Journal of Applied Biology & Biotechnology Vol. 8(02), pp. 41-44, March-April, 2020
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2020.80207

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Factors affecting the chitinase activity of *Trichoderma asperellum* isolated from agriculture field soils

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ARTICLE INFO

Article history:
Received on: August 19, 2019
Accepted on: September 26, 2019
Available online: March 26, 2020

Key words:
Trichoderma,
Chitinase,
Optimization,
Tomato rhizosphere

ABSTRACT

In the present study, 20 fungal strains were isolated from tomato rhizosphere of Senegal. Of 20 strains, five showed the chitinolytic activity on chitin agar medium. Of the five strains, NG4 showed the maximum solubilization zone. This strain was identified by preliminary biochemical and 18S rRNA sequencing analysis. Enzyme production started after 3 days of incubation and maximum was observed after 5 days of incubation. Culture filtrate amended with 0.1% colloidal chitin was used in the production medium. The optimum conditions for maximum chitinase activity are – 6 days of growth and temperature of 30°C at pH 6.0. The chitinase activity was also influenced by the addition of carbon and nitrogen sources in the production medium.

1. INTRODUCTION

Chitin, the second most abundant polysaccharide in nature after cellulose, is a linear polymer of β-1, 4-N-acetylglucosamine. Bacteria, fungi, yeasts, actinomycetes, and plants are the main sources of chitin [1] Complete hydrolysis of chitin can be carried out by a group of chitinolytic enzymes including exochitinase, endochitinase, and chitobiase and release *N*-acetyl-d-glucosamine subunits [2]. Chitinases have a wide range of applications such as biocontrol agents and biopesticides [3]. They were considered as the best biological control agents of plant pathogens due to their ability to degrade fungal cell walls [4,5]. *Trichoderma* species were reported to be act as mycoparasites against soil-borne pathogens such as *Rhizoctonia solani, Sclerotinia rolfsii,* and *Fusarium* sp. [6-8]. In the present study, the optimal culture conditions for maximum production of chitinase production by *Trichoderma* strains under solid-state fermentation were studied and results were discussed.

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2. MATERIALS AND METHODS

2.1. Isolation of Trichoderma

For this study, *Trichoderma* strains isolated from rhizosphere soil samples collected from tomato fields of five different areas of Niaye zone, the main area of horticulture production in Senegal: UCAD, Sangalkam, Gorome, Notto Gouye Diama, and Mboro was used. Identification of *Trichoderma* isolates was based on culture characters as well as microscopic parameters (conidiophores branching, phialides shape and position, spore size, and shape) [9]. The pure cultures maintained at 4°C were used for further studies. The best producer of enzyme was identified by 18S rRNA sequencing (Macrogen, South Korea) as *Trichoderma asperellum.*

2.2. Screening of Isolates for Chitinase Production

The chitinase activities were determined using chitinase detection medium [10] with slight modifications (colloidal chitin 10 g, $MgSO_4$ 0.3 g, NH_4SO_4 3.00 g, KH_2PO_4 2.00 g, agar 20.00 g, and distilled water 1000 ml). Colloidal chitin prepared from commercial chitin was used as a carbon source in the above medium. Four days after incubation, a clear zone surrounding the colony indicates the positive chitinase activity. For positives strains, diameter of hyaline zone around the colony (mm) at different incubation periods (days) was measured.

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2.3. Chitinase Assay

For the determination of chitinase activity, the reaction mixture containing 1 ml each of crude enzyme and 1% colloidal chitin in 0.05 M phosphate buffer (pH 7.0) was incubated at 35°C for 1 h [2]. After incubation, 1 ml of reaction mixture was taken and to this, 1 ml of distilled water was added. The contents were then boiled in a centrifuge tube for 10 min and centrifuged. An aliquot of 0.5 ml of the supernatant was taken and to this, 0.1 ml of potassium tetraborate was added and boiled for exactly 3 min in a water bath. After cooling, 3 ml of P-Dimethylaminobenzaldehyde reagent was added to this reaction mixture and the absorbance was measured at 585 nm. A blank was maintained without chitin or enzyme. The amount of N-Acetyl D-Glucosamine released in the supernatant was determined using N-Acetyl D-Glucosamine as the standard. One unit of the chitinase activity was defined as the amount of the enzyme producing 1 µ mole of N-Acetate glucosamine per minute in 1 ml of reaction mixture under the standard assay conditions [11].

2.4. Optimization of Parameters for Chitinase Production by *T. asperellum*

The isolate *Trichoderma* TG4, highest producer of chitinase activity, was used for optimization using various single parameters. It included incubation period, pH, temperature, carbon sources, nitrogen sources, and colloidal chitin concentration. For each parameter, biomass production (mg/100 ml) and chitinase activity (U/ml) were determined. The effect of incubation period on chitinase production was estimated at different incubation periods (24, 48, 72, 96, and 120 h). Effect of initial pH was studied by adjusting medium pH from 4 to 10 using 0.1 N HCl/NaOH. Six different temperatures (4°C, 15, 20°C, 25°C, 30°C, and 35°C), five carbon sources (sucrose, maltose, galactose, glucose, and colloidal chitin) and five nitrogen sources ($NaNO_3$, $(NH_4)SO_4$, yeast extract, beef extract, and peptone) and five chitin concentrations (0.1, 0.5, 1.0, 1.5, and 2.0) were also evaluated. For each parameter optimization, three sets of independent experiments were carried out and the average values are reported.

2.5. Statistical Analysis

Three replicates were maintained for each treatment. Values were given as means ± standard deviation for triplicate samples.

3. RESULTS AND DISCUSSION

3.1. Chitinase Screening

Five of the 20 strains screened showed the positive results for chitinase activity by the formation of clear zone surrounding the colony on chitin agar plates after 72 h of incubation [Table 1]. Maximum diameter of hyaline zone around colony was observed after 5 days of incubation with *T. asperellum* TG4 (16 mm).

3.2. Effect of Incubation Period on Chitinase Production

Our results [Table 2] elucidated that the incubation period influences the enzyme activity and biomass production. This results showed that a progressive increase production of biomass and enzyme activity by *T. asperellum* was observed between 3 and 5 days of incubation and a drastic reduction of the two parameters 6 and 8 days. Maximum biomass production (353.3 mg/100 ml) and chitinase activity (2.81 U/ml) were reached at 5 of days of incubation.

3.3. Effect of pH on Chitinase Production

The biomass production and enzyme activity were estimated between pH 3 and 10 [Table 3]. The pH of the medium showed a significant influence on the biomass production and chitinase activity by TG4 *Trichoderma* strains. The maximum biomass production (453.3 mg/100 ml) and enzyme activity (2.81 U/ml) were observed at pH 6. Our results showed a progressive increase of enzyme activity from pH 4 to 6. In basic pH (8–10), a drastic reduction of biomass production and enzyme activity was observed.

3.4. Effect of Temperature on Chitinase Production

For the effect of different temperature on biomass and chitinase production by TG4 *Trichoderma* strains, our investigations showed that the maximum biomass production (378.2 mg/100 ml) and enzyme activity (2.99 U/ml) were obtained at 30°C followed by 25°C [Table 4]. Biomass and chitinase activity are low at 4° and increased at 15°C, 20°C, and 25°C. At 35°C and 40°C, a reduction of the different parameters was observed.

3.5. Effect of Carbon Source on Chitinase Production

All the carbon sources (sucrose, maltose, galactose, glucose, and colloidal chitin) increase the biomass production and enzyme activity compares to control [Table 5]. Addition of colloidal chitin in the medium gave maximum biomass production (553.3 mg/100 ml) and chitinase activity (2.01 U/ml) followed by sucrose (1.76 U/ml

Table 1: Screening for chitinase production by fungi isolated from Senegal.

Fungal isolates	Diameter of zone around the colony (mm) at different incubation periods (in days)				
	3	4	5	6	7
TS1	6	8	10	10	10
TN1	4	6	6	6	6
TG4	8	12	16	16	16
TG3	6	8	12	12	12
TM1	4	6	6	6	6

Table 2: Effect of incubation period on chitinase production by *Trichoderma asperellum.*

Incubation period (days)	Biomass production (mg/100 ml)	Enzyme activity (U/ml)
3	148.6±0.24	1.80±0.01
4	227.3±0.49	1.92±0.02
5	353.3±0.6	2.81±0.02
6	250±0.33	1.50±0.03
7	113.3±0.31	0.85±0.02
8	86.6±0.58	0.83±0.01

Table 3: Effect of pH on chitinase activity by *Trichoderma asperellum.*

pH	Biomass production (mg/100 ml)	Enzyme activity (U/ml)
4.0	136±0.47	0.66±0.05
5.0	143.6±0.42	1.88±0.41
6.0	453.3±0.38	2.81±0.29
7.0	353.3±0.53	2.53±0.40
8.0	290±0.73	2.01±0.07
9.0	130±1.04	1.30±0.04
10.0	85.3±0.49	0.35±0.03

for enzyme activity and 411.6 mg/100 ml for biomass production). The galactose showed the lowest chitinase production (0.94 U/ml) compared to control.

3.6. Effect of Colloidal Chitin Concentration on Chitinase Production

Different concentrations of colloidal chitin (0.1%, 0.5%, 1.0%, 1.5%, and 2.0%) were selected to study the effect on biomass and chitinase production [Table 6]. Our results showed a gradual increase of the two parameters with the increase of colloidal chitin concentration up to 1% that showed the maximum biomass production (353.3 mg/100 ml) and enzyme activity (2.81 U/ml). After that, a reduction of biomass and chitinase activity was observed.

3.7. Effect of Nitrogen Sources on Chitinase Production

Effect of nitrogen sources on IAA production by *Trichoderma* strains was studied by the addition of various nitrogenous compounds ($NaNO_3$, $(NH_4)_2SO_4$, yeast extract, beef extract, and peptone). These different nitrogen sources have a significant effect on biomass production and enzyme activity compare to control [Table 7]. Among all the nitrogen sources used, yeast extract was found to be the best nitrogen source for biomass production (456.6 mg/100 ml) and enzyme activity (2.89 U/ml). It is followed for enzyme activity, respectively, by peptone (2.72 U/ml) and beef extract (2.35). Between the nitrogen sources used, $(NH4)_2$ SO_4 showed the lowest biomass production (180 mg/100 ml) and chitinase activity (1.04 U/ml).

Several *Trichoderma* species are able to produce many enzymes like chitinase involved on biocontrol of many soil-borne pathogenic fungi. However, the production of these enzymes depends on many factors such as incubation periods, pH, temperature, carbon sources, and nitrogen sources. Among the 20 strains screened, the results of this study showed that the strains TG4 identify by 18S rRNA sequencing *T. asperellum* showed the maximum zone of inhibition after 5 days of incubation. Optimization of chitinolytic activity of TG4 showed a maximum chitinase and biomass production at 5 days of incubation at 30°C, pH 6. The decrease of chitinase yield after the optimum period of incubation was probably due to the reduced nutrient level of the medium affecting the enzyme synthesis by the fungus [12,13]. Variation of chitinase production of pH and temperature was observed by many authors. Similarly, to our results Mallikharjuna Rao *et al*. [14], Ulhoa and Peberdy [15] found maximum chitinase production at 30°C and pH 6. Sandhya *et al*. [13] found maximum chitinase production after 96 h of incubation at pH 4 using *Trichoderma harzianum* in submerged fermentation. Nampoothiri *et al.* [12] also observed maximum chitinase after 96 h of incubation at 30°C, pH 4.5 with *T. harzianum* in solid-state fermentation. Aida *et al*. [16] showed high level of chitinase production in culture medium with pH 5 at 30°C for 5 days at shaking condition using *Aspergillus terreus* species. Increase in chitinase production at acidic pH in the present study was in coincidence with the results of El-Katatny *et al.* [17], in *T. harzianum* Rifai. Among the various carbon sources tested, colloidal chitin showed the maximum biomass and chitinase production. Same results were observed by Sandhya *et al*. [13] and Nampoothiri *et al*. [12]. Rao *et al*. [16] also showed that maximum chitinase production was observed when chitin or dried cell walls of *S. rolfsii* were incorporated in the media. The production of extracellular enzymes like chitinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls [18,19]. Our results showed that yeast extract was the best nitrogen source for biomass and chitinase production by *Trichoderma*

Table 4: Effect of temperature on chitinase activity by *Trichoderma asperellum.*

Temperature (°C)	Biomass production (mg/100 ml)	Enzyme activity (U/ml)
4	88.0±0.33	0.45±0.11
15	161±0.73	0.88±0.05
20	201±0.47	1.39±0.08
25	353.3±0.31	2.54±0.04
30	378.2±0.33	2.99±0.08
35	333.3±0.42	1.86±0.13
40	203.3±0.44	1.08±0.07

Table 5: Effect of carbon sources on chitinase activity by *Trichoderma asperellum.*

Carbon sources	Biomass production (mg/100 ml)	Enzyme activity (U/ml)
Control	46.6±1.33	0.15±0.01
Sucrose	411.6±1.57	1.76±0.08
Maltose	280±1.53	1.12±0.03
Galactose	180±0.86	0.94±0.05
Glucose	246.6±0.77	1.23±0.05
Colloidal chitin	553.3±0.46	2.01±0.01

Table 6: Effect of colloidal chitin concentration by *Trichoderma asperellum.*

Colloidal chitin concentration (%)	Biomass production (mg/100 ml)	Chitinase activity (U/ml)
0.1	80±0.51	0.19±0.01
0.5	233.3±1.40	1.96±0.03
1.0	353.3±1.11	2.81±0.02
1.5	280±0.76	1.24±0.01
2.0	130±0.47	0.74±0.01

Table 7: Effect of nitrogen sources on chitinase activity by *Trichoderma zasperellum.*

Nitrogen sources	Biomass production (mg/100 ml)	Enzyme activity (U/ml)
Control	40±0.37	0.45±0.06
$NaNO_3$	303.3±0.51	1.90±0.04
$(NH_4)_2SO_4$	180±0.73	1.04±0.02
Yeast extract	456.6±0.53	2.89±0.03
Beef extract	410±0.4	2.35±0.02
Peptone	436.6±0.18	2.72±0.02

strains. Similar results was reported by Nampoothiri *et al.* [12], while Sandhya *et al*. [13] reported that peptone and tryptone showed maximum enzyme production by *T. harzianum.*

4. SUMMARY

The present study reports the factors affecting chitinase production by *Trichoderma* strains. Of the 20 *Trichoderma* strains isolated, five showed chitinolytic activity. Among the five strains, TG4

(*T. asperellum*) showed maximum chitinase activity. Optimization studies on *T. asperellum* showed maximum chitinase activity at 5 days of incubation, pH 6.0, and 30°C temperature. Addition of colloidal chitin and yeast extract as carbon and nitrogen sources, respectively, greatly influenced the chitinolytic activity of *Trichoderm*a strains. These observations, thus, confirmed that *T. asperellum* (TG4) could be a potential fungus for the production of extracellular chitinase.

5. ACKNOWLEDGMENTS

The authors are thankful to CV Raman International Fellowship for African Researcher of the Department of Science and Technology, Government of India, for the financial assistance under Fellowship Project (DO NO. DST/INT/CVRF/2016). We are also thankful to the Department of Botany and Microbiology, Acharya Nagarjuna University, for providing the facilities to do this work.

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How to cite this article:
Gueye N, Kumar GK, Ndiaye M, Sall SYD, Ndiaye MAF, Diop TA, Ram MR. Factors affecting the chitinase activity of *Trichoderma asperellum* isolated from agriculture field soils. J App Biol Biotech. 2020;8(02):41-44. DOI: 10.7324/JABB.2020.80207