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Exploring the industrially important extracellular enzymes from different nematode-trapping fungi

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ABSTRACT

Nematophagous fungi are potential bio-control agent against the plant and animal parasitic nematodes. The extracellular hydrolytic enzymes are one of the important virulence factors for these fungi. In the present study, we investigated industrially important extracellular enzymes from two different genera of nematode-trapping fungi. Duddingtonia flagrans produced the highest amount of amylase (261 U/mL/min) and xylanase (76mU/mL/min) compared to Arthrobotrys conoides (186 and 39 U/mL/min) while it was reversed in case of cellulase, highest (214 U/mL/min) in A. conoides. Protease, chitinase and collagenase activity (236, 19.83 and 2.46 U/mL/min) was higher during induction in D. flagrans RPAN-10 as compared to A. conoides (118.6, 8.3 and 1.2 U/mL/min). D. flagrans RPAN-10 showed 3.14 fold and A. conoides 2.37 fold change in protease activity when induced with nematodes. However, there was no apparent difference in control and induced fungus for chitinase and collagenase. In summary, D. flagrans RPAN-10 was found to be a suitable candidate for further exploring the protease, amylase, chitinase and xylanase while A. conoides is suitable for cellulase production for further application. However, further research is required in order to optimization of growth conditions as well as suitable nutrients in order for large scale production for industrial applications.

Key words: Nematophagousfungi. *Arthrobotrys conoides*, *A. conides*, *Duddingtonia flagrans* and *D. flagrans.* Bio-control, , Extracellular enzymes

INTRODUCTION

Due to the rapid advancement in biotechnology, apart from research, the field of biotechnology is also growing as an important sector for bio-business during the last decade. Use of various enzymes in several industries, especially food and beverages, detergent, textile, animal feed supplement, baking, paper and pulp, leather and biomedical products is now a day rapidly increasing. Microbes represent highest diversity on the earth and they are the virtuous source of industrially important enzymes. Several enzymes from various bacteria (Husain *et al.*, 2015; Kale *et al.*, 2016) and fungi have been cloned, expressed, characterized, engineered and few of them also commercialized (Duza and Mastan, 2013; Choi *et al.*, 2015; Joshi and Satyanarayana, 2015; Singh *et al.*, 2016). Still, the race is not over and researchers are always looking for more and more potential enzymes from the microbes.

Among the large number of non-pathogenic microorganisms capable of producing valuable enzymes, filamentous fungi are particularly interesting as one can grow them on very inexpensive substrates like agro waste and high production of extracellular enzymes. Nematophagous fungi are an important group of soil fungi and one of the bio-control agents against the plant and animal parasitic nematodes (Nagee *et al.*, 2001; Nordbring-Hertz *et al.*, 2006). Fundamental studies regarding their characterization, efficacy to trap nematodes under *in vivo* and *in vitro* as well as genomics and transcriptomics of several fungi from this group have been accomplished (Yang *et al.*, 2011; Meerupati *et al.*, 2013; Wang *et al.*, 2018). It has been now well understood that the penetration of the nematode cuticle and digestion involves the activity of various hydrolytic enzymes such as proteases, chitinase and collagenase secreted by the nematophagous fungi (Yang *et al.*, 2007b; Lopez-Llorca *et al.*, 2008; Nagee *et al.*, 2008).

Among these, proteases are the most studied enzyme from different nematophagous fungi (Liu *et al.*, 2011). Another pathogenicity related enzyme, chitinase has been studied mainly in egg parasitic nematophagous fungi (Tikhonov *et al.*, 2002; Khan *et al.*, 2004). Collagenase have been identified in *Arthrobotrys* spp. (Tosi *et al.*, 2002). Moreover, lipase and esterase have been studied in *Pochonia chlamydosporia* (Esteves *et al.*, 2009). However, much less has been done for the commercially important enzymes such as amylase, cellulase, and xylanase from the nematophagousfungi.

Novel enzyme with high catalytic activity, more flexibility to various parameters such as pH, temperature etc. and low cost production are the main the targets for any industry. Moreover, the producing organism with no risk at all is preferable. Therefore, the main objective of the present study was to explore industrially important extracellular enzymes of the nematode-trapping fungi which we have already isolated and characterized previously (Pandit *et al.*, 2014c).

METHODOLOGY

Microorganisms and culture maintenance

Nematophagous fungi *Arthrobotrys conoides* isolates RPAN-12 (GenBank Accession JX979095) and *Duddingtonia flagrans* RPAN-10 (GenBank Accession JX979096) used in the present study was originally isolated from the agricultural soil of Anand district of Gujarat and characterized (Pandit *et al.*, 2014b; Pandit *et al.*, 2014c; Ramesh *et al.*, 2015). Two other isolates of *D. flagrans* RPAN13&14 (GenBank accession KF741374-75) were also previously characterized by our group (Unpublished work). Cultures were maintained on Corn Meal agar plates (CMA; Hi-Media, 1.7%W/V) by inoculating it on freshly prepared medium followed by allowing them to grow at 28ºC for 7 days and stored at 4ºC. The isolates were sub cultured regularly (every 30 days) following the establishing microbiological procedure.

Medium for amylase, cellulase and xylanase production

For amylase, cellulases and xylanase production, fungi were inoculated into modified Czapek Dox medium. This modification includes 0.3% dextrose and 0.2% $NaNO_2$ instead of 3.0% and 2.0%, respectively. Further, 1.0% Starch (Hi-media), cellulose (Hi-media), Birchwood xylan (Sigma) and chitin (Colloidal suspension) was added into the medium as a carbon source of carbon for the respective enzyme.

Fungus inoculation and growth condition

For all the isolates, spore suspension was prepared by adding 5mL sterilized distilled water containing 0.1% tween 80 (Mishra *et al.*, 2013) on a 7 days old culture grown at 28ºC on a CMA (Hi-media, India). Sixty mL medium was prepared in 250mL Erlenmeyer flask for each enzyme and it was inoculated with 1.0mL of above spore suspension. Tetracycline (30µg/mL) was added

into each flask in order to prevent the bacterial contamination. Fungi were allowed to grow at 28ºC and 125rpm on a rotary shaker. For amylase and cellulase, fungi were allowed to grow for 7 days and for xylanase 15 days. For all enzymes, uninoculated medium served as a control. For enzyme activity measurement, the growth medium was centrifuged at 10K rpm for 10 minutes and the supernatant was used as crude enzyme.

Enzyme assay for amylase, cellulase and xylanase

Amylase, cellulase and xylanase activity were measured using substrate 1% soluble starch, 1% CMC and 1% Birchwood xylan prepared in 20 mM sodium phosphate buffer pH 6.7, 50 mM sodium acetate buffer, pH 5.0 and 50 mM sodium acetate buffer, respectively. Enzyme activity was measured from 1mL of culture supernatant by incubating with respective substrate for 30min. Liberated sugars were measured using DNS reagent (Gomes *et al.*, 2001). Absorbance was measured at 540nm. For amylase and cellulase D-glucose and for xylanase D-xylose was used as a standard.

Effect of nematode on protease, chitinase and collagenase activity of the nematode-trapping fungi.

To study the effect of presence of nematodes on protease, chitinase and collagenase enzyme activity, all the isolates were grown in nutritionally poor Czapek Dox medium as described above. For collagenase only *A. conoides* and *D. flagrans* (RPAN-10) was used. For chitinase, colloidal chitin was prepared as described by (Hsu and Lockwood, 1975) with some modification. 20gm chitin (Hi-media) was dissolved in 100mL concentrated HCl by stirring for 1hr. Chitin from the suspension was precipitated by slowly adding 500mL of water at 4ºC. The colloidal chitin was filtered through Whatman filter paper, collected in Petri plates and washed four times using water until the pH of the suspension become near to neutral and then dried in oven at 50-60 ºC.

In our previous study (Pandit *et al*., 2014b), the effect of nematode on protease activity was accessed using medium described by (Braga *et al.*, 2012). In this study, the medium was formulated with 0.3% sucrose and 0.2% sodium nitrate, which is 10 times less than the original content of the medium%. 250mL flask containing 60 mL of the above medium was inoculated in triplicate with a definite amount of spore suspension prepared as described above for each fungus. Fungi were allowed to grow at 28ºC on a rotary shaker at speed 125rpm for four days. After four days, 1mL suspension containing approximately 1000 nematodes were added to each flask and further allowed to grow at 28ºC without shaking. For protease, chitinase flasks were incubated for 48hr while for collagenase for 72hr. Flask without nematodes was used as a control.

Protease activity was measured as described in our previous study (Pandit *et al*., 2014b). Chitinase activity was measured using 1% colloidal chitin as substrate as described by (Tikhonov *et al*., 2002). Briefly, 1mL of culture supernatant was added to 2mL of colloidal chitin. All the tubes were incubated for 30min at 37°C on a rotary platform at a speed sufficient to keep the chitin in suspension. After 2hr, vials were placed in boiling water bath for 5minutes to inactivate the enzyme. It was allowed to cool to room temperature and then the suspension was centrifuged to retain the supernatant liquid. 1mL of this supernatant was used for color development using DNS reagent. Absorbance was measured at 540nm and N-Acetyl-D-Glucosamine was used as standard.

Collagenase activity was measured using collagen (Sigma, USA) as a substrate by following the Sigma's standard protocol (Catalog No. C9891). In brief, collagen was prepared in 50mM TES buffer with 0.36mM calcium chloride. 1mL culture supernatant was mixed with 1mL of substrate and incubated at 37°C for 5hr. Tubes were swirled for 10-15 seconds at an interval of one hour. 1mL of mixture was used for color development using 2mL of ninhydrin reagent. Absorbance was measured at 570nm and standard curve was prepared using leucine as a substrate.

RESULTS AND DISCUSSION

The use of microorganisms and their products nowadays is rapidly increasing. Due to heterotrophic nature, most fungi secrete hydrolytic enzymes in order to gain nutrition from the variety of substrates available in the surroundings. The enzymes isolated from fungi have wide range of industrial applications (Østergaard and Olsen, 2011). Nematophagousfungi are predacious fungi found in the subphylum Pezizomycotina a largest of Ascomycota. Within this group, nematode-trapping fungi are one of the important sub-group which forms special devices of mycelia in order to trap nematodes and then degrade the host cuticle via secretion of hydrolytic enzymes. The characterized enzymes from this group include proteases and chitinase. However,

when nematodes are unavailable, fungi survive as a saprophyte in the soil. In order to survive as a saprophytes, fungi must be able to degrade a wide range of substrates available. This clearly make a sense that, these fungi are able to utilize a broad range of substrates available in the soil. In order to do this, fungi must secrete hydrolytic enzymes. Industrial application of fungal enzymes has been intensively reviewed. Therefore, in the present study, we explored the extracellular hydrolytic enzymes from *A. conoides* and *D. flagrans*.

Amylase, cellulase and xylanase in nematode-trapping fungi

Nematode-trapping fungi are generally found in agricultural soil. Most of the agricultural soil contain the debris of crop which is a rich source of cellulose, xylan and starch. The highest amylase activity 261 U/mL/min was obtained in fungal strain *D. flagrans* RPAN-10 and minimum 186mU/mL/min in *A. conoides*. Two other isolates, of *D. flagrans* RPAN-13 & 14 showed 226 and 258 U/mL/min activity, respectively (Fig. 1A). Hence, *D. flagrans* which is proficient in trapping of gastrointestinal nematodes produce more amylase compared to *A. conoides* under the tested condition. Similarly, xylanase activity was also higher in *D. flagrans* compared to *A. conoides*. The highest xylanase activity was obtained with RPAN-10 (76mU/mL/min) and minimum in *A. conoides* (39 U/mL/min). The xylanase activity of RPAN-13 and RPAN-14 were 69 U/ml/min and 65 U/mL/min, respectively (Fig. 1B). While for cellulase, highest activity was measured in *A. conoides* (214 U/mL/min) and lowest in RPAN-13 9103mU/mL/min). The other two isolate, RPAN-10 and RPAN-14 showed moderate cellulase activity, 143 U/mL/min and 113 U/mL/min, respectively. All these enzymes are very essential for saprophytic fungi as well as the pathogenesis of fungal plant pathogens. To our knowledge, there is no direct evidence for the activity of these enzymes in nematophagous fungi. Very recently, (Lan *et al 2016.*) have reported that *Arthrobotrys* spp. CX1 could gelatinize filter paper cellulose via decrystallization however, no cellulase activity was detected in the culture supernatant. Apart from this, genome sequencing and annotation of nematophagous fungi has reported several genes of glycosyl hydrolase family (Lebrigand *et al.*, 2016), however, direct assay based study is lacking.

Fungal amylases have wide scale industrial applications including starch processing (Souza, 2010), food industry (Dey and Banerjee, 2015), paper industry (Singh *et al.*, 2014) etc. Cellulases are also used in various industries including paper pulp, textile and biofuel industry as well as food and bio remediation (Han and He, 2010; Singh *et al.*, 2012). Likewise, fungal xylanases is also having widespread industrial applications (Ahmed *et al.*, 2016).

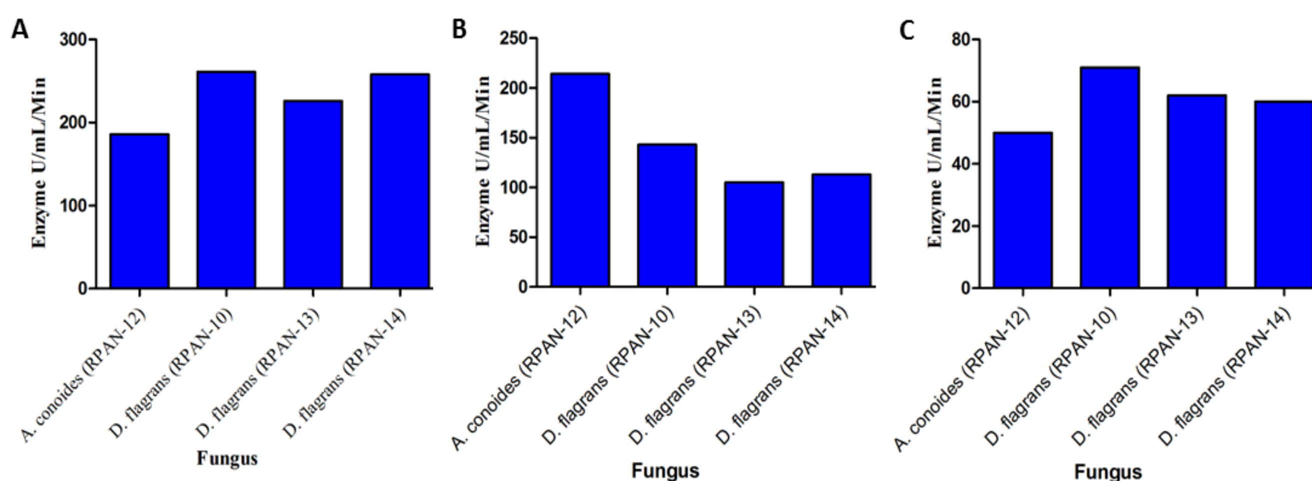

Fig. 1: Amylase, cellulase and xylanase activity in *A. conoides* and different iodates of *D. flagrans*. A-amylase, B-cellulase and C-xylanase.

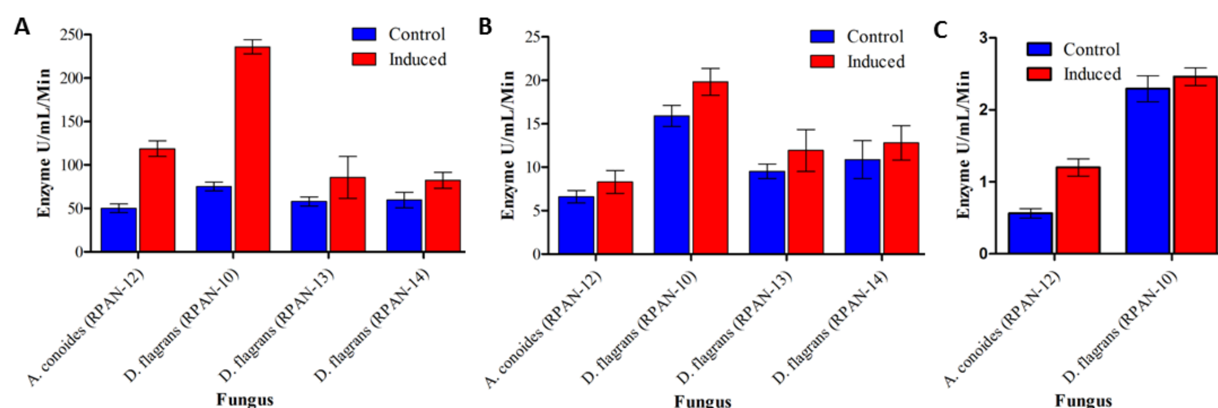

Fig. 2: Protease, chitinase and collagenase activity of nematode-trapping fungi after induction with nematodes. A –protease, B- chitinase and C- collagenase.

Protease, chitinase and collagenase activity

The basic mechanism of nematode-trapping fungi involves the formation of trapping structures, catching nematodes into the traps, penetration of nematode cuticle and consume whatever available. Thus, once nematodes are trapped by the fungus, it starts secreting various enzymes in order to break the nematode cuticle. It's been reported that secretion of hydrolytic enzymes secreted by parasitic fungi depends on the composition of the host surface (Sahai and Manocha, 1993). As the nematode cuticle is mainly made up of proteins, carbohydrates and lipids (Spiegel and McClure, 1995), proteases, Chitinases and collagenases play a very important role during penetration (Yang *et al*., 2007b).

In our previous study, we have confirmed induction in protease activity in *D. flagrans* (RPAN-10) and *A. conoides* (RPAN-12), however in that we have used half strength potato dextrose medium. In contrast to this, this time we used modified medium. If readily available carbon or nitrogen source is accessible, naturally microorganisms prefer it (Fernandes *et al.*, 2012). Hence, in order to make fungi ready to utilize different carbon and nitrogen source, reduce the amount both the carbon (dextrose) and nitrogen $NaNO_2$ into growth medium. Almost all the isolates showed an increased protease activity when induced with nematodes (Fig. 2). Moreover, in contrast to our previous study, this time we got a quite good amount of protease activity. Among the various industrial enzymes, 75% market is of hydrolytic enzymes, protease covers 2/3 of this (Savitha *et al.*, 2011). Like amylase and cellulases, proteases have wide scale application in various industries (Tavano, 2013; Novelli *et al.*, 2016). Secretion of protease by *D. flagrans*, its characterization and optimum growth have been reported (Meyer and Wiebe, 2003; Braga *et al.*, 2011; Braga *et al*., 2012). However, there is only a single report for *A. conoides* (Yang *et al.*, 2007a).

Chitinase and collagenase activity were also found to be increased when nematodes were added to growing fungi. In this case too, *D. flagrans* RPAN-10 was found to be superior in terms of activity (Fig. 2). Chitinase can be used as biopesticide, single cell protein for feed supplement, bio waste management etc. (Stoykov *et al.*, 2015). Chitinase of *D. flagrans* have been reported to be involved nematode infection. Collagenase have several applications, especially in medicine (Alipour *et al.*, 2016) and researchers are continuously searching for new microbial collagenases during the last few years.

In this study, amongst all the four isolates of nematode-trapping fungi, *D. flagrans* RPAN-10 was found to better for further study and characterization of enzymes. However, for large scale production the growth parameters and cultural conditions need to be optimized (Chandrasekaran *et al.*, 2015; Yadav *et al.*, 2016). (Soares *et al.*, 2013; de Freitas Soares *et al.*, 2015) have optimized conditions for protease from *Monacrosporium thaumasium* and chitinase from *Monacrosporium thaumasium Monacrosporium sinense*. As nematode-trapping fungi are one of the potential bio-control agent, the purified enzymes from these fungi offers a safe as well as eco-friendly approach for enzyme production. Solid state fermentation is one of the best option of for *D. flagrans* which can then directly use for animal feed supplement. Hence, use of *D. flagrns* for solid state fermentation can serve a dual purpose of animal feed supplement as well bio-control of gastrointestinal parasitic nematodes. Thus, *D. flagrans*

isolate RPAN-10 is an appropriate candidate for animal feed suppliers and for the production of protease, amylase, chitinase, cellulase and xylanase. Cellulase production was higher in *A. conoides* compared to *D. flagrans*. In our previous study, *A. conoides* was found to be the superior candidate to control *Melodgyne* spp. root-knot nematodes *in vitro* (Pandit *et al*., 2014c) as well as a phosphate solubilizer (Pandit *et al.*, 2014a) hence, for bio based agriculture industries, *A. conoides* could serve multiple purpose i.e. bio-control of nematodes and biofetilizer via phosphate solubilisation and degradation of cellulose rich agriculture waste. However, the condition for maximum production, physical and chemical parameters growth needs to be optimized. Hence, this study provides a base for further research to explore industrially important enzymes of nematophagousfungi.

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