IMPACT OF VARIOUS CULTURAL PARAMETERS FOR EXTRACELLULAR PECTINASE PRODUCTION BY SOME *FUSARIUM OXYSPORUM* ISOLATES IN SURFACE BATCH BROTH FERMENTATION

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Abstract

Pectinase has tremendous industrial application. Filamentous fungi should be exploited as a commercial source of pectinase. Three Fusarium oxysporum isolates were isolated from rhizosphere of grass pea (Lathyrus sativus), tomato (Solanum esculentum) and potato (S. tuberosum) and identified through conidial morphology and rDNA sequence analysis. On pectin agar plate they showed transparent zone around their colony indicating they were pectinase positive. In surface batch broth culture, they were subjected to various culture parameters such as incubation time, temperature, pH, pectin concentration, different culture media, carbon and nitrogen sources for optimal production of pectinase. Crude enzyme was prepared by filtering the fungal biomass and activity was determined by assaying polygalacturonase activity. 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 5% pectin. Among the nine culture media studied, maximum activity was observed in Czapek's Dox broth (3.37, 3.83 and 2.968 U/ml), moderate activity in malt extract and other natural broth media and least activity in Asthana and Hawker's broth medium. In the 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 with various biotechnological applications.

Keywords: *Fusarium oxysporum*, pectinase, incubation time, pH, temperature, nutritional supplement.

Received: 12.07.2020 Reviewed: 19.11.2020 Accepted: 18.12.2020

INTRODUCTION

Pectin is an important component of plant cell wall and accounts for 0.5-4.0% of the fresh weight plant material depending upon their sources (Reddy and Sreeramulu, 2012). Its highest concentration is detected in the middle lamella where it acts as a cementing material between adjacent cells and provides rigidity to plant. Chemically, it is a heterogenous polysaccharide with the linear structure in which few hundred to thousand mono-galacturonic acid units are linked via α-(1→4)-glycosidic bond forming a backbone. The backbone is substituted at certain regions with α-(1→2) rhamnose units from which side chains of arabinose, galactose and xylose may occur. Many of the carboxyl groups are esterified with methanol to form methoxy groups. On the basis of number of methylated unit, pectin substances are of three types: pectic acid (non methylated units), pectinic acid (<75% methylated galacturonan units) and pectin(>75% methylated units**).**

Pectinase are the group of enzymes which degrade pectin substances resulting maceration of the 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 polymethyl 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 classified as endo-liquefying or depolymerizing enzyme or exo-saccharifying enzyme. 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). Acidic pectinases are useful in extraction, clarification and liquefaction of fruit juices, maceration of vegetables to produce various products like pastes and purees, preparation of poultry feed and to improve chromaticity and stability of red wine; whereas alkaline pectinases are widely used in the textile industry for retting of plant 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 (Sharma and Satyanarayana, 2012).

Many bacteria, fungi and higher plants are known to produce pectinase. However, major portion of commercial pectinase is obtained from filamentous fungi because of their rapid growth on the substrate, and their huge biomass can secrete a good amount of enzyme within a short period of time. The members of Fusarium oxysporum are soil-borne, filamentous fungi that cause vascular wilt diseases in a wide variety of economically important crops. To gain entrance into the plant cells, they generally secrete a mixture of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases. Many workers have reported role of Fusarium oxysporum as a useful source of extracellular pectinase which can be exploited in commercial applications (Al-Najada et al., 2012; Sunitha et al., 2013; Reddy and Saritha, 2015). Different pectinolytic enzymes (PG1, PG2 PG3 and PL) of Fusarium oxysporum f.sp lycopersici causing vascular wilt of tomato has been purified and characterized (Di Pietro and Roncero 1996a, b, c; Garcia et al., 1997).

Submerged (SMF) and solid state fermentation (SSF) techniques have been widely used for pectinase production by different microorganisms. However, microbial pectinases are produced mostly by SMF and the process is influenced by various physical and chemical parameters which are crucial for increasing the yield. The present study was conducted to optimize growth parameters like incubation time, pH of growth medium, incubation temperature, initial pectin concentration and other nutritional requirements (culture media, carbon and nitrogen sources) for the maximum production of pectinase in surface batch broth fermentation. The long-term goal of the study is develop a low cost technology for high production of pectinase which can fulfill the need of the enzyme for biotechnological purposes.

MATERIAL AND METHODS

Isolation, screening and identification of pectinolytic Fusaria

The rhizospheric soil samples of three wilted plants viz., grass pea (Lathyrus sativus), tomato (Solanum esculentum) and potato (Solanum tuberosum) 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 [composition (g/l): pectin5, peptone 3, yeast extract 2, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $MnSO_4.5H_2O$ 0.01, $(NH_4)_2SO_4$2,agar 20; ampicilin0.1%, trace mineral solution 1.0 ml [composition of trace mineral solution (g/l): $CuSO_4.5H_2O$ 0.4, $FeSO_4$0.08, $Na_2MoO_4$0.08, $ZnSO_4$0.8, $Na_2B_4O_7$0.04, $MnSO_4$0.008],pH 6.5 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). 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. The primers ITS4 (5' - TCCTCCGCTTATTGATATGC- 3') and ITS5 (5' -GGAAGTAAAAGTCGTAACAAGG- 3') were used to amplify a DNA region containing internal transcribed spacer (ITS) 1, 5.8S rDNA and ITS 2 sequences. Primers LROR (5' - ACCCGCTGAACTTAAGC- 3') and LR5 (5' - TCCTGAGGGAAACTTCG- 3') were used to amplify partial 28S rDNA portion (Ghosal et al., 2020).The PCR products were sequenced and the sequences were used for homology search through BLAST (Basic Local Alignment Search Tool) software of NCBI. The rDNA sequences were submitted inGenbank to get accession numbers.

Preparation of crude enzyme and quantitative assay of enzyme activity

Pectinase positive isolates were inoculated in pectin broth medium [composition (g/l): pectin 5, sucrose 5, tryptone 3, yeast extract 2, KCl 0.5, $MnSO_4$ 0.01, $MgSO_4.7H_2O$ 0.5, $(NH_4)_2SO_4$ 2, trace mineral solution 1ml, pH 6](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 eppendrof 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). For this, 1 ml of 1% pectin solution, 3 ml of 50 mM sodium citrate buffer, pH 5.2 and 1 ml of crude enzyme were mixed and incubated at 37±1°C for 30 min. After that, whole reaction mixture or its dilution in the buffer was mixed with equal volume of DNS reagent [1 g DNS, 1.6 g NaOH, 40.3 g Na-K-tartarate in 100 ml distilled water], kept the mixture in a boiling water bath for 5 min, and then placed in an ice-bath. After cooling, absorbance was measured at 540 nm. The enzyme and substrate blanks were run in parallel. Amount of the reducing sugar in the supernatant was estimated by comparing the standard curve of D-galacturonic acid. One unit of pectinase activity (U) was defined as the amount of enzyme required to release 1 µmole 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, pH of the growth media, incubation temperature, substrate concentration, growth media and effects of carbon and nitrogen sources required for optimal production of pectinase were studied. When one parameter was being screened the other parameters kept constant.

Study of effect of incubation time on pectinase production

The fungal isolates were inoculated in 100 ml of pectin broth medium in 250 ml Erlenmeyer flask and incubated at 30±2°C in a static condition. The enzymatic activities were estimated at 2 days interval up to 14 days. The enzymatic activity (U/ml) was plotted in a graph against incubation time (days) and from this, time required for maximum enzyme production was determined.

Study of effect of pH of the growth medium on pectinase production

Different sets of pectin broth medium with pH range 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 were prepared using dilute HCl and NaOH. The fungal isolates were grown in the media at 30±2°C for 7 days and enzyme activity was estimated taking aliquot from each set. The enzymatic activity (U/ml) was plotted in a graph against pH of medium and from this optimal pH of the culture medium required for maximum enzyme production was determined.

Study of effect of incubation temperature on pectinase production

The fungal isolates were inoculated in the pectin broth medium and incubated at four

different temperatures viz., 10, 20, 30 and 40°C. After 7 days of incubation, enzymatic activity was estimated and plotted in a graph against incubation temperature. From this optimal temperature required for maximum pectinase production was determined.

Study of effect of substrate (pectin) concentration on pectinase production

Four sets of modified pectin broth medium were prepared with concentrations of pectin(w/v) 0.0%, 2.5%, 5.0%and 7.5% and pH of the medium was adjusted to 5.5. Then media were inoculated with the fungal isolates and incubated at 30±2°C for 7 days. The pectinase activity was estimated taking aliquot from each set and plotted in a graph against substrate concentration of the medium and from this, suitable pectin concentration of the culture medium required for maximum pectinase production was determined.

Study of effect of different broth media on pectinase production

The fungal isolates were grown in different natural, semi-synthetic and synthetic broth media viz., Czapek's Dox medium, potato dextrose medium, Nutrient broth, Sabouraud's broth, Richard's broth, Potato broth, Pikovoskya's broth, Malt extract broth and Asthana and Hawker's broth media at 30±2°C for 7 days. After growth of the fungal isolates, pectinase production was estimated taking aliquot from each inoculated culture media. The enzymatic activity (U/ml) was plotted in a graph against culture medium and from this, most suitable culture medium required for maximum enzyme production was determined.

Study of effect of carbon sources on pectinase production

Different sets of modified Czapek's Dox broth medium were prepared where sucrose (3%,w/v) was replaced by other carbon sources viz., dextrose, lactose, mannitol, maltose, starch, sorbitol and pectin. One control set without carbon source was also prepared. The fungal isolates were inoculated in each sets and incubated at 30±2°C for 7 days. After that, pectinase production was estimated taking aliquot from each set. The pectinase activity was plotted in a graph against each carbon source and from this, most suitable carbon source required for maximum pectinase production was determined.

Study of effect of nitrogen sources on pectinase production

Different sets of modified Czapek's Dox broth medium were prepared where sodium nitrate (0.2 %, w/v) was replaced by other nitrogen sources viz., glycine, glutamine, peptone, sodium nitrite and potassium nitrate. One control set without nitrogen source was also prepared. The fungal isolates were grown in each set at 30±2°C for 7 days. After that, pectinase production was estimated taking aliquot from each set. The pectinase activity was plotted in a graph against each nitrogen source and from this, most suitable nitrogen source required for maximum pectinase production was determined.

RESULTS AND DISCUSSION

Isolation, screening and identification of pectinase producing Fusaria

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 was designated as F. *oxysporum f.sp. lycopersici* FT and the potato isolate was designated as F. *oxysporum f.sp. tuberos*i FPo.

For molecular identification, primer pair ITS5 and ITS4 was used to amplify 600 bp DNA fragment containingITS1, 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 pectinsolubilization 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).

Effect of incubation period on pectinase production

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, but decreased at stationary phase. *Fusarium oxysporum* isolates being fast-growing nature reached highest growth within a week in pectin broth medium at 30±2° C under static condition. Pectinase production increased with increasing incubation time up to 6thday (Figure 2). At that time, F. *oxysporum f.sp. lentis* FL showed maximum production of pectinase (0.831U/ml) followed by F. *oxysporum f.sp. lycopersici* FT(0.617 U/ml) and F. *oxysporum f.sp. tuberosi* FPo (0.610U/ml).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 (Abdullah et al., 2018; Htwe et al., 2017). Thus, the optimal incubation time for pectinase production by the F. *oxysporum* isolates was 6thday in surface batch broth culture.

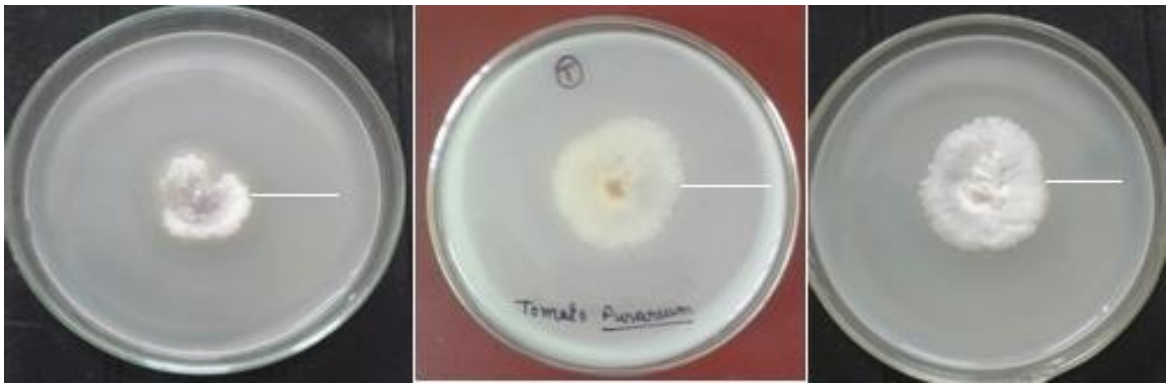

Figure 1. Transparent zone around the fungal colony indicating pectinolytic activity of the fungal isolates, F. *oxysporum f.sp. lentis* FL(left),F. *oxysporum f.sp. lycopersici* FT(middle), F. *oxysporum f.sp. tuberosi* FPo (right); white line indicates radius of the zone

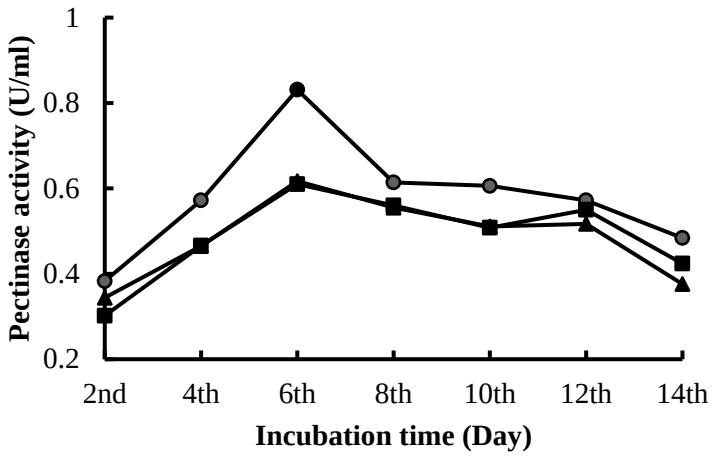

Figure 2. Effect of incubation time on pectinase production of the fungal isolates, F. *oxysporum f.sp. lentis* FL( ),F. *oxysporum f.sp. lycopersici* FT( ), F. *oxysporum f.sp. tuberosi* FPo ( )

Effect of pH on pectinase production

The pH of the culture medium is an important factor for growth and production of metabolites. In this study, F. *oxysporum* isolates preferred acidic pH for production of pectinase (Figure 3). At the pH 5.5of the fermentation medium, highest pectinase production was observed by F. *oxysporum f.sp. lentis* FL(0.856 U/ml) and F. *oxysporum f.sp. tuberosi* FPo (0.513U/ml). 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). These observations were in congruent with reports of Banu et al. (2010) and Abdullah et al. (2018). 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.

Effect of temperature on pectinase production

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 at 30°C.

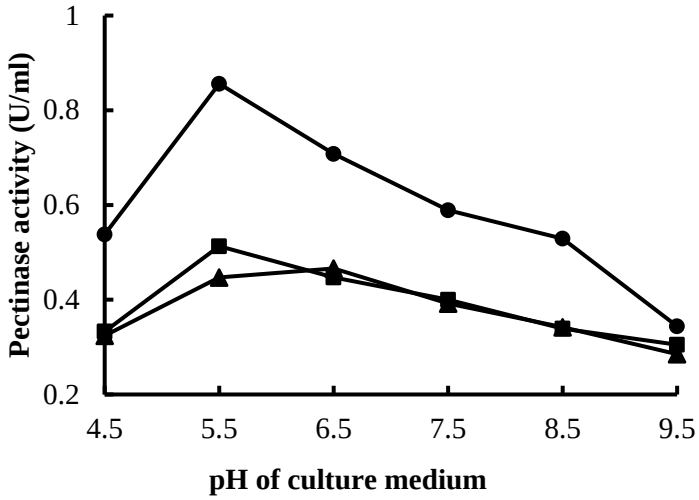

Figure 3. Effect of pH of fermentation medium on pectinase production of the fungal isolates, F. *oxysporum f.sp. lentis* FL( ),F. *oxysporum f.sp. lycopersici* FT( ), F. *oxysporum f.sp. tuberosi* FPo ( )

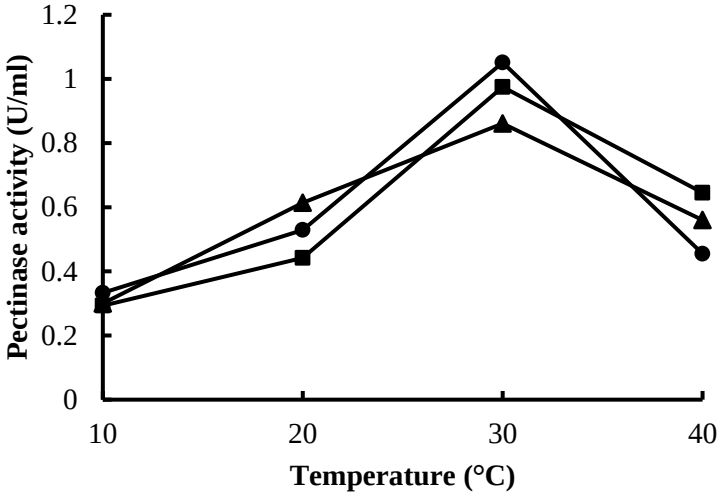

Figure 4. Effect of incubation temperature on pectinase production of the fungal isolates, F. *oxysporum f.sp. lentis* FL( ),F. *oxysporum f.sp. lycopersici* FT( ), F. *oxysporum f.sp. tuberosi* FPo ( )

At this temperature highest pectinase production was observed by F. *oxysporum f.sp. lentis* FL (1.051 U/ml), F. *oxysporum f.sp. lycopersici* FT (0.861 U/ml) and F. *oxysporum f.sp. tuberosi* FPo (0.975 U/ml) (Figure 4). Thus, 30°C was found to be optimum temperature for pectinase production by the isolates. Abdullah et al. (2018) noted similar observation where 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).

Effect of pectin concentration in the culture medium on pectinase production

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 (Figure 5) and its presence in the medium enhanced pectinase production. This indicated that pectinase of the F. *oxysporum* isolates were of inducible type. Highest pectinase activity was observed when they were grown in medium supplemented with 5% pectin (Fig 5). The isolates FL, FT and FPo produced pectinase 1.018, 0.783 and 0.804 U/ml, respectively. Pectin concentration 2.5% exhibited lesser pectinase production due to lower availability of the substrate. Pectin concentration 7.5% also resulted lesser pectinase production because 5% is the optimum concentration for maximum pectinase production by the isolates and higher than 5% pectin might inhibit their growth and pectinase production. Similar result was also reported in other pectinolytic microorganisms (Manal et al., 2016).

Effect of different culture media on pectinase production

It was very significant to select an appropriate fermentation medium for production of enzyme. Nine different media were tested for pectinase production by the F. *oxysporum* isolates. All the three isolates produced highest pectinase when they were grown in Czapek's Dox broth (Figure 6). The isolates FL, FT and FPo produced pectinase 3.37, 3.83 and 2.968 U/ml, respectively. The reason might be due to the fact that Czapek's Dox broth 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, isolates produced least pectinase in Asthana and Hawker's broth medium. However, isolates produced moderate level of pectinase in natural broth media viz., malt extract broth and potato broth media.

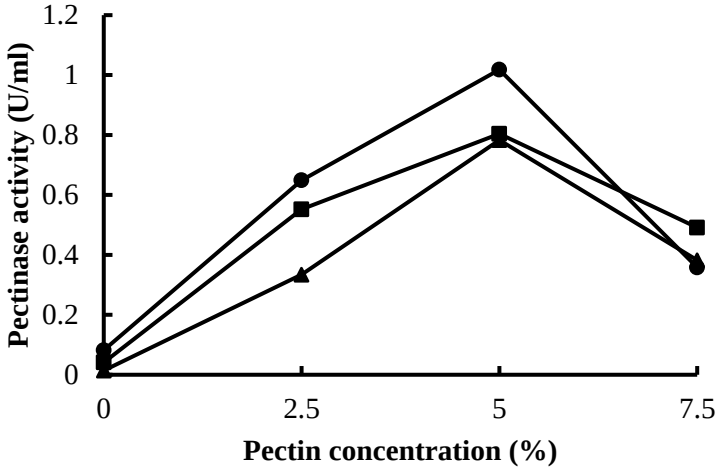

Figure 5. Effect of substrate concentration in the culture medium on pectinase production of the fungal isolates, F. *oxysporum f.sp. lentis* FL( ),F. *oxysporum f.sp. lycopersici* FT( ) , F. *oxysporum f.sp. tuberosi* FPo ( )

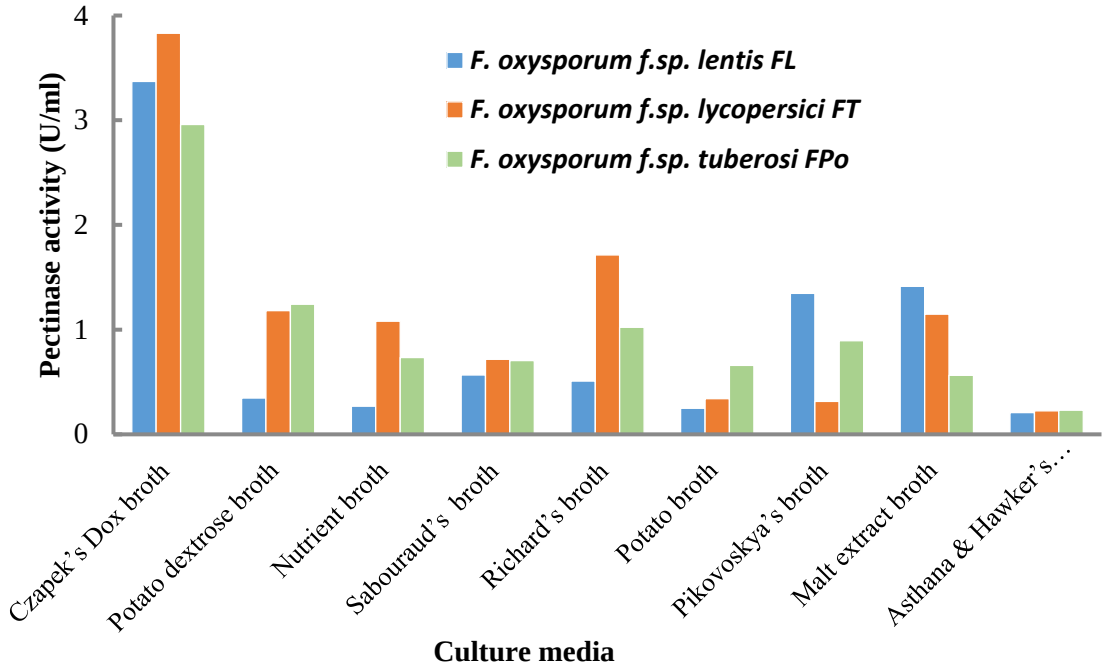

Figure 6. Effect of different culture broth media on pectinase production by the three F. *oxysporum* isolates

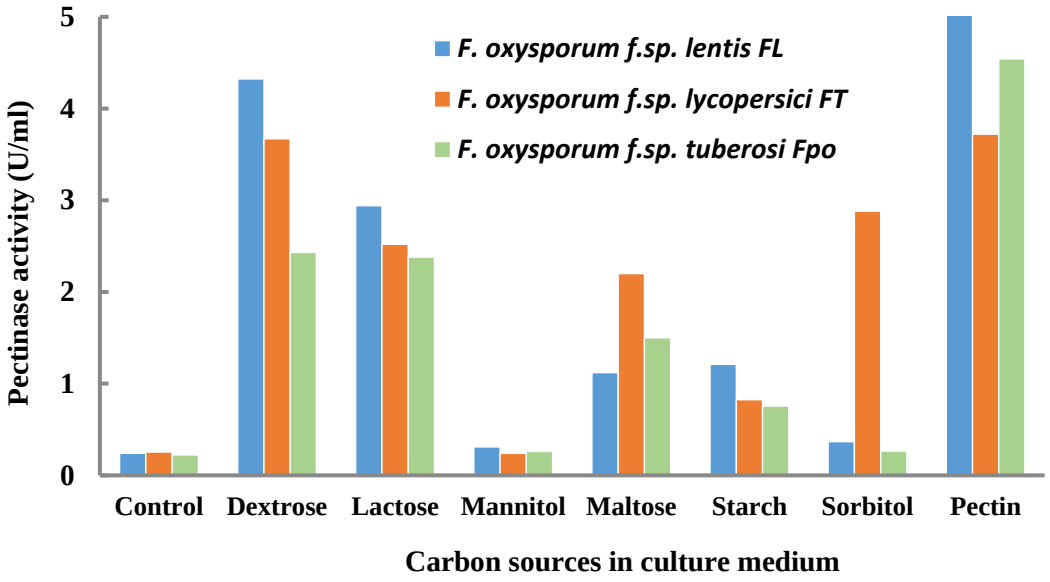

Figure 7. Effect of different carbonsourcesin modified Czapek's Dox broth on pectinase production by the three F. *oxysporum* isolates

Effect of different carbon sourceson pectinase production

Carbon source present in the fermentation medium had played an important role in growth and metabolite production of the microorganisms. 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) (Figure 7). Rashmi et al. (2008) also showed highest pectinase activities by Aspergillus niger isolates when they were grown in pectin rather than other carbon sources. 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 1% dextrose as a carbon source showed better

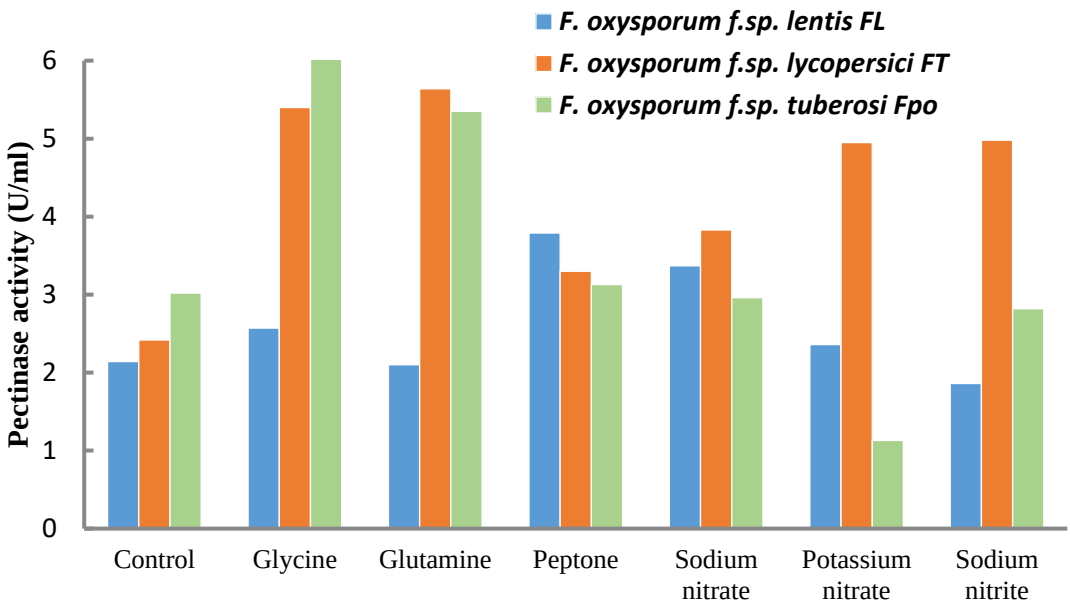

Figure 8. Effect of different nitrogen sources in modified Czapek's Dox broth on pectinase production by the three F. *oxysporum* isolates

results in pectinase production (Reddy and Saritha, 2015). The presence of other monosaccharide 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.

Effect of different nitrogen sources on pectinase production

Nitrogen sources in the fermentation medium also played a determining factor for growth and development of microorganisms. In this study, 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. F. *oxysporum f.sp lycopersici* FT also showed enhanced pectinase production in presence of nitrogenous salts such as sodium nitrate, potassium nitrate and sodium nitrite. However, F. *oxysporum f.sp lentis* FL showed highest pectinase production in presence of peptone (3.79 U/ml).Reddy and Saritha (2015)reported that addition of 0.1% of peptone as a nitrogen source enhanced production of pectinlyase by *Fusarium* sp.

CONCLUSION

Three F. *oxysporum* isolates of grass pea (*Lathyrus sativus*), tomato (*Solanum esculentum*) and potato (*S. tuberosum*) were found to be good producer of pectinase. The best circumstances for the tested enzymes were 6th day of incubation time, medium pH 5.5-6.5, incubation temperature 30°C and 5% pectin supplement in the growth medium. Secretion of pectinase in acidic ranges had a great significance for their application in fruit juice industry. In addition, suitable broth medium (Czapek's Dox medium) and some nutritional amendments like replacement of sucrose with pectin as carbon source and added nitrogen sources such as peptone, glycine and glutamine showed effective pectinase production. This optimization of the cultural parameters was useful to develop low cost pectinase production technology using the fungal isolates.

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.

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