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PRODUCTION AND OPTIMIZATION OF PECTINASE AND CHITINASE PRODUCING BACTERIAL STRAINS ISOLATED FROM MANGROVE SEDIMENTS OF NIJAMPATNAM

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ABSTRACT

A total of ten bacterial isolates were used for the production of chitinase and protease and the soil samples were collected from mangrove areas of Nijampatnam, Andhra Pradesh. Isolation procedures were carried out by using nutrient agar medium (NAM). Marine water was used for the enzyme production and bacterial isolation. Chitin broth media and pectin broth media were used for this study. The present ten isolates are *Pseudomonas, Staphylococcus, Micrococci, Klebsiella sp.1 Klebsiella sp.2, E. coli, Enterobacter, Proteus sp.1, Proteus sp.2, and Citrobacter are morphologically different. All these strains were tested for chitinase* and *Pectinase* production. Maximum chitinase production (1.21 IU/ml) was observed by Enterobacter sps. The other isolate pseudomonas produces maximum amount (1.19 IU/ml) of pectinase. These two isolates were selected for the further studies like bio control agents. The optimum conditions pH 7.0, temperature 30 C and incubation period 48 hours for maximum production of enzyme. The carbon and nitrogen sources glucose and peptone greatly influenced the enzyme production. These enzymes have received increased attention due to its wide range of biotechnological applications, especially in agriculture for bio control of phytopathogenic fungi and harmful insects.

KEYWORDS: Chitin, Pectinase, Mangrove soils, Nutrient Agar Medium.

INTRODUCTION

Chitin is a homopoloymer of N-acetyl-D-glucosamine residues related to β 1-4 binding. The derivatives of chitin are oligosaccharides of 2 to 20 residues of N-acetyl glucosamine at length and low molecular weight, offer chemical and biological properties other than those of original polymer, such as water solubility and signalling functions during symbiotic interactions in plants. Chitin and all its derivatives share a high nitrogen content (6.14-8.3%) and high thermal and chemical stability (Yen and Mav, 2007).

Chitinase activity is stimulated in the nodule cortex and in the infected zone of some ineffective soybean nodules (Staehelin*et al*., 1992; Pasniske*et al*., 1994). The major source of chitinase (E.C. 3.2.1.14) is microorganisms and it represents a vast renewable fermentation feedstock of both carbohydrate and nitrogen sources. Enzymes capable of bio converting chitin to low molecular weight fermentable products potentially have significant commercial value (Kumar and Ram 2016).

There is an increasing demand to replace chemical based industries with biotechnological processes involving microorganisms and use of enzymes such as amylases, lipases, xylanases, cellulases, chitinases and pectinases *etc*. (Underkofler *et al*., 1957; Bajpai, 1999; Bruhlmann *et al*., 2000; Demirijan *et al*., 2001).

Pectinases are the enzymes, which break down pectic materials. Microbial pectinases have high potential and commercial applications for serve mankind. Pectinases may be alkaline and acidic. The acidic pectinases are employed in juices and beverages industries for purification and clarification of wines and juices; whereas the alkaline pectinases are applied in wastewater treatment of vegetables processing origin having the pectinicious materials.

Pectinases are enzymes that degrade pectin substances. Pectinases are essentially hydrolases, lyases and stands. (Danielle *et al*., 2009). Microbial pectinases are secreted by fungi, bacteria and yeast. Pectins and chitins are the variety of substances, which are present in higher plants and are macromolecules of high molecular mass. They are basically the component of plant cell wall and middle lamella, and very thin extracellular layer, which joins the young, cells together. The function of the pectic substances is to give the structural integrity to the cells

and make them cohesive. There are three major groups of pectic substances, all of these contains D-galacturonic acid as the common component which may be in lesser or greater extent depending upon the group. The three major groups of pectic substrates include: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). However there were limited reports on these important enzymes by bacterias isolates from mangrove sediments of nijampatnam. The enzyme production and optimization studies were conducted.

MATERIALS AND METHODS

Isolation of bacteria

One gram representative mangrove soil sample was suspended in 10 ml of sterile distilled water and shaken thoroughly for 10 minutes. The microorganisms were isolated from collected samples by the serial dilution plate technique using Nutrient Agar Medium (NAM). Serial dilutions up to 10^{-5} of each sample were prepared by using sterilized water (Sneath, 1986). Sample dilutions were plated (in triplicates) on NAM and incubated at 35°C for 24 to 48 h. Pure Colonies were picked and maintained on NAM slants at 4° C and further assessed for production and characterization of Chitinase and Pectinase (Silpa *et al*., 2018).

Biochemical tests for bacterial isolates

Biochemical tests were conducted for all the isolates studied and the methods with slight modifications followed by (Kumar and Ram, 2016; Silpa *et al*., 2018; Cheesbrough, 1991).

Oxidase test

Tested bacterial colony was smeared on the filter paper previously saturated with freshly prepared oxidase reagent. Positive oxidase test was recorded as the development of a blue-purple colour within 10 minutes.

Catalase test

Gas bubbles detecting within 10 s after added purified bacterial culture to 5 ml of hydrogen peroxide solution, considered as a positive catalase test.

Urease test

Slanted two millilitres of urea medium which placed in bijou bottles applied for the incubated bacterial colony at room temperature. Red-pink colour in the medium was considered as a positive test for urease induction.

Indole test

Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35 °C for 24 h on SIM media indicated a positive and negative results respectively.

Simmons Citrate test

Simmons Citrate test was performed via inculcate Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures then, incubated at 37 °C up to 48 h. changing media colour from green to bright blue indicate positive reaction.

Methyl red (MR) test

After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35 °C for up to 4 days, changing color to red indicate MR test positive- appearance of tested bacteria (Color Atlas and Textbook of Diagnostic Microbiology, 2016).

Chitinase production

Enzyme assay

The chitinase activity was determined colorimetrically by detecting the amount of N-acetyl-D-glucosamine (NAG) released from a colloidal chitin substrate (Vyas and Deshpande 1989). A 48-h grown culture was harvested and centrifuged in a microfuge (Biofuse Primo-R) at 10,000 rpm for 10 min to obtain cell-free culture supernatant. The reaction mixture (1.0 ml) for enzyme assay consisted of enzyme (340 µl) with CC (3 mg) as a substrate in 50 mM sodium acetate buffer (pH 5.0). This mixture was incubated at 55 °C for 1 h; the remaining colloidal chitin of the reaction was removed by centrifugation at 10,000 rpm for 10 min, and the chitinase activity was assayed in the supernatant by measuring the released sugar from colloidal chitin (Nelson 1944). Briefly, the above supernatant was reacted with dinitrosalicylic acid (DNS) by boiling for 5 min, and absorbance of the released NAG was measured in reaction mixture at 540 nm after cooling to room temperature. One international unit (IU) was defined as the activity that produced one µmol of the product per hour. Ndiogou Gueye *et al*., (2020)

PECTINASE PRODUCTION

Estimation of enzyme activity

Qualitative enzyme analysis

The enzyme was checked by making spot of a loopful of a 24 h grown bacterial culture on 2.5% pectin suphlented LB agar plates. The plates were incubated at 37ºC at dark for 24 h. The diameter of clear zone of hydrolysis around the spot after 24 and 48 h, followed by staining with Lugel, siodine solution. The methods followed by Ravi *et al*., (2019).

Quantitative enzyme analysis

Pectinolytic activity of the all thirteen strains was determined by a modified (colori-metric method of Miller, 1859 modified by Aguillar and Huitron (1990). Pectinase enzyme 100µl was taken in Eppendorff and 100µl of 1% pectin solution added to enzyme. Pectin solution of 1% was prepared by dissolving 1 g of pectin in 100 ml of sodium citrate buffer at pH 6.5. The enzyme substrate mixture was incubated at 40°C for 15 min. Then 400µl of dintrosalicylic acid (DNS) was added to the mixture to terminate the reaction and it was kept in the boiling water bath for 15 min. Blank was prepared along with by adding the 100µl of distilled water in place of enzyme. Absorbance was recorded at 570nm

wavelength in spectrophotometer in glass cuvettes against the blank Standard curve of glacturonic acid was prepared by reading its 1,2.....5 mg/ml concentrations as mentioned above. Concentrations of reducing sugars in the sample were then measured with the help of following equation:

Conc. of reducing sugars (mg/ml) = O.D. of sample x 0.4 x *dilution factor*

The amount of reducing sugars was then converted into the enzyme's units according to the method referred above.

Optimization studies

The optimization of culture conditions for Chitinase production by bacterial isolates were carried out by keeping all the factors constant except the one that was being studied. Experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml CC medium incubated in an orbital shaker at 180 rpm, and samples were harvested after 48 h because this isolate showed maximum rate of enzyme production within the first 48 h of incubation. Effect of initial media pH on chitinase production was studied by varying media pH from 3.0 to 9.0, and cultures were grown at different temperatures ranging from 28 to 65°C.

In order to determine the effect of different nitrogen sources on enzyme production, various inorganic nitrogen supplements (ammonium sulphate, AS; ammonium chloride, AC; sodium nitrate, SN; potassium nitrate, PN or di-ammonium hydrogen phosphate, DHP) were used in the growth medium at equivalent nitrogen levels of 0.1, 0.2 and 0.3 % (w/v). The effect on chitinase production was also evaluated using organic supplements (yeast extract, malt extract, beef extract or peptone at 1.5 g/l) and carbon sources (sucrose, maltose, lactose, fructose, glycerol or starch at 1.0 g/l). In this case, the medium was separately supplemented with each compound and incubated at optimum culturing conditions. For each optimization parameter, three sets of independent experiments were carried out and the mean values are reports (Silpa *et al*., 2018).

Statistical Analysis

All measurements were carried out in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range tests. Differences at $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

A total of twenty three isolates were isolated from mangrove soils of Nijampatnam. Soil samples were collected for every two months and air dried. For the successful isolated serial dilution was used for this study to isolate the bacteria, NAM plates were used and incubated at 48Hrs. After the successful incubation colonies were observed. Morphological and preliminary studies like colony colour, colony size, shape of the colony, spore production. Gram's test was conducted. On the basis of preliminary and morphological characters isolates were designated as *Pseudomonas, Staphylococcus, Micrococci, Klebsiella sp.1 Klebsiella sp.2, E. coli, Enterobacter, Proteus sp.1, Proteus sp.2, and Citrobacter*.

Further all the ten bacterial strains were tested for biochemical characters like oxidase, citrate, and urease tests. Half of the isolates showed positive results on Simmons citrate tests and Methyl red test. Out of the ten there are three isolates Staphylococcus coccus, Klebsiella-2, Proteus-1, showed positive results on Indole tests.

Biochemical characters reveal that to know the nature of bacterial activity on various tests. The potentialities of bacterial isolates, diversity of bacterial maybe differ in their biochemical studies (Table-1).

Table 1: Biochemical characterization of bacterial isolates.

Sl.No.	Name of the isolate	Oxidase test	Catalase test	Simmons citrate test	Urease test	Indole test	Methyl Red test
1	*Pseudomonas*	+	+	-	+		+
2	*Staphylococcus*	+	+	-	+	+	+
3	*Micrococci*	+	+	-	+		+
4	*Klebsiella sp.1*	+	+		+		
5	*Klebsiella sp.2*	+	+		+	+	
6	*E. coli,*	+	+	-	+		+
7	*Enterobacter*	+	+	-	+		
8	*Proteus sp.1*	+	+		+	+	
9	*Proteus sp.2*	+	+		+		+
10	*Citrobacter*	+	+		+		

***Each data is an average of three replicates**

Table 2: Production of Chitinase and pectinase (IU/ml) by bacterial isolates.

Sl.No.	Name of the isolate	Chitinase production (IU/ml)	Pectinase production(IU/ml)
1	*Pseudomonas*	1.11	1.10
2	*Staphylococcus*	0.98	0.65
3	*Micrococci*	0.92	0.73
4	*Klebsiella sp.1*	1.02	0.94
5	*Klebsiella sp.2*	1.03	0.75
6	*E. coli,*	0.94	0.68
7	*Enterobacter*	1.21	1.19
8	*Proteus sp.1*	0.88	0.75
9	*Proteus sp.2*	0.94	0.63
10	*Citrobacter*	0.75	0.72

*The overall model is significant with $p<0.05$

The isolated bacterial strains were tested for the enzyme productions. All the ten bacterial strains showed the Chitinase and pectinase productions. Of the ten bacterial strains the isolate Enterobacter which produced maximum (1.21 IU/ml) amount of Chitinase and 1.19 IU/ml of pectinase respectively. The range of Chitinase production was 0.75 IU/ml to 1.12 IU/ml by all the isolates studied. The production of pectinase was ranged 0.63 to 1.12 IU/ml by all the strains in the work (Table-2 & Figure-1).

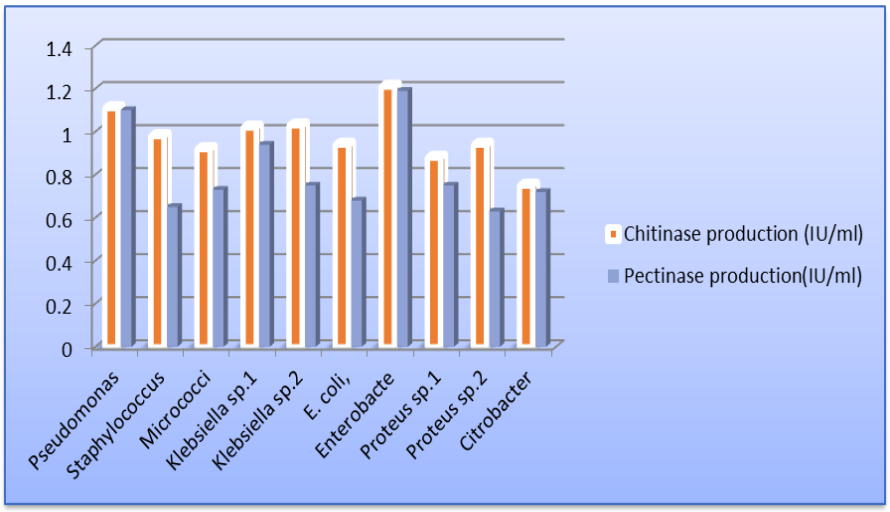

Figure-1: Production of Chitinase and pectinase (IU/ml) by bacterial isolates.

Factors effecting the Pectinase and Chitinase production by bacterial isolates

Various factors like pH, incubation period and temperature may affect the enzyme production in bacteria and fungi. The present study mainly focusing on enzyme production by all the ten bacterial isolates. All the ten bacterial strains were tested for the enzyme productions. The incubation period of 48 to 72 hours were tested for all the isolates. The maximum enzyme production was observed at 48 hours by all the ten isolates. The two pH ranges pH6 and pH7 were used in this study. All the isolates showed maximum enzyme production on pH 7.0. In low pH the ten isolates showed slow growth and enzyme production in neutral pH. The enzyme production of all the isolates were tested for two temperatures ranges 30°C. This is suitable for the growth of bacteria (Table 3, 4& 5).

Table 3: Effect of incubation period on Chitinase and Pectinase production(IU/ml) by bacterial isolates.

Sl.No.	Name of the isolate	Chitinase production (IU/ml) 48 Hours	Chitinase production (IU/ml) 72 Hours	Pectinase production (IU/ml) 48 Hours	Chitinase production (IU/ml) 72 Hours
1	*Pseudomonas*	0.67	0.67	0.88	0.88
2	*Staphylococcus*	0.92	0.92	0.75	0.75
3	*Micrococci*	0.78	0.78	0.91	0.91
4	*Klebsiella sp.1*	0.74	0.74	0.45	0.45

5	*Klebsiella sp.2*	0.99	0.99	0.56	0.56
6	*E. coli,*	0.58	0.58	0.77	0.77
7	*Enterobacter*	1.21	1.21	1.19	1.19
8	*Proteus sp.1*	0.32	0.32	0.67	0.67
9	*Proteus sp.2*	0.35	0.35	0.79	0.79
10	*Citrobacter*	0.77	0.77	0.93	0.93

*The overall model is significant with $p<0.05$

The enzyme production started from 24 hours of incubation period and it reached maximum at 72 h of incubation. Various researches reveal that the enzyme production varies from species to species. Enzyme production started after 3 days of incubation and maximum was observed after 5 days of incubation. (Gueye *et al*., 2020).

Table 4: Effect of pH on Pectinase and Chitinase and Pectinase production (IU/ml) by bacterial isolates.

Sl.No.	Name of the isolate	Chitinase production (IU/ml) pH-6	Chitinase production (IU/ml) pH-7	Chitinase production (IU/ml) pH-6	Chitinase production (IU/ml) pH-7
1	*Pseudomonas*	0.45	0.55	0.75	0.75
2	*Staphylococcus*	0.65	0.67	0.89	0.89
3	*Micrococci*	0.77	0.38	0.97	0.97
4	*Klebsiella sp.1*	0.78	0.89	0.91	0.91
5	*Klebsiella sp.2*	0.98	0.78	0.88	0.88
6	*E. coli,*	0.65	0.88	0.65	0.65
7	*Enterobacter*	1.21	1.21	1.19	1.19
8	*Proteus sp.1*	0.88	0.76	0.76	0.76
9	*Proteus sp.2*	0.68	0.87	0.74	0.74
10	*Citrobacter*	0.75	0.65	0.62	0.62

*The overall model is significant with $p<0.05$

Table 5: Temperature on Chitinase and Pectinase production (IU/ml) by bacterial isolates.

Sl.No.	Name of the isolate	Chitinase production (IU/ml)		Pectinase production (IU/ml)	
		30°C	35°C	30°C	35°C
1	*Pseudomonas*	0.67	0.62	0.78	0.67
2	*Staphylococcus*	0.87	0.80	0.89	0.77
3	*Micrococci*	0.97	0.82	0.88	0.73
4	*Klebsiella sp.1*	0.81	0.75	0.91	0.89
5	*Klebsiella sp.2*	0.82	0.75	0.77	0.67
6	*E. coli,*	0.91	0.88	0.85	0.75
7	*Enterobacter*	1.21	1.10	1.19	1.05
8	*Proteus sp.1*	0.72	0.65	0.65	0.61
9	*Proteus sp.2*	0.67	0.61	0.62	0.57
10	*Citrobacter*	0.58	0.55	0.77	0.63

*The overall model is significant with $p<0.05$

Effect of carbon and nitrogen sources

Carbon and nitrogen sources greatly affect the enzyme productions. The presence of glucose as carbon source the strain Enterobacter which produces maximum enzyme production. In this study various mono, di, and poly saccharides were used for the production of enzymes (Table-6). Chitinase production was maximum increased with the presence of carbon source glucose as suitable was also reported by Kumar and Ram (2016).

The nitrogen source peptone is suitable for maximum enzyme production by Enterobacter. All the isolates showed moderate enzyme production in peptone containing media (Table-7). Carbon and nitrogen sources greatly affect the secondary metabolite productions like Exo polysacharides and enzymes (Kumar and Ram; 2014).

Table 6: Effect of carbon sources on Chitinase and Pectinase production by bacterial isolates.

Sl.No.	Name of the isolate / Carbon Source	Chitinase production (IU/ml)				Pectinase production (IU/ml)			
		Glucose	Fructose	Galactose	Maltose	Glucose	Fructose	Galactose	Maltose
1	*Pseudomonas*	0.85	0.81	0.78	0.89	0.89	0.88	0.83	0.99
2	*Staphylococcus*	0.91	0.88	0.75	0.87	0.91	0.78	0.84	0.75
3	*Micrococci*	0.78	0.77	0.74	0.78	0.94	0.85	0.85	0.78
4	*Klebsiella sp.1*	0.98	0.78	0.91	0.97	0.99	0.75	0.88	0.72
5	*Klebsiella sp.2*	0.78	0.75	0.89	0.96	0.78	0.65	0.75	0.75
6	*E. coli,*	0.81	0.91	0.79	0.70	0.98	0.96	0.71	0.86
7	*Enterobacter*	1.21	0.82	0.77	0.85	1.19	0.75	0.65	0.85
8	*Proteus sp.1*	0.88	0.83	0.85	0.81	0.88	0.75	0.89	0.90
9	*Proteus sp.2*	0.78	0.88	0.79	0.78	0.79	0.79	0.88	0.92
10	*Citrobacter*	0.75	0.82	0.88	0.68	0.90	0.88	0.78	0.86

*The overall model is significant with $p<0.05$

Table 7: Effect of nitrogen sources on Chitinase and Pectinase production by bacterial isolates.

Sl.No.	Name of the isolate / Nitrogen Source	Chitinase production (IU/ml)				Pectinase production (IU/ml)			
		Peptone	Yeast Extract	Beef Extract	Glycine	Peptone	Yeast Extract	Beef Extract	Glycine
1	*Pseudomonas*	0.76	0.88	0.85	0.79	0.88	0.98	0.75	0.81
2	*Staphylococcus*	0.89	0.67	0.99	0.85	0.87	0.91	0.89	0.72
3	*Micrococci*	0.77	0.89	0.78	0.76	0.92	0.88	0.87	0.74
4	*Klebsiella sp.1*	0.85	0.85	0.81	0.79	0.85	0.85	0.85	0.71
5	*Klebsiella sp.2*	0.76	0.82	0.83	0.80	0.80	0.87	0.76	0.73
6	*E. coli,*	0.78	0.85	0.84	0.84	0.79	0.74	0.75	0.82
7	*Enterobacter*	1.21	0.75	0.77	0.82	1.19	0.83	0.73	0.87
8	*Proteus sp.1*	0.98	0.78	0.75	0.84	0.78	0.88	0.77	0.90
9	*Proteus sp.2*	0.91	0.81	0.76	0.97	0.75	0.75	0.72	0.88
10	*Citrobacter*	0.88	0.83	0.78	0.92	0.64	0.76	0.84	0.75

*The overall model is significant with $p<0.05$

CONCLUSIONS

In this study we observed the maximum Chitinase and pectinase production (1.21 IU/ml) and (1.19 IU/ml) by Enterobacter. The other isolate pseudomonas also produces maximum amount of pectinase. These two isolates were selected for the further studies like bio control agents. The optimum conditions like pH 7.0, temperature 30 C and incubation period 48 hours for maximum production of enzyme. The carbon and nitrogen sources glucose and peptone greatly influenced the enzyme production.

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