KAVAKA 56 : 8-12 (2021) doi: 10.36460/Kavaka/56/2021/8-12

Impact of various cultural parameters for extracellular pectinase production by some *Fusarium oxysporum* isolates in surface batch broth fermentation

Pinki Tikadar and Bejoysekhar Datta*

Mycology and Plant Pathology Research Laboratory, Department of Botany, University of Kalyani-741245, West Bengal, India.

**Corresponding author Email : bejoy.datta@gmail.com*

(Submitted on April 13, 2021; Accepted on May 19, 2021)

ABSTRACT

Pectinase has tremendous industrial application. Filamentous fungi such as *Fusarium oxysporum* might be exploited as a commercial source of pectinase. Three *F. oxysporum* isolates from rhizosphere of grass pea (*Lathyrus sativus* L.), tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) were used in the study. They showed transparent zone around their colony on pectin agar plate. Crude enzyme was prepared by growing them in pectin broth and various culture parameters were optimized for maximum production of pectinase. All the three isolates showed highest pectinase production after 6th day of incubation at 30°C in the medium having pH range 5.5 - 6.5 and supplemented with 0.5% pectin. Among the nine culture media studied, maximum activity was observed in Czapek's Dox broth (3.37, 3.83 and 2.96 U/ml), moderate activity in malt extract broth and least activity was observed in Asthana and Hawker's broth. In the CDB medium when sucrose was replaced with other carbon sources, maximum activity was obtained in presence of pectin followed by dextrose and least activity in mannitol. Among nitrogen sources, amino acids such as glycine and glutamine supported maximum production of pectinase in tomato and potato isolates and peptone in grass pea isolate. Thus, the isolates could be treated as effective producer of pectinase enzymes with various biotechnological applications.

Keywords: Incubation time, temperature, pH, nutritional supplement

INTRODUCTION

Pectinase are the group of enzymes which break pectin, the main structural polysaccharides found in the middle lamella of plant cell. Thus they act as macerating enzymes that cause softening of plant tissue. There are basically three types of pectinase enzymes depending on mode of action. Pectin methyl esterase (PME) catalyses the hydrolysis of the methoxyl group of pectin forming pectic acid and methanol. Hydrolases include polygalacturonases (PG) and poly methyl galacturonases (PMG) which catalyses the hydrolytic cleavage of α-(1→4)-glycosidic bond in pectic acid and pectin, respectively. Lyases include pectate lyase and pectin lyase (PL) which catalyse the cleavage of α-(1→4)-glycosidic linkage in pectic acid and pectin, respectively by trans-elimination reaction and forming unsaturated galacturonates and methyl galacturonates, respectively. Both hydrolase and lyase can be further divided into endo- and exo-forms. Generally, PG and PMG showed optimum activity at acidic pH whereas PL showed highest activity at alkaline pH. Commercially available pectinase preparations are mixtures of these enzymes.

Investigation of pectinases is a central issue in industry due to their wide biotechnological applications (Garg *et al*., 2016). Pectinases share 25% in the global sales of food enzymes (Mehmood *et al*., 2018). Acidic pectinases are useful in fruit juice industry, preparation of vegetables pastes and purees, poultry feed and to improve chromaticity and stability of red wine; whereas alkaline pectinases are widely used in the textile industry for retting of fibres, manufacturing of cotton fabrics, pulp and paper industry and in improving the quality of black tea, extraction of vegetable oil and waste water treatment (Chand and Satyanarayana, 2012).

Various microorganisms are chief source of commercial pectinase. However, filamentous fungi are used widely because of their rapid growth on the cheap agro-wastes and secretion of good amount of enzyme within a short period of time. The members of *Fusarium oxysporum* are soil-borne, filamentous fungi that have been reported to produce a number of hydrolytic enzymes including pectinases (Reddy and Saritha, 2015). Submerged (SMF) and solid state fermentation (SSF) techniques are generally used for commercial production of enzymes including pectinase. However, various physical and nutritional parameters of growth are crucial to increase the yield. The aim and objective of the present study was to optimize growth parameters such as incubation time and temperature, pH and initial pectin concentration of the growth medium and other nutritional requirements such as culture media, carbon and nitrogen sources for the maximum production of pectinase in surface batch broth fermentation. This information will be useful to scale-up the pectinase production and to develop a low cost pectinase manufacture technology for biotechnological purposes.

MATERIALS AND METHODS

Isolation, screening and identification of pectinolytic Fusaria: The rhizospheric soil samples of three wilted plants *viz*., grass pea (*Lathyrus sativus* L.), tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) were collected from two agricultural fields of West Bengal, India and were used to isolate fungi by dilution plate technique on potato dextrose agar (PDA) medium supplemented with 0.025% Rose Bengal, 0.1% pentachloronitrobenzene (PCNB) and 100 mg/l streptomycin. The pectin degrading activity of the isolates was tested by growing them on pectin-agar medium at 30±2°C for 3-5 days (Rajendran *et al*., 2011). After sufficient growth, plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide (HDTMA) and kept for 30 min. HDTMA could precipitate the unused pectin in the medium and formation of a transparent zone around the fungal colony on an opaque background confirmed the presence of pectinolytic activity by the isolates (Sunitha *et al.,* 2013). The solubilization index was calculated as the ratio of total diameter (colony + zone) to the colony diameter.

Pectinase positive isolates were identified on the basis of morphological characteristics according to 'The *Fusarium*

Laboratory Manual (Leslie and Summerell, 2006).

Molecular identification of the isolates was performed through rDNA sequence analysis (Ghosal *et al*., 2020).

Preparation of crude enzyme and its quantitative assay: Pectinase positive isolates were inoculated in pectin broth medium (Rajendran *et al*., 2011) and incubated at 30±2 °C for 7 days. Cell-free culture supernatant obtained by filtration using Whatman filter paper No.1 was centrifuged to remove conidia. The crude enzyme was used immediately or stored in sterile tubes at -20°C for a month.

Pectinase activity was evaluated by assaying polygalacturonase (PG) activity by measuring amount of reducing sugar released using 3,5-dinitrosalicylic acid (DNS) reagent (Htwe *et al.*, 2017). One unit of pectinase activity (U) was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute under standard assay condition (Okonji *et al*., 2019).

Optimization of cultural conditions for extracellular pectinase production: Cultural conditions such as incubation time (at 2 days interval up to 14 days), incubation temperature (10-40°C), media pH (4.5-9.5), pectin concentration (0 - 0.75%, w/v) of broth media, fermentation media such as Potato broth (PB), Malt extract broth (MEB), Potato Dextrose broth (PDB), Nutrient broth (NB), Pikovoskya's broth (PKVB), Sabouraud's broth (SB), Czapek's Dox broth (CDB), Richard's broth (RB) and Asthana and Hawker's broth (AHB) and carbon and nitrogen sources required for highest pectinase production were studied. When one parameter was being screened the other parameters kept constant. To study effect of carbon sources, different sets of modified CDB medium were prepared where sucrose (3%, w/v) was replaced by anyone of the dextrose, lactose, maltose, mannitol, sorbitol, starch and pectin. Effect of nitrogen source was studied by replacing sodium nitrate (0.2%, w/v) in CDB medium by anyone of the glycine, glutamine, peptone, sodium nitrite and potassium nitrate. Control sets without carbon or nitrogen source were also prepared. Enzyme activity was recorded after 7 days of incubation at 30±2°C. All experiments were repeated at least thrice and the data were subjected to statistical analysis.

RESULTS

Following dilution plating of the rhizosphere soil samples, a number of fungal colonies were observed on PDA plates of which colonies with white cottony mycelia were selected. Fungal samples of these colonies were stained with cotton blue and mounted in lactophenol and microscopic observation revealed that all the three fungal isolates had sickle-shaped macroconidia, unicellular microconidia and septate hyphae. This indicated that the fungi belong to the genus *Fusarium*. Further, morphological characterization confirmed that all the three isolates belong to *F. oxysporum*. The fungal isolate isolated from grass pea was designated as *F. oxysporum* f.sp. *lentis* FL, the tomato isolate as *F. oxysporum* f.sp. *lycopersici* FT and the potato isolate as *F. oxysporum* f.sp. *tuberosi* FPo.

For molecular identification, primer pair ITS5 and ITS4 was used to amplify 600 bp DNA fragment containing ITS1, 5.8S rDNA and ITS2 region of *F. oxysporum* f.sp. *lentis* FL and *F. oxysporum* f.sp. *lycopersici* FT whereas primer pair LROR and LR5 was used to amplify 1000 bp fragment containing partial 28S rDNA region of *F. oxysporum* f.sp. *tuberosi* FPo. According to NCBI BLAST, all the isolates showed more than 99% similarity with *F. oxysporum* strains and their GenBank accession numbers were MT020426, MT020427 and MT020430, respectively.

All the three *F. oxysporum* isolates were able to degrade pectin and produced transparent zone on pectin agar medium (**Fig. 1**). Based on pectin solubilization index isolates are arranged as *F. oxysporum* f.sp. *lentis* FL (1.43)> *F. oxysporum* f.sp. *lycopersici* FT (1.36) > *F. oxysporum* f.sp. *tuberosi* FPo (1.30).

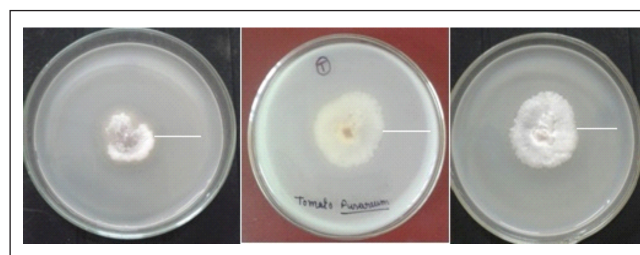

Fig. 1: Transparent zone around the fungal colony indicating their pectinolytic activity, *F. oxysporum* f.sp. *lentis* FL (left), *F.oxysporum* f.sp. *lycopersici* FT (middle), *F. oxysporum* f.sp. *tuberosi* FPo (right); white line indicates radius of the zone.

Fusarium oxysporum isolates being of fast-growing nature reached the highest growth within a week in pectin broth medium at 30±2°C under static condition. Pectinase production increased with increasing incubation time upto 6th day (**Fig. 2**). At that time, *F. oxysporum* f.sp. *lentis* FL showed maximum production of pectinase (0.831 U/ml) followed by *F. oxysporum* f.sp. *lycopersici* FT (0.617 U/ml) and *F. oxysporum* f.sp. *tuberosi* FPo (0.610 U/ml). Further increase in the incubation time decreased the enzyme activity.

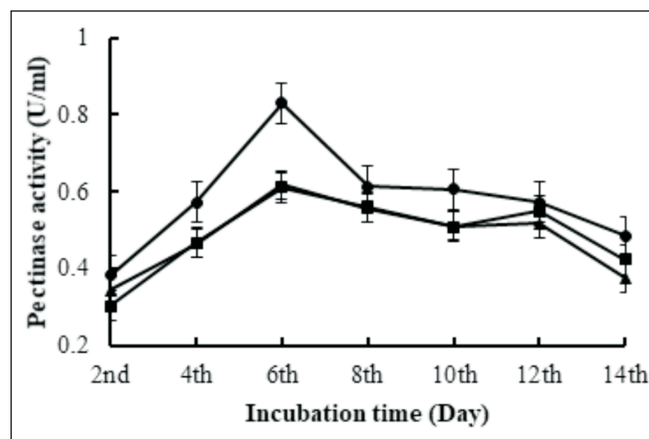

Fig. 2: Effect of incubation time on pectinase production of the fungal isolates, *F. oxysporum* f.sp. *lentis* FL, *F. oxysporum* f.sp. *lycopersici* FT, *F. oxysporum* f.sp. *tuberosi* FPo.

Pectinase production by the three isolates was lowest at 10°C, slightly increased at 20°C and highest at 30°C (**Table 1**). With further increase in incubation temperature (up to 40°C) pectinase production decreased. At optimum temperature (30°C), *F. oxysporum* f.sp. *lentis* FL produced highest pectinase (1.051 U/ml), followed by *F. oxysporum* f.sp. *tuberosi* FPo (0.975, U/ml) and *F. oxysporum* f.sp. *lycopersici* FT (0.861 U/ml).

Pectinase production by the *F. oxysporum* isolates decreased at alkaline pH of the fermentation medium and increased at acidic pH (**Table 1**). At pH 5.5 of the medium, highest pectinase production was observed by *F. oxysporum* f.sp. *lentis* FL (0.856 U/ml) and *F. oxysporum* f.sp. *tuberosi* FPo (0.513 U/ml). However, *F. oxysporum* f.sp. *lycopersici* FT showed highest pectinase production at pH 6.5 (0.466 U/ml) and almost similar pectinase production at pH 5.5 (0.447 U/ml). Highest pectinase activity was observed when the isolates were grown in medium supplemented with 0.5% pectin (**Table 1**). In the medium the isolates FL, FT and FPo produced pectinase 1.018, 0.783 and 0.804 U/ml, respectively. At higher (0.75%) or lower (0.25 to 0%) concentration of the substrate pectinase production halted.

Table 1: Pectinase activity (U/ml) of three *F. oxysporum* isolates at optimum culture conditions

F. oxysporum isolates	6th day of growth	Temperature (30 °C)	Media pH (slightly acidic) [a]	Pectin concentration (0.5%)	Culture medium (CDB)	Carbon source (pectin) [b]	Nitrogen source [c]
FL	0.831	1.051	0.856	1.018	3.37	5.02	3.79 [d]
FT	0.617	0.861	0.466	0.783	3.83	3.72	5.64 [e]
Fpo	0.610	0.975	0.513	0.804	2.97	4.54	6.35 [f]

[a]pH 5.5 for FL and FPo, pH 6.5 for FT
[b]by replacing sucrose (3%, w/v) in Czapek's Dox broth
[c]by replacing sodium nitrate (0.2%, w/v) in Czapek's Dox broth; [d] peptone; [e] glutamine; [f] glycine
Mean standard deviation for all the values is $<\pm 5.0\%$.

Among the nine culture media tested for pectinase production by the *F. Oxysporum* isolates, all of them produced highest pectinase when they were grown in Czapek's Dox broth (**Fig. 3**). The isolates FL, FT and FPo produced pectinase 3.37, 3.83 and 2.97 U/ml, respectively. However, isolates produced moderate level of pectinase in Malt extract broth, Potato Dextrose broth, and Richard's broth and least pectinase in Asthana and Hawker's broth.

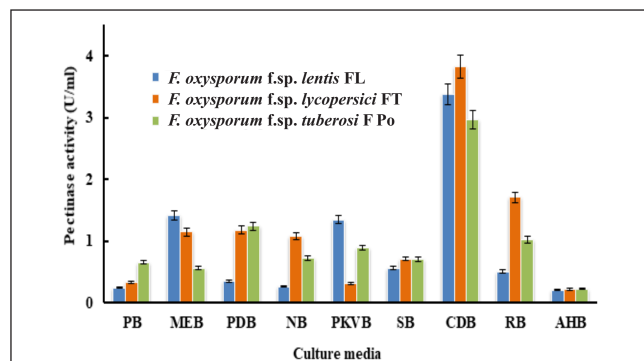

Fig. 3: Effect of different culture broth media on pectinase production by the three *F. oxysporum* isolates.

Among different carbon sources, pectin supplemented Czapek's Dox broth supported highest pectinase production (5.02, 3.72 and 4.54 U/ml by the isolates FL, FT and FPo, respectively) **(Fig. 4)**. Next to pectin, dextrose also supported increased production of pectinase (4.322, 3.67 and 2.43 U/ml by the isolates FL, FT and FPo, respectively). The presence of other monosaccharides such as lactose and maltose also yielded moderate pectinase. However, presence of sugar alcohol such as mannitol halted their pectinase production due inhibition of growth. The *F. oxysporum* isolates also utilized starch due to their amylolytic activity and produced moderate level of pectinase in presence of starch as a carbon source.

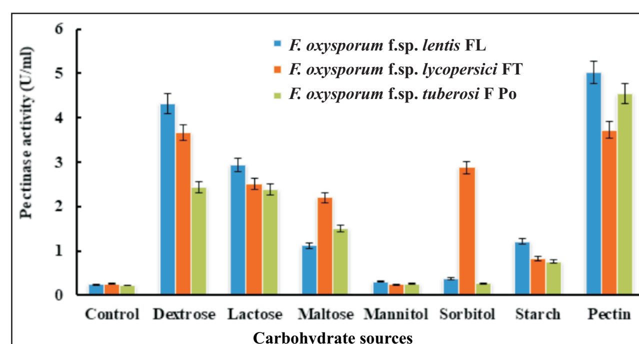

Fig. 4: Effect of different carbon sources in modified Czapek's Dox broth on pectinase production by the three *F. oxysporum* isolates

Supplementation of amino acids such as glycine and glutamine supported highest pectinase production by *F. Oxysporum* f.sp *lycopersici* FT and *F. oxysporum* f.sp. *tuberosi* FPo (**Fig. 5**). *F. oxysporum* f.sp *lycopersici* FT also showed enhanced pectinase production in presence of inorganic salts such as sodium nitrate, potassium nitrate and sodium nitrite. However, highest pectinase production by *F. oxysporum* f.sp *lentis* FL was observed in the presence of peptone (3.79 U/ml).

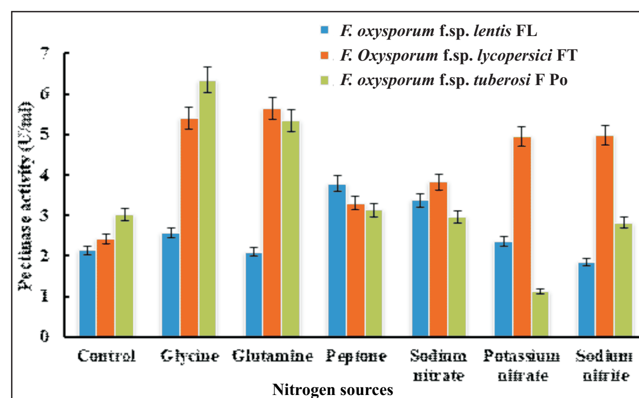

Fig. 5: Effect of different nitrogen sources in modified Czapek's Dox broth on pectinase production by the three *F. oxysporum* isolates.

DISCUSSION

Pectinase, like other enzymes, is a primary metabolite. Fungi produce this enzyme to degrade complex polymer of pectin to obtain simple sugar for their growth. Hence, pectinase

production increased along with growth and reached its highest peak at the late log phase of growth. The highest pectinase production by the *F. oxysporum* isolates was observed on the 6th day of growth (**Fig. 2**) when the isolates showed maximum biomass production in surface batch broth culture. Beyond this period the pectinase activity started to decrease due to stoppage of growth as a result of exhaustion of essential supplements in the medium and/or accumulation of toxic auxiliary metabolites (Htwe *et al.*, 2017; Abdullah *et al.*, 2018).

Temperature is one of the crucial factors that regulate the fermentation process. It has a great impact on growth and metabolic activity of fungal strain. Since the *F. oxysporum* isolates were isolated from the agricultural fields of sub-tropical region and being their mesophilic nature, they grew best and produced highest amount of pectinase at 30°C (**Table 1**). Abdullah *et al.* (2018) noted similar observation where the optimum temperature for pectinase enzyme production was 30°C. Above and below the optimum temperature the enzyme activity decreased which might be due to growth reduction or enzyme inactivation or suppression of cell viability (Htwe *et al.*, 2017). The pH of the culture medium is an important factor for growth and production of metabolites. In this study, pectinase production was highest at acidic pH of the fermentation medium (**Table 1**). These observations are in congruent with reports of Banu *et al.* (2010) and Abdullah *et al.* (2018). Secretion of pectinase in acidic ranges had a great significance for their application in fruit juice industry. However, at the alkaline pH of the medium pectinase production of all the three isolates declined sharply. This might be due to lowering of growth, disturbance of membrane permeability and enzyme stability at the alkaline pH.

Pectin is an easily degraded polymer and its concentration in the culture medium influences growth and development of pectinolytic microorganisms. In the study, least pectinase activity was observed in absence of pectin and its presence in the medium enhanced pectinase production. This indicated that pectinase of the *F. oxysporum* isolates were of inducible type. Pectinase production was highest when they were grown in synthetic medium supplemented with 0.5% pectin (**Table 1**). Pectin concentration 0.25% exhibited lesser pectinase production due to lower availability of the substrate. Pectin concentration 0.75% also resulted in lesser pectinase production because 0.5% was the optimum concentration for maximum pectinase production by the isolates and higher than 0.5% pectin might inhibit their growth and pectinase production. Similar result was also reported in other pectinolytic microorganisms (Manal *et al.*, 2016). Sudeep *et al.* (2020) reported that 48 h incubation period, 1% pectin concentration and 30°C temperature were the optimum culture conditions for *Aspergillus* sp. Gm strain to produce maximum pectinase enzyme.

It was very significant to select an appropriate fermentation medium for production of enzyme. Different natural, semi-synthetic and synthetic broth media were tested for pectinase production by the *F. oxysporum* isolates and Czapek's Dox broth was found to be most suitable medium for highest pectinase production (**Fig. 3**). The reason might be due to the fact that CDB medium supported maximum growth of the fungal isolates than the other growth media. Abdullah *et al.* (2018) reported that nutrient present in Czapek's Dox broth favoured the fungal growth and secretion of enzyme. Conversely, Asthana and Hawker's broth medium was not preferred by the isolates for growth and pectinase production. However, in natural broth media *viz*., Malt extract broth, moderate level of pectinase was produced.

Carbon source present in the fermentation medium played an important role in growth and metabolite production of the microorganisms. Modified Czapek's Dox broth where sucrose was replaced with pectin resulted in highest pectinase production by the isolates (**Fig. 4**). Rashmi *et al*. (2008) also showed highest pectinase activities by *Aspergillus niger* isolates when they were grown in pectin rather than other carbon sources. The *F. oxysporum* isolates also showed substantial pectinase production in the presence of dextrose. The presence of 1% dextrose as a carbon source showed pectinase production by *Fusarium* sp. (Reddy and Saritha, 2015). Nitrogen sources in the fermentation medium also played a determining factor for growth and development of microorganisms. In this study, three *F. oxysporum* isolates showed varied preference of nitrogen source for pectinase production (**Fig. 5**). *F. oxysporum* f.sp *lycopersici* FT could utilize both organic and inorganic nitrogenous sources while *F. oxysporum* f.sp. *tuberosi* FPo preferred amino acids glycine and glutamine. *F. oxysporum* f.sp *lentis* FL produced the highest pectinase in presence of peptone. Reddy and Saritha (2015) reported that the addition of 0.1% of peptone as a nitrogen source enhanced production of pectin lyase by *Fusarium* sp.

CONCLUSION

The pectinase activity of the three *F. oxysporum* isolates increased many-fold under optimized culture conditions (30°C temperature and pH 5.5 and 0.5% pectin supplement in the growth medium). In pectin broth the grass pea isolate (FL), tomato isolate (FT) and potato isolate (FPo) produced pectinase 0.831, 0.617 and 0.610 U/ml, respectively but in pectin (3%) supplemented Czapek's Dox broth (CDB) FL produced pectinase 5.02 U/ml (enzyme activity increased six folds); in glutamine (0.2%) supplemented CDB medium, FT produced pectinase 5.64 U/ml (enzyme activity increased nine-fold); and in glycine (0.2%) supplemented CDB medium, FPo produced pectinase 6.35 U/ml (enzyme activity increased ten folds). Further work is necessary for the purification and characterization of the pectinase of the *F. oxysporum* isolates for their industrial utilization.

ACKNOWLEDGEMENT

The work has been supported by the funds received from University of Kalyani and DST-FIST and DST-PURSE programme II, Govt. of India.

REFERENCES

Abdullah, R., Farooq, I., Kaleem, A. *et al*. 2018. Pectinase production from *Aspergillus niger* IBT-7 using solid state fermentation. *Bangladesh Journal of Botany* **47** (3): 473-478.

Banu, A.R., Devi, M.K., Gnanaprabhal, G.R. *et al*. 2010. Production and characterization of pectinase enzyme from *Penicillium chrysogenum*. *Indian Journal of Science and Technology* **3** (4): 377-381.

Chand, S.D. and Satyanarayana T. 2012. Biotechnological potential of agro residues for economical production of thermoalkali-stable pectinase by *Bacillus pumilus* dcsr1 by solid-state fermentation and its efficacy in the treatment of ramie fibres. *Enzyme Research* **7**: 1-7.

Garg, G., Singh, A., Kaur, A. *et al*. 2016. Microbial pectinases: an ecofriendly tool of nature for industries. *3 Biotech* **6**: 47.

Ghosal, D., Tarafder, M. and Datta, B. 2020. *Fusarium oxysporum* isolates showed varied benzimidazole fungicides sensitivity under low temperature and heavy metal stress. *Rhizosphere* **15**: 100227.

Htwe, Z.M., Bo, B. and Mya, K.M. 2017. Effect of various parameters on pectinase producing activity of two bacterial isolates and application of crude enzyme in apple juice extraction. *International J. Sci.Res.* **6** (8): 1670-1678.

Leslie, J.F. and Summerell, B.A. 2006. *The Fusarium Laboratory Manual*. New York, Wiley.

Manal, S., Sahar, S.M., Manal, G.M. and Mohsen, M.A. 2016. Purification and kinetics of pectinase production from *Paenibacillus lactis* NRC1 locally isolated from Egyptian mangrove habitat. *Der Pharma Chemica* **8**: 150-159.

Mehmood, T., Saman, T., Irfan, M. *et al*. 2018. Pectinase production from *Schizophyllum commune* through central composite design using citrus waste and its immobilization for industrial exploitation. *Waste and Biomass Valorization* **10**: 2527-2536; doi: org/10.1007/s12649-018-0279-9

Okonji, R.E., Itakorode, B.O., Ovumedia, J.O. and Adedeji, O.S. 2019. Purification and biochemical characterization of pectinase produced by *Aspergillus fumigatus* isolated from soil of decomposing plant materials. *J. App. Biol. Biotech.* **7** (3): 1-8.

Rajendran, R., Sundaram, K.S., Radhai, R. *et al*. 2011. Production and optimization of fungal pectinase from *Fusarium* sp. *International Journal of Current Research* **3** (4): 254-258.

Rashmi, R., Murthy, K.R.S., Sneha, G. *et al*. 2008. Partial purification and biochemical characterization of extracellular pectinase from *Aspergillus niger* isolated from groundnut seeds. *Journal of Applied Biosciences* **9** (1): 378-384.

Reddy, M.P. and Saritha, K.V. 2015. Bio-catalysis of mango industrial waste by newly isolated *Fusarium* sp. (PSTF1) for pectinase production. *3 Biotech* **5**: 893-900.

Reddy, P.L. and Sreeramulu, A. 2012. Isolation, identification and screening of pectinolytic fungi from different soil samples of Chittor district. *International Journal of Life Sciences Biotechnology and Pharma Research* **1** (3): 186-193.

Sudeep, K.C., Upadhyaya, J., Joshi, D.R. *et al.* 2020. Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. *Fermentation* **6**: 59; doi: 10.3390/ fermentation 6020059.

Sunitha, V.H., Nirmala Devi, D. and Srinivas, C. 2013. Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. *World Journal of Agricultural Sciences* **9** (1): 1-9.