BEST: International Journal of Humanities, Arts, Medicine and Sciences (BEST: IJHAMS)
ISSN (P): 2348–0521, ISSN (E): 2454–4728
Vol. 9, Issue 1, Jun 2021, 1-8
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Best Journals
Knowledge to Wisdom

OPTIMIZATION OF EXTRACELLULAR GLUCANASE PRODUCTION FROM FUNGI ANTAGONISTIC TO PHYTOPATHOGEN *FUSARIUM OXYSPORUM* MTCC 4162 IN VITRO

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ABSTRACT

In order to find out the potential biological control fungal agent against *Fusarium oxysporum* MTCC 4162, various isolated fungal species from local rhizosphere showing antagonistic activity were optimized for extracellular glucanase production. The locally isolated antagonists included *Rhizopus oryzae*, *Penicillium notatum, Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger, Mucor racemosus, Curvularia lunata, Alternaria alternata* and *Fusarium equiseti.* Results revealed that all tested fungal species except *Rhizopus oryzae* (pH 7.5) produces maximum glucanase at neutral pH (pH 7.0). Incubation temperature 25°C and 35°C were found optimum for production of glucanase enzyme. *A. alternata* showed maximum glucanase activity (112.22 µg glucose min^{-1} mg protein^{-1}) at 25°C followed by *A. flavus. Aspergillus niger* produced maximum glucanase enzyme in presence of starch (59.21 µg glucose min^{-1} mg protein^{-1}) followed by galactose (50.25 µg glucose min^{-1} mg protein^{-1}). *Alternaria alternata* produced maximum glucanase enzyme in presence of peptone (123.98 µg glucose min^{-1} mg protein^{-1}) followed by tryptone (63.90 µg glucose min^{-1} mg protein^{-1}). The study advocates that for effective biological control, optimization of fungal strains for glucanase must be performed to get better results.

KEYWORDS: Biological control, Phytopathogen, Antagonism, *Fusarium* & Glucanase

1. INTRODUCTION

Antagonism among fungi is a long established strategy for biological control of the pathogenic fungi [1]. Plant pathogens, i.e. *Fusarium oxysporum* are among the most important biotic agents causing serious losses and damages to agricultural products. Further, development of more effective antagonistic agents is the need of the hour, looking at the various deleterious effects of chemical agents on crops and environment [2]. It is important for the antagonistic fungi to penetrate the cell wall of the phytopathogen effectively to exert the biological control, as the fungal cell wall is composed of polysaccharides, including α-1,3-glucan, α-1,4-glucan, β-1,3-glucan, β-1,3-1,4-glucan, and β-1,6-glucan as main constituents [3]. Glucanases are able to hydrolyse their substrates by cleaving β-linkages at random sites along the polysaccharide chain, leading to the release of smaller oligosaccharides and glucose. Production of extracellular glucanase is the key to biological control and can be affected by various factors including media conditions and nutrients [4]. This provokes that for isolation of a more effective biological control agents, expression and optimization of extracellular glucanase in one of the key features. During our hunt for effective biological control agent against *F. oxysporum* MTCC 4162, we earlier identified nine fungal strains from local area that have shown more than 50% reduction of radial mycelial growth of *Fusarium oxysporum* MTCC4162 during *in vitro* trials. The present study deals with the optimization of culture

conditions for maximizing the production of extracellular glucanase for various fungal isolates.

2. MATERIALS AND METHODS

Isolation of Fungi Antagonistic to *Fusarium oxysporum* MTCC 4162

The fungi antagonistic to *F. oxysporum* MTCC 4162 was obtained from Microbial Type Culture Collection, Chandigarh, India, originally isolated from soil. Antagonistic activity was established using the dual culture method and the suppression of radial growth of phytopathogen was recorded. These fungi were identified earlier using macroscopic and microscopic features [5].

Culture Conditions and Glucanase Assay

The fungal species with antagonistic activity were screened for their ability to produce extracellular glucanase. For this 100 ml of potato dextrose broth (pH 7.5) was inoculated with fungal spore suspension containing 10^4-10^6 spores and incubated at 25 ± 2°C with shaking at 100 rpm for 7 days. The culture was filtered through Whatman No.1 filter paper, before the proteins (enzymes) were precipitated using 70% ammonium sulphate saturation at 4°C followed by dialysis (10 KDa cut-off, HiMedia, India) overnight in 50 mM phosphate buffer (pH 7.0). The dialysate was used to evaluate β-glucanase activity (EC 3.2.1.6) using the method of Gautam *et al*. [6]. Briefly, to 0.5 ml Carboxy Methyl Cellulose solution (1% w/v), 0.5 ml of partially purified enzyme extract was added and were incubated at 37°C for 30 minutes in water bath. Controls devoid of enzyme extract were also run simultaneously. To the reaction mixture, added 3 ml of 3, 5-dinitrosalicylic acid reagent followed by 1.0 ml of sodium potassium tartrate (40% w/v). Reaction mixture was then placed in a boiling water bath (70°C) for 5 minutes for colour development and cooled immediately on an ice bath. The solution was then diluted to 15 ml using distilled water and absorbance was noted at 550 nm against a reagent blank. Rate of enzyme activity was determined in terms of amount of glucose produced per minute, calculated by using a standard curve of D-glucose. Specific enzyme activity was calculated as rate of enzyme activity per mg protein in reaction mixture, calculated using the standard curve of bovine serum albumin using Bradford's method [7].

Optimization of Growth Conditions in Shake Flask

Optimization of the production medium was done by classical method [8]. Culture condition namely physical parameters (pH and temperature) and nutritional parameters (carbon source and nitrogen source) were optimized for maximum yield of glucanase enzymes. Effect of pH on production of enzyme was determined by adjusting the pH in the range of 4.0-9.0 (with a difference of 0.5) of the potato dextrose with 0.1 N HCl and 0.5 N NaOH. Medium with pH 7.5 was set as a control. Seed inoculum of all studied fungal strain was inoculated in 100 ml of potato dextrose medium and incubated at 25°C for 7 days at 100 rpm. After incubation, enzyme extract was prepared and specific enzyme activity was estimated by following standard assay method as mentioned above in 2.2To study the effect of temperature on enzyme production, the seed inoculum was inoculated in 100 ml of potato dextrose medium (pH 7.0) and incubated for 7 days at 100 rpm. Effect of temperature on enzyme production was assessed by incubating medium at different temperature 15-85 °C with a difference of 10 °C. Medium with temperature 25 °C was set as a control. After incubation, enzyme extract was prepared and specific enzyme activity was estimated.

To study the effect of carbon source on enzyme production, the seed inoculum was inoculated in 100 ml of potato dextrose medium (pH 7.0) and incubated for 7 days at 100 rpm. Effect of various carbon sources on enzyme production was assessed by substituting fructose, sucrose, maltose, galactose, soluble starch and cellulose in place of glucose in the

potato dextrose medium. Medium with glucose was served as control. Effect of nitrogen sources on enzyme production was assessed by adding peptone, tryptone, ammonium nitrate and ammonium sulphate in potato dextrose medium. The seed inoculum was inoculated in 100 ml of potato dextrose medium (pH 7.0) and incubated for 7 days at 100 rpm. After incubation, specific enzyme activity was estimated.

3. RESULTS

To isolate fungal species rhizospheric soil samples were collected from different sites of Jabalpur (M.P.) region during the year 2018-2019. Dual culture technique was utilized to determine the *in vitro* antagonistic potential of isolated fungal species (test fungus) to suppress the growth of phytopathogen *F. oxysporum* MTCC 4162 on PDA. Out of 22 tested fungi, only 9 could show some inhibition of the *F. oxysporum* MTCC 4162 (data not shown). The fungal species showing antagonistic activity against *F. oxysporum* MTCC 4162 included *Aspergillus flavus*, *A. niger, A. fumigatus*, *Rhizopus oryzae*, *Penicillium notatum, Mucor racemosus, Curvularia lunata, Alternaria alternata* and *Fusarium equiseti.* All these fungus were optimized to extracellular glucanase production and compared.

Effect of pH on Glucanase Production

In order to determine the effect of medium pH on glucanase production by fungal species, liquid state fermentation was conducted. Isolated fungal species were incubated in medium with different pH ranging 4.0 - 9.0 at 25° C for 7 days and results are presented in Fig 1(A-I). Results revealed that all tested fungal species except *Rhizopus oryzae* (pH 7.5) produces maximum glucanase at neutral pH (pH 7.0). Among all studied strains *A. alternata* showed maximum glucanase activity (134.14 μg glucose min^{-1} mg $protein^{-1}$) followed by *M. racemosus* (132.92 μg glucose min^{-1} mg $protein^{-1}$) > *P. notatum* (130.90 μg glucose min^{-1} mg $protein^{-1}$) > *A. fumigatus* (117.07 μg glucose min^{-1} mg $protein^{-1}$) > *C. lunata* (110.35 μg glucose min^{-1} mg $protein^{-1}$) > *F. equiseti* (109.51 μg glucose min^{-1} mg $protein^{-1}$) > *R. oryzae* (82.20 μg glucose min^{-1} mg $protein^{-1}$) > *A. flavus* (80.34 μg glucose min^{-1} mg $protein^{-1}$) > *A. niger* (66.75 μg glucose min^{-1} mg $protein^{-1}$).

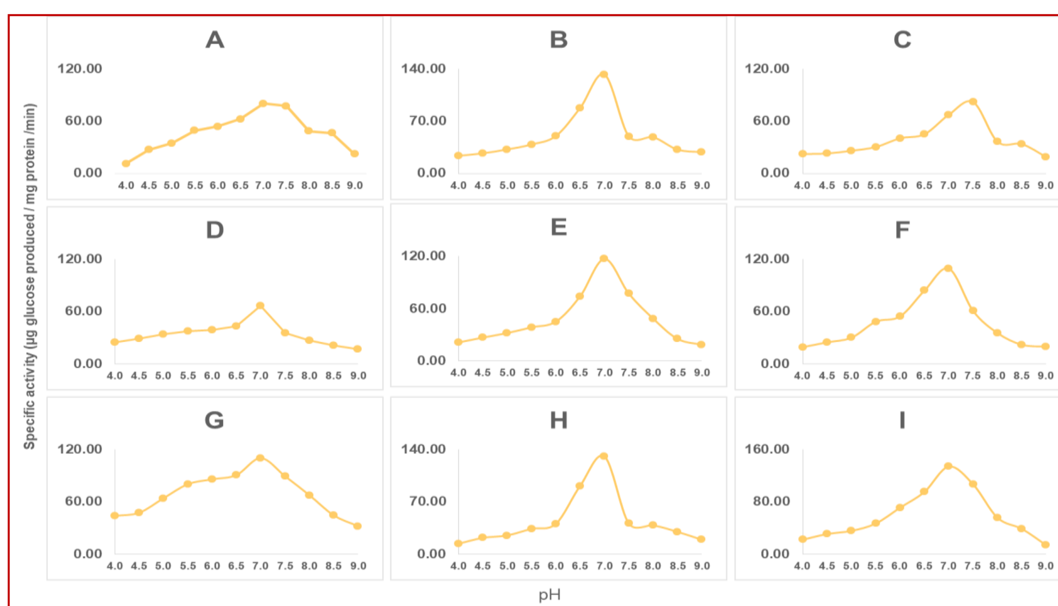

Figure 1: Specific Glucanase Activity (μg glucose produced min^{-1} mg $protein^{-1}$) of different fungi antagonistic to *Fusarium oxysporum* MTCC 4162 at varying pH. A: *Aspergillus flavus*, B: *Mucor racemosus*, C: *Rhizopus oryzae*, D: *Aspergillus niger*, E: *Aspergillus fumigatus*, F: *Fusarium equiseti*, G: *Curvularia lunata*, H: *Penicillium notatum*, and I: *Alternaria alternata*

Effect of Temperature on Glucanase Production

To determine the effect of incubation temperature on production of glucanase enzyme, the fungal species were grown in batch culture by incubating them at different temperature ranging from 15-85° C for 7 days. Data revealed that temperature 25°C and 35°C were found optimum for production of glucanase enzyme. *Alternaria alternata* showed maximum glucanase activity (112.22 µg glucose min^{-1} mg protein^{-1}) at 25°C followed by *A. flavus* (95.32 µg glucose min^{-1} mg protein^{-1}), *R. oryzae* (89.39 µg glucose min^{-1} mg protein^{-1}), *A. fumigatus* (88.42 µg glucose min^{-1} mg protein^{-1}), *F. equiseti* (63.06 µg glucose min^{-1} mg protein^{-1}) while *C. lunata* possess maximum glucanase activity (96.50 µg glucose min^{-1} mg protein^{-1}) at 35°C followed by *A. niger* (55.06 µg glucose min^{-1} mg protein^{-1}), *M. racemosus* (54.69 µg glucose min^{-1} mg protein^{-1}) and *P. notatum* (50.86 µg glucose min^{-1} mg protein^{-1}) Fig 2 (A-I).

Figure 2: Specific Glucanase Activity (µg glucose produced min^{-1} mg protein^{-1}) of different fungi antagonistic to *Fusarium oxysporum* MTCC 4162 at varying temperatures. A: *Aspergillus flavus*, B: *Mucor racemosus*, C: *Rhizopus oryzae*, D: *Aspergillus niger*, E: *Aspergillus fumigatus*, F: *Fusarium equiseti*, G: *Curvularia lunata*, H: *Penicillium notatum*, and I: *Alternaria alternata*

Effect of Carbon Source on Glucanase Production

The use of different carbon sources viz. glucose, fructose, sucrose, maltose, galactose, starch and cellulose on production of glucanase enzyme by the isolated fungal species revealed that *A. niger* produces maximum glucanase enzyme in presence of starch (59.21 µg glucose min^{-1} mg protein^{-1}) followed by galactose (50.25 µg glucose min^{-1} mg protein^{-1}) while all other tested fungal species produces maximum amount of glucanase in presence of glucose followed by starch. In presence of glucose, *A. alternata* showed maximum glucanase activity (120.19 µg glucose min^{-1} mg protein^{-1}) followed by *R. oryzae* (109.51 µg glucose min^{-1} mg protein^{-1}), *A. flavus* (101.90 µg glucose min^{-1} mg protein^{-1}), *F. equiseti* (97.67 µg glucose min^{-1} mg protein^{-1}) and *C. lunata* (95.95 µg glucose min^{-1} mg protein^{-1}). *A. fumigatus* (90.56 µg glucose min^{-1} mg protein^{-1}) and *P. notatum* (90.53 µg glucose min^{-1} mg protein^{-1}) showed almost same amount of glucanase activity in

presence of glucose while minimum amount of glucanase was produced by *M. racemosus* (67.49 µg glucose min^{-1} mg protein^{-1}) (Fig 3 (A-I).

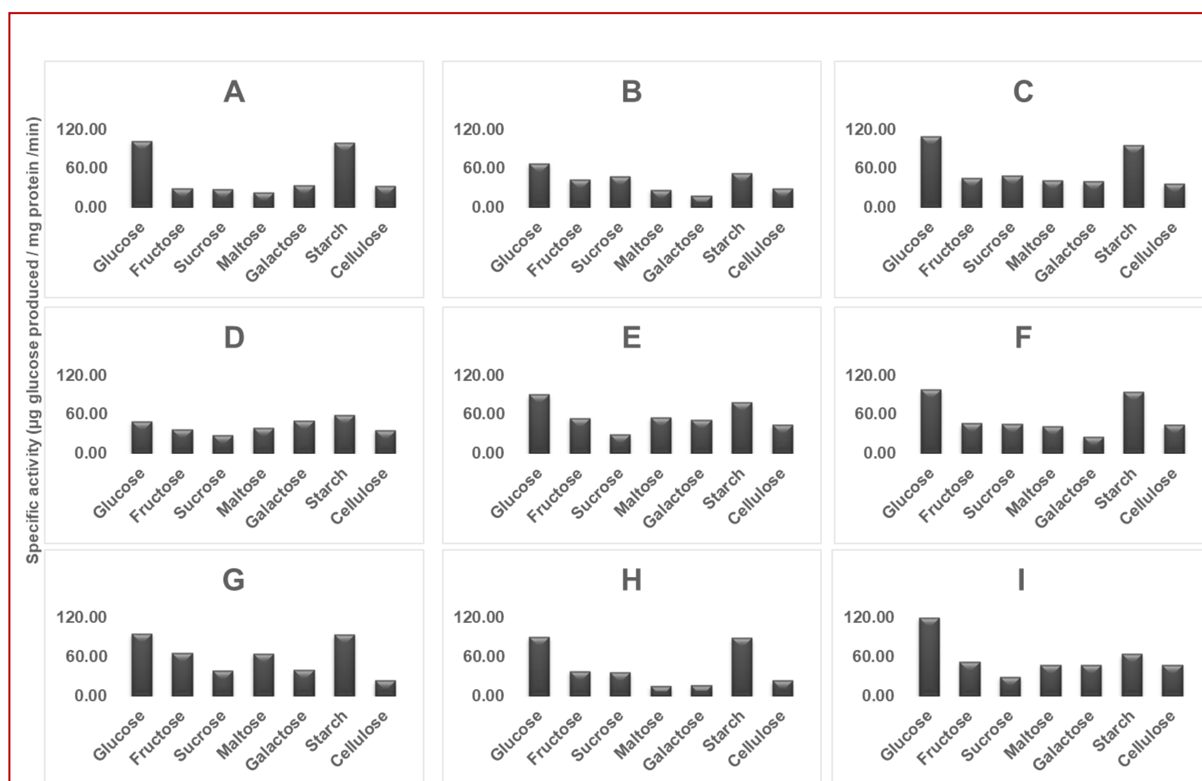

Figure 3: Specific Glucanase Activity (µg glucose produced min^{-1} mg protein^{-1}) of different fungi antagonistic to *Fusarium oxysporum* MTCC 4162 with different carbon sources. A: *Aspergillus flavus*, B: *Mucor racemosus*, C: *Rhizopus oryzae*, D: *Aspergillus niger*, E: *Aspergillus fumigatus*, F: *Fusarium equiseti*, G: *Curvularia lunata*, H: *Penicillium notatum*, and I: *Alternaria alternata*

Effect of Nitrogen Source on Glucanase Production

The effect of different nitrogen sources viz. peptone, tryptone, ammonium nitrate and ammonium sulphate on production of glucanase enzyme by the isolated fungal species was determined and results are presented in Fig 4 (A-I). Results revealed among all studied strains *A. alternata* produces maximum glucanase enzyme in presence of peptone (123.98 µg glucose min^{-1} mg protein^{-1}) followed by tryptone (63.90 µg glucose min^{-1} mg protein^{-1}) while minimum amount of glucanase was produced by *A. fumigatus* in presence of ammonium nitrate (23.42 µg glucose min^{-1} mg protein^{-1}). Along with *A. alternata, R. oryzae* (114.64 µg glucose min^{-1} mg protein^{-1}), *A. flavus* (111.02 µg glucose min^{-1} mg protein^{-1}), *C. lunata* (104.91 µg glucose min^{-1} mg protein^{-1}), *F. equiseti* (99.23 µg glucose min^{-1} mg protein^{-1}), *A. fumigatus* (98.23 µg glucose min^{-1} mg protein^{-1}), *P. notatum* (93.60 µg glucose min^{-1} mg protein^{-1}) and *M. racemosus* (85.88 µg glucose min^{-1} mg protein^{-1}) showed maximum glucanase activity in presence of peptone followed by tryptone. *Aspergillus niger* produces maximum amount in presence of tryptone (64.99 µg glucose min^{-1} mg protein^{-1}) followed by peptone (62.72 µg glucose min^{-1} mg protein^{-1}).

Figure 4: Specific Glucanase Activity (µg glucose produced min^{-1} mg protein^{-1}) of different fungi antagonistic to *Fusarium oxysporum* MTCC 4162 with different nitrogen sources. A: *Aspergillus flavus*, B: *Mucor racemosus*, C: *Rhizopus oryzae*, D: *Aspergillus niger*, E: *Aspergillus fumigatus*, F: *Fusarium equiseti*, G: *Curvularia lunata*, H: *Penicillium notatum*, and I: *Alternaria alternata*

4. DISCUSSIONS

Microbial biological control agents use a great variety of mechanisms to protect plants from pathogens and production of metabolites is one of them [9]. Lytic enzymes are among these metabolites that can break down polymeric compounds, including chitin, proteins, cellulose, hemicellulose and DNA [10,11]. A basic principle in mycoparasitism or in turn biological control can well be attributed to the enzymes that can penetrate the fungal cell wall, and glucanases are one of them, as glucan is one of the important constituent of fungal cell wall. The extensive use of chitinase and glucanase producing microorganism as biological control agents against many fungal pathogens has been reported [12,13]. These lytic enzymes break down cell wall polysaccharides into short oligomers and by this way facilitate the hyperparasite to penetrate into the cytoplasm of the target fungi. Parmar *et al.* [14] reported that a significant positive correlation existed between percentage growth inhibition of phytopathogen and β-1,3-glucanase activity in the culture medium of antagonist treatment.

During the present study, it was found that almost all fungal species produced glucanase at neutral pH (pH 7.0) and temperature 25-35°C. Results are contradictory to the previous report who reported maximum enzyme activity of *Aspergillus niger* and *Penicillium chrysogenum* at pH 5.0 [15], and *Aspergillus niger* at pH 4.0 - 4.5 [16]. Among carbon sources, glucose, followed by starch are best suited for glucanase production while peptone followed by tryptone are found to be most influential nitrogen source for glucanase production by most of the fungal species. In contrast to present finding peptone and yeast extract (1.0% (w/v) was best nitrogen sources for the production of endo-ß-glucanase by *A. niger* [6]. The contradiction may be understood by the use of different strains and other environmental conditions in various studies.

5. CONCLUSIONS

The present study optimizes the glucanase production from various fungi that can be antagonistic to *Fusarium oxysporum* MTCC 4162, a well-known plant pathogen. The study shows that for the biological control of phytopathogens, the locally isolated strains should be used after optimizing the conditions for lytic enzyme production in order to achieve effective results.

6. ACKNOWLEDGEMENT

The authors would like to acknowledge Head, Department of P.G. Studies and Research in Biological Sciences, Rani Durgavati University, Jabalpur for laboratory facilities.

7. CONFLICT OF INTERESTS

The authors have no conflict of interests.

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