl* D glucosamine residues and is the most abundant renewable resource after cellulose. Chitin chain is present in three forms of arrangement, they are α, β, and γ. The α form is dominant and more stable consisting of antiparallel chains. The β forms consist of antiparallel chains and occur only in marine organisms. The γ chitin is a mixture of both α and β. The preparation of chitin includes demineralization and deproteinization of shellfish waste material with the use of strong acids and bases (HCl and NaOH). Chitin is mainly derived from crustaceans such as shrimp and crab shells and is mainly composed of calcium carbonate. Is is a structural component of the cell wall of most fungi. Chitin is closely associated with protein, minerals, lipids and pigments. It is insoluble in water but soluble in organic solvents because of its strong intra and intermolecular hydrogen bonds. Some pre-treated chitin is used as a substrate for microbial chitinases

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production. The chitin degrading enzyme, chitinases (EC 3.2.1.14) are a glycosyle hydrolase group of enzymes that catalyse the hydrolysis of insoluble chitin into monomer and oligomer. The microbial extracellular chitinases play an important role in pharmaceutical industry due to their immense potential application like isolation of protoplast of fungi and yeast, preparation of SCP, control of pathogenic fungi, production of bio-pesticides and control of mosquito propagation (Halder *et al*. 2012). Different types of substrates which contain chitin have been tried for the production of chitinases, which include fungal cell walls, prawn, crab and shrimp shells (Tagawa and Okazaki 1991). Some pre-treated chitin is used as a substrate for microbial chitinases production (Wang and Chang, 1997). Molluscs which are widespread and abundant play an important role in the food chain and are important food sources for larger animals from fish to whales. Shrimp processing industry plays an important role in global economy. The global annual recovery of chitin from the processing of marine crustacean waste is estimated to be around 37,300 metric tons (Sheikh and Deshpande 1993). The shell fish industry is operative among all the costal countries and contribute hugely to to the food delicacies. During the processing of prawns, shrimps and lobsters mostly, the meat is taken, while the shell and head portions are discarded as wastes which results in huge amount of waste generated throughout the world. The main industrial source of chitin is shellfish waste from processing of crab and shrimp. Biological control, or the use of microorganisms or their secretion to prevent plant diseases offers an attractive alternative or supplement for the control of plant diseases without the negative impact of chemical control. Therefore, biological control tactics have become an important approach to facilitate sustainable agriculture (Wang 1999). Chitinases are generally related with the production of antifungal compounds and extracellular hydrolytic enzyme (chitinases and 1, 3-β glucanase). Chitinolytic enzymes are efficient in lysing the fungal cell wall. The microorganisms that produce these enzymes are capable of eradicating fungal diseases that are a problem for global agricultural production.

Materials and Methods:-

Isolation of Fungal Species

Prawn, crab and molluscs shell wastes disposed soil samples were collected from the disposal area in Cochin district, Kerala. The samples were serially diluted and plated on Potato Dextrose Agar medium (PDA) and incubated for seven days at 30°C. After incubation, the plates were observed for fungal growth and were sub-cultured and maintained on PDA slants at 4°C. The fungal isolates were identified based on their morphology, mycelia structure and spore formation (Barron 1968; Ellis 1976; Domsche and Gams 1972).

Screening of Soil Fungal Isolates for Chitinolytic Activity

The identified fungal strains were grown individually for screening for chitinolytic activity on colloidal chitin agar plates (Sherief *et al*. 1991a). The clearance zone formed around the colonies was determined by adding 0.1 percent congored solution and counter stained with 1M NaCl for 15-20 min. A clear zone of chitinase hydrolysis gave an indication of chitinase producing microorganisms. The diameter of the clear zone was measured to provide a quantitative comparison of chitinolytic activity.

Enzyme production

Erlenmeyer flasks containing 100ml of colloidal chitin liquid medium (Sherief *et al*. 1991b), was sterilized at 1 atm for 15 minutes. After cooling, one ml of Streptomycin sulphate (10,000 ppm) was added and incubated for 5, 7, 9 and 11 days at 30°C under static conditions. The clear filtrate obtained through Whatman No. 40 was used as a source of extracellular enzyme. A quantity of 5.0 g of the washed mycelia mat was macerated in five ml of sodium phosphate buffer of pH 7.0 in a pre-chilled mortar and pestle with a pinch of acid washed sand. The homogenate was centrifuged and the supernatant served as crude source of intracellular enzyme.

Chitinase assay

Chitinase activity was determined by the production of *N-acetyl*-D-glucosamine using DNS method. The reaction mixture consisted of 1ml of 0.2 M sodium phosphate buffer (pH 7.0) containing 10 percent of colloidal chitin and 1ml enzyme solution. The mixture was kept in water bath at 50°C for 1 hr. The reaction was terminated by adding 1 ml of 1 percent NaOH and boiled for 5 minutes at 100°C and centrifuged for 10 minutes. 1 ml of the supernatant was mixed with 1ml DNS solution and boiled at 100°C for 10 minutes. Finally, the content was cooled and 5ml of distilled water was added to the mixture. The absorbance was read at 535 nm and the protein content was determined by the method of Lowry *et al.*1951.

Effect of different substrates on enzyme production

The isolated fungal strain was incubated in the production medium containing 0.50g of substrates like prawn shell, crab shell and molluscs shell powder . The enzyme activity for each trial was estimated at 5th, 7th, 9th and 11th day of incubation in triplicates.

Screening of the extracts for antifungal activity

Test fungal strains of *Aspergillus niger*, *A. flavus, A. fumigatus* kept in the fungal culture bank of Department of Botany, Avinashilingam University, Coimbatore were taken and maintained as subculture in slants. Antifungal studies were carried out by agar well and disc diffusion methods.

Agar well diffusion method (Smania *et al.* 1995)

Each fungal suspension of test microorganisms were spread separately over the surface of PDA plates with cotton swap and five wells each of 6mm diameters were made on the agar. The wells were filled with 20µl each of enzyme (obtained from different substrates) along with negative control (phosphate buffer) and positive control (fluconazole) at the centre. The plates were incubated at 30°C for 76 hours. The zone of inhibition was calculated by measuring the diameter of clear zone formed around the well.

Disc diffusion method (Bauer *et al.* 1966)

Plates were prepared by pouring 20 ml of sterile PDA medium into the sterile petriplates and were inoculated with test organisms. Sterile paper disc (Whatman No.1, 3.6 mm diameters) impregnated with 20µl quantity each enzyme, phosphate buffer (as negative control) and positive control (fluconazole) and were air dried and placed on the PDA plates. The plates were incubated at 30°C for 76 hours. The zone of inhibition was calculated by measuring the diameter of clear zone formed around the disc.

Results and Discussion:-

Isolation and Screening of Chitinase Producing Fungi

Among the number of mycoflora like *Rhizopus stolonifer, Aspergillus fumigatus, A. flavus, A. terreus, A. clavatus, A. nidulans, Penicillium chrysogenum,* etc isolated from the prawn, crab and molluscs shell wastes disposal area soil samples, only 4 fungal strains *Aspergillus terreus, A. fumigatus, A. flavus,* and *A. clavatus* showed maximum hydrolyzing zones (Table 1). A significantly highest hydrolyzing zone (clearance zone) of 20 mm (out of colony diameter of 65 mm) was obtained by *Aspergillus terreus* followed by 14 mm (out of colony diameter of 55 mm) by *A. flavus.* Since, *A. terreus* showed remarkably prominent clear zone of chitinolytic activity, it was selected as a potential candidate for the enzyme study. Sharaf 2005 obtained clearance zone of 32.0 mm, 26.0 mm and 18.0 mm respectively by *Aspergillus flavus, A. foetidus* and *A. niger*. Jenin *et al*. 2016 reported that the two fungal species belonging to the genus *Aspergillus* such as *A. niger* and *A. fumigatus* were found to be good chtinase producers.

Table 1:- The diameter of colony and hydrolyzing zone.

S.No	Fungal strains	Colony Diameter (mm)	Hydrolyzing Zone(mm)
1.	Aspergillus terreus	65.0	20.0
2.	Aspergillus flavus	55.0	14.0
3.	Aspergillus fumigatus	30.0	10.0
4.	Aspergillus clavatus	20.0	9.0

Table 2:- Chitinase activity (Umg^{-1} protein) of *Aspergillus terreus* on Prawn, Crab and Molluscs shell powder as substrate.

Substrate	**Enzyme Activity**		**5th day**	**7th day**	**9th day**	**11th day**	**SED**	**CD (p<0.05)**
Prawn shell	**Intra**	**Control**	0.51	0.72	0.58	0.25	0.06	0.12
		with substrate	2.36	4.89	2.51	1.75		

Powder	**Extra**	**Control**	0.42	0.81	0.54	0.27	0.03	0.05
		with substrate	1.17	5.29	2.77	1.36		
Crab shell Powder	**Intra**	**Control**	0.37	0.53	0.47	0.23	0.02	0.03
		with substrate	1.62	3.75	2.92	0.76		
	Extra	**Control**	0.51	0.74	0.50	0.46	0.03	0.07
		with substrate	1.77	4.23	3.22	0.95		
Molluscs shell powder	**Intra**	**Control**	0.47	0.77	0.43	0.38	0.02	0.04
		with substrate	1.34	3.49	2.09	0.92		
	Extra	**Control**	0.55	0.70	0.48	0.36	0.06	0.13
		with substrate	1.45	3.37	1.19	0.73		

Values are mean of three triplicates

Umg^{-1} = 1μ mol of N-acetyl-D-glucosamine released min^{-1} mg^{-1} Protein.

Assessing Chitinase Production on Different Substrates (Table 2)

Among the different substrates (prawn, crab and molluscs shell powder wastes), chitinase activity by *Aspergillus terreus* showed an enhancement from 2.36 Umg^{-1} to 4.89 Umg^{-1} enzyme protein up to 7^{th} day of incubation and it gradually decreased to 1.75 Umg^{-1} enzyme protein in prawn shell powder waste as substrate compared to control with an enhancement from 0.51 Umg^{-1} to 0.72 Umg^{-1} enzyme protein and it declined to 0.25 Umg^{-1} enzyme protein on the 11^{th} day of incubation. The least enhanced activity was observed in molluscs shell powder waste from 1.34 Umg^{-1} to 3.49 Umg^{-1} enzyme protein and it decreased to 0.92 Umg^{-1} than the control (from 0.47 Umg^{-1} to 0.77 Umg^{-1} enzyme protein and declined to 0.38 Umg^{-1} enzyme protein) on the 11^{th} day of incubation at an intracellular level. At an extracellular level, the chitinase activity by *Aspergillus terreus* was increased from 1.17 Umg^{-1} to 5.29 Umg^{-1} enzyme proteins up to 7^{th} day of incubation and it gradually declined to 1.36 Umg^{-1} enzyme protien on the 11^{th} day of incubation than the control from 0.42Umg^{-1} to 0.81 Umg^{-1} enzyme proteins in prawn shell powder waste as substrate. The minimal activity observed was in molluscs shell powder waste from1.45Umg^{-1} to 3.37 Umg^{-1} enzyme protein up to 7^{th} day of incubation and it decreased to 0.73Umg^{-1} enzyme protein than the control (from 0.55 Umg^{-1} to 0.70 Umg^{-1} enzyme protein and decreased to 0.38 Umg^{-1} enzyme protein) on the 11^{th} day of incubation. The present result is on par with the report of (Rattanakit *et al*. 2002) who found that the chitinase production by *Aspergillus* sp. S1-13 was increased to 58% - 65% when, shrimp powder was used as a substrate. (Setthakaillus *et al*. 2008) obtained highest chitinolytic activity of 3.1 Uml-1 by *Aspergillus* sp. Similar finding of highest chitinase production of 26.8 Uml-1 by *Aspergillus flavus* CFR10 was reported by Suresh and Anilkumar, 2012. Similar view was expressed by Krishnaveni and Ragunathan 2014 who reported the maximum chitinase activity in Shrimp wastes (4.7 U/min), followed by Snail shell (4.3 U/min), crab shell (4.2 U/min) and fish scales (3.7 U/min) by *Aspergillus terreus* CBNRKR KF529976.

Screening of the enzyme extracts for antifungal activity (Table 3)

Agar well diffusion method

It was observed from the results of the agar well diffusion method that among the enzyme produced from different substrates by *Aspergillus terreus*, highest zone of inhibition of 28.0 mm was observed against phytopathogenic fungi, *Aspergillus flavus* from the chitinase produced from the prawn powder waste as a substrate. This was higher than that observed (26.0 mm) in positive control (fluconazole). The minimum zone of inhibition observed was 10.0 mm in case of chitinase produced by molluscs powder waste against the phytopathogenic fungus *A.niger*. The moderate inhibition zone was present in *A. fumigatus* (22 mm). The present result is accordance with the result of Brurberg *et al*. 2000 who reported that chitinase showed much stronger inhibitory activity to *Fusarium solani* (83%) compared to *Aspergillus flavus* (63%). El-Katatny *et al*. (2005) found that the purified endochitinases of *Trichoderma* has shown antifungal activity against *Sclerotium rolfsii*. Similar result was observed by Gunalan *et al*. 2012a who found that among the four strains of phytopathogenic fungi tested (*Aspergillus niger, Alternaria solani, Rhizopus stolonifera* and *Fusarium solani*), highest antifungal activity was observed against *Rhizopus stolonifera* from the chitinase produced by *A. flavus*.

Table 3:- Inhibition zone of chitinase against phytopathogenic fungi by *Aspergillus terreus* (agar well and disc diffusion method).

Method	Pathogenic Fungal strains	Zone of inhibition(mm)				
		Prawn	**Crab**	**Molluscs**	**Positive control (Fluconazole)**	**Negative control (Phosphate buffer)**
Agar well diffusion	*Aspergillus flavus*	28.0	24.0	18.0	26.0	8.0
	Aspergillus fumigatus	22.0	15.0	14.0	24.0	10.0
	Aspergillus niger	17.0	13.0	10.0	24.0	7.0
Disc diffusion	*Aspergillus flavus*	24.0	22.0	13.0	23.0	10.0
	Aspergillus fumigatus	21.0	11.0	11.0	22.0	8.0
	Aspergillus niger	16.0	15.0	10.0	20.0	6.0

Disc Diffusion Method

After 5 days of incubation, a crescent growth inhibition was observed around the disc containing chitinase produced by *Aspergillus terreus* on different substrates (prawn shell, crab shell and molluscs shell powders) against the pathogenic fungi like *Aspergillus flavus, A. fumigatus* and *A. niger.* The results of the antifungal activity of chitinase against pathogenic fungi by disc diffusion method revealed that among the chitinase produced by *Aspergillus terreus* from different substrates, maximum zone of inhibition was obtained in prawn shell powder as substrate (24.0 mm) against the pathogenic fungi, *A. flavus* compared to positive control (23.0 mm). The least antifungal activity of 10.0 mm was recorded in molluscs powder waste against the pathogenic fungus, *A. niger*. The present finding is in accordance with the result of Sandhya *et al*. 2004 who found that the extracellular chitinase produced by *Trichoderma harzianum* TUBF781 showed antifungal activity and with Nampoothiri *et al*. 2004 who reported that chitinase by *Aspergillus niger* NCIM563 showed antifungal activity against a fungal strains like *Rhizopus, Mucor* and *Aspergillus* sp. Similar view was expressed by Gunalan *et al*. 2012b that the chitinase produced from *Aspergillus flavus* acted as a biocontrol agent against the phytopathogenic fungi (*A. niger, Alternaria solani, Rhizophus stolonifer and Fusarium solani*). The present result is on par with the results of Halder 2013 who also reported the chitinase enzyme exhibited high degree of antifungal activity particularly against pathogenic fungi *Aspergillus flavus* and *Fusarium oxysporum* by dissolving their cell wall components.

Conclusion:-

Thus, it can be deduced from the present investigation, that the candidate *Aspergillus terreus*, a chitinolytic fungus can be effectively harnessed for its maximum production by using cost effective, cheap substrates (shell fish wastes). The chitinase produced from *A. terreus* might have synthesized some antifungal compounds which may be responsible for killing pathogenic fungi. From the sea food waste, commercially important enzyme chitinase could be produced and it not only solves the environmental problem of disposal of waste in an ecofriendly manner but also promotes the production of value added products of economic benefits at a cheaper rate.

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