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Research Article

ISOLATION AND SCREENING OF SOIL MICROBES FOR EXTRACELLULAR CHITINASE ACTIVITY

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ABSTRACT

Present investigation was aimed to isolate and screen microbiota from various soil samples collected from different regions of Kalaburagi, Karnataka, India for chitinase production. In simultaneous isolation and screening of microbes from fifty four soil samples, a total of seventy five microbial isolates were found with chitinolytic activity. These 75 isolates include 51 bacteria, 11 fungi and 13 actinomycetes. Among these, nineteen isolates exhibited sound, thirty three isolates exhibited moderate and twenty three isolates shown poor chitinase activity on chitin agar plate. Further, two bacterial, two actinomycetes and a fungal isolate with good chitinase activity were selected and subjected for chitinase production under submerged fermentation. As a result, comparatively an actinomycete KLSL-55 emerged as the highest enzyme producer with the activity of 84.66 IU.

Keywords: Chitinase, Actinomycetes, Chitinolytic microbes and rhizospheric soil

1. INTRODUCTION

Chitinases (EC.3.2.1.14) are group of enzymes responsible for the hydrolysis of chitin, a nitrogen containing linear polymer of B-1-4 linked N-acetyl glucosamine residues. They play a pivotal role in performing many biological functions and widely distributed in plants [1] and microbes including bacteria [2-4], fungi [5] and actinomycetes [6-7]. In plants chitinases involved in defense against pathogens [8-12]. Chitin is the second most abundant natural polymer and widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae [13].

On the other hand plant diseases are a major problem facing by plant cultivators and are responsible for the loss of 10 % of the total global crop production [14]. Molds, one of the most hostile plant pathogens, are conventionally controlled with chemical fungicides. The extensive use of chemical fungicides, which has tripled over the last four decades, has accelerated environmental pollution and bioaccumulation. Moreover, chemical fungicides may be lethal to beneficial insects and microorganisms populating the soil and may enter the food chain. Despite their high effectiveness and ease of use, chemical fungicides have many disadvantages. Biological control, the use microorganisms to control plant diseases offers an alternative and at this juncture many microbial enzymes were reported as biocontrol agents [15-16].

Biological control of plant pathogens by microorganisms has been considered to be more natural and an environmentally acceptable alternative to the existing chemical treatment methods [17]. The main concept involved in the biocontrol of plant pathogens are mycoparasitism, competition for space and nutrients, stimulation of the plant's defensive capacity, and secretion of bioactive compounds such as antibiotics and cell wall degrading enzymes [18-22]. Chitinolytic enzymes have been considered for the control of plant pathogenic fungi owing to their ability to degrade chitin, which is a major structural component of most fungal cell wall [23-24]. In the present study attempt was made to isolate and screen the soil microbiota for chitinase enzyme production.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

All the chemicals and reagents used in this experimental design were procured from HiMedia Ltd Mumbai India.

2.2. Sample Collection and Isolation of Chitinolytic Microbes

A total of 54 different soil samples were collected from different regions of Kalaburagi, India. The location of soil samples was rhizospheric soil of wheat, maize, and rice, fish market. For screening of chitinase producing microbes, selective media supplemented with 1% colloidal chitin were used. For chitinolytic bacteria, Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4Cl, 1.0; NaCl, 0.5; yeast extract, 0.05; agar, 15; colloidal chitin, 10; pH, 7 (g/l). To isolate chitinolytic fungi, $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; agar, 15; colloidal chitin, 10; pH, 5.6; (g/l). Whereas, to isolate actinomycetes with chitinolytic activity, colloidal chitin, 10; yeast extract, 0.5; $(NH_4)_2SO4$, 1.0; KH_2PO_4, 1.36; $MgSO_4.6H_2O$, 0.3; agar, 15; pH, 7.2; (g/l). Then the cultures were incubated at 37°C for 48 h. The isolates possessing chitinolytic activity which produced the zone of chitin hydrolysis were then selected. The screening was

conducted based on chitinolytic index that was defined as a ratio of a clear zone and colony diameter.

2.3. Preparation of Colloidal Chitin

Colloidal chitin was prepared from the chitin by the modified method of Hsu and Lockwood [25]. In brief, powdered chitin (40g) was dissolved in 600 ml of concentrated HCl with vigorous stirring for 60 min at 30°C. Chitin was precipitated as a colloidal suspension by adding it slowly to 2 l of water at 4–10° C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5 l of distilled water. Washing was repeated thrice until the pH of the suspension becomes to 3.5. After the above treatment, the loose colloidal chitin was used as a substrate.

2.4. Production of Chitinase under Submerged Fermentation (SmF)

As many as 19 isolates with good zone of chitin hydrolysis were further subjected for chitinase production under submerged fermentation. Three different production media supplemented with 1% colloidal were exploited for chitinase production by different isolates of fungi, bacteria and actinomycetes. For fungal isolate, 100ml production medium of pH 6.5 consisted (g/l) of $(NH_4)_2SO_4$ -4.2, NaH_2PO_4-6.9, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$-0.3, Tween-80-0.2, $FeSO_4 \cdot 7H_2O$-0.005, $MnSO_4$-0.0016, $ZnSO_4$-0.0014, $CaCl_2 \cdot 2H_2O$ 0.002 [26], for bacteria chitin broth consisting (g/l) of Na_2HPO_4, 0.65; KH_2PO_4, 1.5; NaCl, 0.25; NH_4Cl, 0.5; $MgSO_4$, 0.12; $CaCl_2$, 0.005; with pH 7 [27] and for actinomycetes, the cultivation media containing (g/l) yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.36; $MgSO_4.6H_2O$, 0.3; with pH 7.2 were used. The Erlenmeyer flask containing 100ml of different production media were inoculated with 1ml culture of selected fungal, bacterial and actinomycetes isolates separately. Thus inoculated flasks were kept for incubation; the incubation temperature was maintained at 32°C, 37°C and 40°C for fungi, bacteria and actinomycetes respectively. There after chitinase assay was performed.

2.5. Enzyme Assay

Chitinase activity was measured with colloidal chitin as substrate. Enzyme solution (1.0 ml) was allowed to react with 1.0 ml of 0.5% colloidal chitin in 0.1M citrate buffer (pH 7.0). The mixture was incubated at 37°C in shaking water bath for 30min. After incubation the reaction was terminated by adding 2ml of DNS reagent, followed by heating in boiling water bath for 5min. After cooling the coloured solution was centrifuged at 10,000 rpm for 5min at room temperature and the absorption of the supernatant was measured at 540nm against the control. The supernatant was used for analysis of reducing sugar using the dinitrosalicylic acid (DNS) method [28]. One unit of chitinase activity was defined as the amount of enzyme that liberates 1µmol of reducing sugar per min per ml.

2.6. Identification of Microorganism

The selected isolate KLSL-55 was subjected for cultural characterization wherein the isolate was streaked on chitin agar plates and there after cultural characters were observed and recorded. Gram's staining of the organism was performed and results were recorded.

3. RESULTS

3.1. Simultaneous Isolation and Screening of Microbes for Chitinase Activity

A total of 75 different chitinolytic microbes were isolated from 54 soil samples collected from different habitats of Gulbarga, Karnataka, India (Table 1). All 75 isolates were subjected for microscopic, cultural and morphological examination. Among the 75 chitinolytic isolates, 51 isolates were found to be bacteria, 11 were found to be fungi and the remaining 13 were identified as actinomycetes. On the basis of colloidal chitin hydrolysis and zone of clearance (>15 mm) on CCA plate, nineteen colonies including 13 bacteria, 1 fungi and 4 actinomycetes were considered with good chitinolytic activity and selected for chitinase production under SmF.

Soil represents a favorable habitat for microorganisms and is inhabited by a wide range of microorganisms, including bacteria, fungi, algae and actinomycetes. There were plenty of microbes were isolated from soil and reported owing to their capability to produce enzymes such as cellulase [29], protease [30], chitinase [2, 31-32], amylase [33-35], lipase [36] and Pectinase [37]. In the presented investigation a total of 75 microbial isolates were isolated from various soil samples with chitinolytic activity. Similar type of results was reported by Pranee and Rillapat [32], wherein they have isolated 65 strains of actinomycetes with chitinolytic activity. Further, Thirteen strains were recognized with antagonistic activity against the fungus *Sclerotium rolfsii*. In another study 54 actinomycetes were isolated from rhizospheric soil with chitinolytic activity; further few isolates were found with antifungal activity against *Colletotrichum capsici* and *Fusarium oxysporum* [37]. Soil bacteria are also predominant producers of chitinase. Saima and Roohi isolated [38] 58 morphologically different bacteria with chitinolytic activity from different soil samples. All these investigation suggests that, soil exhibits diverse microorganisms with chitinolytic activity and it will be serves as the best sources of microbes with diverse metabolic and physiological activities.

3.2. Production of chitinase under SmF

Submerged fermentation was performed with the selected nineteen isolates to analyze the chitinase productivity. Table.2 represents the chitinase production by all nineteen isolates. Comparatively, the isolates KLSL-55, KLSL-6, KLSL-42, KLSL-18 and KLSL-50 found with good enzyme yield with the activity of 84.66 IU, 80.54 IU, 66.66 IU, 65.58 IU and 60.41 IU respectively. As the highest enzyme producer, the isolate KLSL-55 was consider for further optimization studies. Fig.1. represents the chitinase production pattern by 5 selected

isolates. Plate1 shows the zone of chitin hydrolysis by KLSL- 55.

Table.1 Zone of chitin hydrolysis by various isolates

Sl.No.	Isolate	Zone of Chitin Hydrolysis	Sl.No.	Isolate	Zone of Chitin Hydrolysis
	Bacteria			**Bacteria**	
1	KLSL1	30	39	KLSL58	5
2	KLSL2	29	40	KLSL59	10
3	KLSL4	10	41	KLSL60	3
4	KLSL11	10	42	KLSL61	3
5	KLSL13	12	43	KLSL62	22
6	KLSL14	12	44	KLSL63	3
7	KLSL15	10	45	KLSL65	22
8	KLSL16	13	46	KLSL70	9
9	KLSL17	10	47	KLSL71	3
10	KLSL18	28	48	KLSL72	7
11	KLSL19	17	49	KLSL73	5
12	KLSL20	7	50	KLSL74	3
13	KLSL22	23	51	KLSL75	4
14	KLSL23	23		**Actinomycetes**	
15	KLSL24	21	52	KLSL3	14
16	KLSL25	12	53	KLSL5	10
17	KLSL26	14	54	KLSL6	29
18	KLSL27	21	55	KLSL7	24
19	KLSL28	13	56	KLSL8	10
20	KLSL29	3	57	KLSL9	11
21	KLSL30	19	58	KLSL10	10
22	KLSL31	15	59	KLSL12	26
23	KLSL33	7	60	KLSL21	5
24	KLSL34	3	61	KLSL32	5
25	KLSL35	7	62	KLSL55	30
26	KLSL36	8	63	KLSL64	5
27	KLSL37	17	64	KLSL67	6
28	KLSL38	10		**Fungi**	
29	KLSL43	7	65	KLSL39	3
30	KLSL45	10	66	KLSL40	7
31	KLSL46	13	67	KLSL41	10
32	KLSL47	13	68	KLSL42	15
33	KLSL48	10	69	KLSL44	3
34	KLSL49	5	70	KLSL51	5
35	KLSL50	29	71	KLSL53	3
36	KLSL52	18	72	KLSL54	3
37	KLSL56	7	73	KLSL66	3
38	KLSL57	3	74	KLSL68	5
			75	KLSL69	8

Table.2 Chitinase production pattern by various isolates

Isolate	Nature of organism	Chitinase Activity [IU]
KLSL1	Bacteria	56.95
KLSL2	Bacteria	51.33
KLSL18	Bacteria	65.58
KLSL19	Bacteria	31.58
KLSL22	Bacteria	10.16
KLSL23	Bacteria	8.45
KLSL24	Bacteria	7.66
KLSL27	Bacteria	35.54
KLSL30	Bacteria	25.75
KLSL37	Bacteria	13.45
KLSL50	Bacteria	60.41
KLSL52	Bacteria	56.41
KLSL62	Bacteria	40.37
KLSL65	Bacteria	57.83
KLSL6	Actinomycetes	80.54
KLSL7	Actinomycetes	57.66
KLSL12	Actinomycetes	47.70
KLSL55	Actinomycetes	84.66
KLSL42	Fungi	64.66

Plate1: Chitin hydrolysis by actinomycetes KLSL-55

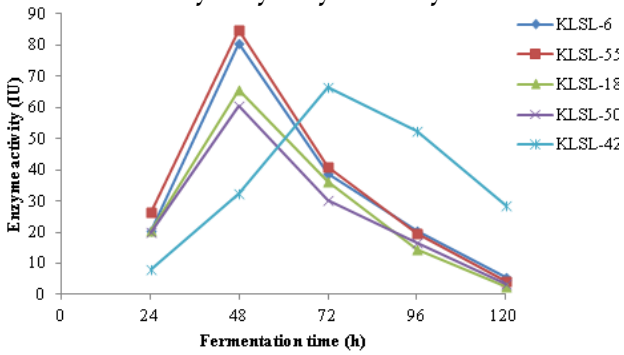

Fig.1: Chitinase productivity pattern by the selected isolates

3.3. Identification of Microbial Isolate

The microbial isolate KLSL-55 was identified as an Actinomycete on the basis of cultural characteristics and microscopic observations. The isolate was produced powdery small sized round colonies with rhizoidal margin on chitin agar media. The colonies were found with white coloration upto five days of incubation, thereafter the colonies imported with gray color pigmentation (Plate.2). Gram's staining of the isolate revealed that, it is Gram positive organism with branched structures (Plate.3).

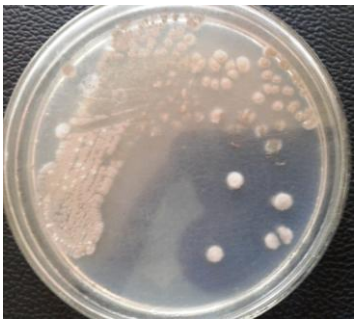

Plate 2: Growth of KLSL-55on Chitin agar plate,

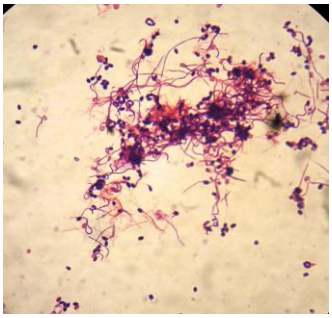

Plate 3: Grams nature of the isolate KLSL-55

4. DISCUSSION

Cultivation of microbes in liquid medium allows high control of the processes and increased mass transfer, as well as enhanced oxygen delivery which will helps in the good growth of the organism as well as its enzyme productivity [39]. In the present investigation SmF was employed for chitinase production by five selected isolates including 2 bacteria, 2 actinomycetes and a fungus. SmF supported preferable yield of chitinase by all selected isolates. The actinomycetes and bacterial isolates were shown highest chitinase productivity after 48 h of fermentation course, whereas fungus took 72 h for the highest productivity of chitinase (Fig. 1). In the present investigation 55 bacterial isolates were obtained from soil samples with chitinolytic property. There are several reports on production of chitinase from soil microbes. Twenty five bacteria were isolated from rhizospheric soil and reported for their chitinolytic property [40]. Further it was found that, chitinase from *Bacillus* sp. I-5 and *Bacillus cereus* I-21 produced under SmF was more effective in hydrolyzing exoskeleton of whitefly (*Bemicia tabaci*). Sharmistha et al., [41] isolated 18 bacterial isolates from sand with chitinolytic property. In similar type of studies Pranee and Rillapat in the year 2012 screened as many as 283 actinomycetes from soil for chitinolytic activity [32]. As the result a total of 68 actinomycetes identified with well chitinolytic activity. 97

isolates of genus *Streptomyces* and 6 isolates of *Microbiospora* from soil were reported for chitinolytic activity [42]. An indigenous fungus *Trichoderma* isolated from soil was exploited for chitinase production and process was optimized [43]. The submerged bioprocess method was optimized for chitinase production by *Beauveria bassiana* isolated from marine sediment soil. The isolate was reported to produce chitinase with activity of 246·6 units/g [44]. In our studies, there were 13 actinomycetes were isolated from soil with chitinolytic activity and among these, few isolates are recognized with good yield under SmF. All these investigations justify that, soil is the best source for chitinolytic microbes specially actinomycetes with comparatively considerable chitinolytic activity. It also reveals that, there are many more actinomycetes in soil which can be isolated and exploited for chitinase production.

5. CONCLUSION

This presented study and relevant data revealed that, soil harbors the vast kind of microbiota with chitinolytic activity. Among all isolates, comparatively actinomycetes showed highest enzyme activity than bacterial and fungal isolates. Comparatively, isolate KLSL-55 found as highest enzyme producer under SmF. Further optimization studies for the enhanced chitinase production will be continued with KLSL-55.

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