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ENZYMATIC AND PHYTOCHEMICAL ANALYSIS OF ENDOPHYTIC FUNGI ON AEGLE MARMELOS FROM WESTERN GHATS OF TAMIL NADU, INDIA.

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ABSTRACT

Fungal species expands an endophytic role inside the tissues of medicinal plants and are known to produce metabolites, phytochemicals and enzymes in a wide range with biological properties. In the present investigation, the most dominant and potent endophytic fungal strains of Aeglemarmelos, an ethno medicinal plant prevalent in the forest of Western Ghats, Tamil Nadu, India were screened for ability to produce exoenzymes like amylase, protease, lipase, cellulase, laccase and xylanase. The representative potent strains were Curvulariaaustraliensis(FC2AP)and Alternariacitrimacularis(FC8ABr) belonged to the same order Pleosporales. The growth of the endophytic fungi was similar with the highest mycelia formation in the respective media. These endophytic fungal strains produced extracellular enzymes in different pH at different incubation period in a considerable range. Furthermore, the endophytes produced phytochemicals constituents flavonoids and phenols, and this unveils that the strains have highest antioxidant properties. The production of enzymes and phytochemicals provided the insights into their origin and ecological role in the host plant. Hence, this study explains the production and biological activity of enzymes and phytochemicals in endophytic fungi.

KEYWORDS: *Amylase, cellulase, endophytic fungi, lipase, protease, Western Ghats, xylanase.*

INTRODUCTION

Enzymes are the biocatalysts produced by the living cells for specific biochemical reactions generally forming parts of the metabolic processes of the cells. They are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. The practical application and industrial use of enzymes to accomplish certain reactions apart from the cell dates back many centuries and was practiced long before the nature or function of enzymes was understood. Comparing to all nonpathogenic microbes, filamentous fungi are supposed to be an interesting one for production of enzymes because of their easy cultivation process and large scale production of enzymes for industrial purposes. In this, endophytic fungi are relatively unexplored area among microbe isolation and it represents a new source for retrieving the enzymes with different potentialities.[1]Theendophytic fungi on medicinal plant are proposed to have unique and extreme habitats possessing novel metabolic and enzyme systems which may help for the host protection and colonization. Furthermore, the endophytes are the economical sources in discovering the novel enzymes for medical purposes and this interest has been drawn after the discovery of taxol which is an anticancer drug used in medicinal fields.[2] Interestingly, knowing the mineral wealth of Western Ghats regions (hotspot of India), we aimed our research to isolate endophytes from this unexplored area. The *present* study was undertaken to investigate the prevalence

of endophytic fungi and to assess the ability of these fungi in the production of industrially relevant enzymes and phytochemicals.

MATERIALS AND METHODS

Isolation and identification of endophytic fungi

The samples from Aeglemarmelos (Vivlam tree) collected in Western Ghats regions, Coimbatore, TN, India were processed and sterilized by modified method of Mani et al [3] and the sterile samples were placed on fungal isolation media to isolate the endophytic fungi. The potent strains were identified morphologically through SEM analysis and molecularly by amplification of ITS 1 and ITS 4 regions of rDNA sequencing. The growth characterization of the potent strains was measured spectrophotometrically at 450 nm in Sabouraud's Dextrose Broth (SDB) and Malt Extract Broth (MEB) for 21 days incubation period in shaking conditions.

Extracellular enzyme characterization

The extracellular enzyme production was estimated by the absorbance readings taken at 7^{th}, 11^{th}, 15^{th} and 19^{th} day of incubation at different pH range of 5 to 9. Each isolate were inoculated at different range of pH in basal media and the enzyme activity was quantified.

Estimation of proteases

The qualitative analysis of protease were carried out by growing the isolate in GYP agar with 0.4% gelatin and after the incubation period the culture were flooded with saturated aqueous ammonium sulphate, the clear zone around the colony indicate the hydrolysis reaction. The zone was calculated using the formula 1: QEA= DCC- DC (DCC: diameter of the colony plus clearing zone; DC: diameter of the colony). The quantitative estimation of protease enzyme will be followed by the protocol of Saba et al.[4] and one unit of protease was defined as the amount of enzyme required to decrease the OD value by 0.001 U/ hr/ mL of broth.

Estimation of cellulase and xylanase

The plate based method for cellulase and xylanase were tested on basal media by supplementation of 1% carboxymethyl cellulose in YPA (Yeast Peptone Agar) medium and 1% xylan in GYP medium respectively. The cellulose activity was determined by congo red solution and destained with NaCl after incubation, a clear zone was measured with formula 1. The xylanase activity was measured by the clear zone around the respective fungal colony. Similarily, the quantitative estimation was determined by culturing the isolates in the respective broth media and enzyme was quantified by DNS (dinitrosalicyclic acid) method of Miller [5]and the absorbance was read at 600nm. One unit of cellulase and xylanase was defined as the amount required for liberating one microgram of glucose from the soluble substrate/ hr/ mL of media.

Estimation of amylase

The amylase enzyme was qualitatively measured with formula 1 by clear halos around the colony formed by the flooding of 1% iodine solution in 2% KI and the colony was grown in GYP media amended with 2% starch. The enzyme was quantified by following the method of Saba et al.[4] and one unit of amylase was defined as the amount required for liberating one microgram of glucose from the soluble substrate/ hr/ mL of medium.

Estimation of lipase

The qualitative analysis of lipase also observed by a clear halo around the colony in peptone agar amended with 1% tween 20 and the zone was calculated using the formula 1. For estimating the lipase activity titrimetric method was followed.[4]One ml of titration volume is equal to 2.5 units of lipase.

Phytochemicals characterization

The qualitative and quantitative phytochemical analysis in potent endophytic fungal strains was screened by following the descriptions and protocols of Nirjanta Devi et al.[6] The phytochemical constituents such as alkaloids, flavonoids, phenols, tannins, saponins, steroids and cardiac glycosides were analysed for the presence in different crude metabolite extracts of endophytic fungal isolates. The crude metabolite extracts were prepared in hexane, petroleum ether, ethyl acetate and methanol for both the endophytes and concentrated for characterization.

RESULTS AND DISCUSSION

Endophytes produce considerable variation in extracellular enzymes and in this study two potential endophytes produced variable amount of extracellular enzymes. Both the endophytes FC2AP and FC8ABr have been isolated from AeglemarmelosatVellingiri hills, Coimbatore, India and they have been identified as CurvulariaaustraliensisandAlternariacitrimaculairsb elonging to the order Pleosporales. The growth

pattern of these two strains was shown in fig 1 by the optical density units in different days of incubation. The growth of FC2AP was highest from the starting day of incubation but later on FC8ABr growth was higher from 15th day of incubation reaching 3.72 OD units (Fig 1). Both the strains produced extracellular enzyme in a medium range. On qualitative enzymatic analysis, the strain C. australiensisFC2AP and A. citrimacularisFC8ABr showed high production of cellulase with the values of 2.2 and 2.1 respectively whereas no production was observed in laccase.The isolate FC2AP showed intermediate enzyme production in protease and lipase of 1.7 and 1.8 whereas lower production was observed in amylase enzyme with 0.6 values in plate based analysis. Similarly, FC8ABr exhibited intermediate enzyme production in protease and xylanase with the values of 1.4 and 1.6 whereas lower production was noticed in amylase enzyme with the value of 0.4 (Table 1). The endophytes exhibited the highest cellulase activity and this is because, the two strains belonged to the same order. The profile of endophytic fungal extracellular enzyme production suggests their ecological role as latent pathogens/ endophytes or saprobes in natural environment. Same as both the strains showed intermediate proteolytic activity and this unveiled their possible role as endophytes. It is also interesting to note that both the strains did not show any activity for laccase enzymes in agar plate assays and also showed lesser activity for amylolytic enzymes. This was similar to the results of Bhagobaty and Joshi [7] exhibiting the non amylolytic and non laccolytic activity in endophytic fungi from medicinal plants. Lumyonget al.[8] investigated that 26 strains of entophytic fungi from Thailand had the ability of producing mannose, xylanase, proteinase and cellulase was distributed among the test strains and further the research of Lumyong[8] revealed that the ability to produce the enzymes may be related to the lifestyle of endophyticfungi.The quantitative production of exoenzymes amylase, protease, lipase, cellulase and xylanase in different pH range in different incubation period in liquid media was depicted in fig 2, 3, 4, 5 and 6 respectively. The amylolytic activity was measured spectrophotometrically and on comparing the two strains FC8ABr was found to produce more amylases than the other one by exhibiting 0.79 OD U/ hr/ mL at pH 9 on 11th day incubation. Interestingly, the strain produced amylase at alkaline pH, and this was controversy to the report of Maccheroniet al.[9] who observed the amylase activity was not seen at an alkaline pH and starch was degraded at neutral and acidic pH. But FC2AP strain produced about 0.66 OD U/ hr/ mL (Fig 2) at neutral pH on 11th day of incubation, there was only a little variation in the production of amylases between two strains and this may be due to their difference in class. According to the study of Maria et al. [10], the enzymatic activity of mangrove endophytic fungi of Indian southwest coast, investigated that the amylase and protease were present in lesser quantities when compared to other enzymes and this was noted in our research. The highest protease activity was seen in pH 7 on 7th and 11th day of incubation for FC2AP and FC8ABr respectively. Both the strains produced 1.1 OD U/ hr/mL and 0.92 U/ hr/ mL (Fig 3). In this activity, FC2AP produced more proteases when compared to FC8ABr and also we can see the enzyme production was increased in acidic pH and reached the maximum at neutral pH thereafter it started to decline in alkaline pH.The lipase activity was estimated by titrimetric method and FC8ABr does not produce any traces of lipase whereas FC2AP revealed significant amount of lipases. About 59 U/ hr/ mL (Fig 4) enzyme was produced at pH 9 at 7th day incubation. The enzyme production was increased gradually from acidic pH 3 to alkaline pH 9 till 7th day of incubation and after the stated day the production declined. This declination was similar to the research of Maccheroni et al.[9] which showed the result of absence of lipase in acidic pH and secreted at neutral and alkaline pH. Few investigations suggested that the lipases can be used for degradation process in food industries. The endophytic fungi produce highest units of lipases in submerged culture conditions when compared to solidified agar media. FC2AP and FC8ABr produced highest cellulase enzyme activity of 0.91 U/ hr/ mL and 1.2 U/ hr/ mL at neutral and alkaline pH on 11th day of incubation respectively (Fig 5). The enzyme production was increased with the increasing pH range and after attaining alkaline range of pH 9, the cellulases started to decrease in their count. The decline of enzymes after 11th day of incubation is due to utilization of media constituents by the endophytic fungi. Cellulases are mainly produced by fungi were taken for paper industries to degrade cellulose which is a pollutant. In accordance with this, FC2AP and FC8ABr produced cellulase at a considerable amount quantitatively.Theendophytic fungal strains in this investigation produced xylanase at a certain range and on comparing these two strains, FC8ABr produced highest value of enzyme activity 0.79 OD U/ hr/ mL on 15th day of incubation at neutral pH, whereas FC2AP produced enzyme of 0.61 OD u/

hr/ mL at pH 5 on 7th day incubation and after that it does not produce xylanase. On comparison of plate based assays and liquid medium, the production of extra cellular enzyme differs variably. Enzymes that were not detected on plate based assays were quantified in liquid medium cultural conditions; this may be due to the availability of substrates per surface area which were increased accordingly. Moreover, the enzyme production in the plate based assay is relative to the diameter of the colony with the formation of clear halo zone around the fungal colonies and the strain which have a higher growth rate in terms of diameter of the colony, the clear halo zones created in the agar plates with the substrates specific to the enzyme activity may be masked and thereby it may prevent the detection of the particular enzyme. The enzyme was detected in solid medium by the release of enzyme from the mycelium and activity in the medium after the production, thus the lack of a positive result could be meant as the enzyme may not be produced, or it may be produced but not released from the mycelium, or it may be produced and released but inhibited by the medium. However, absence of enzyme in the solid media does not confirm the enzyme activity for the particular strain.[7]In this investigation, FC2AP does not produce xylanase in agar plate method but it produced considerable amount of xylanases in liquid medium till 7th day of incubation and after the stipulated day the production was declined. The endophytic fungal isolates exhibited typical growth characteristics in Sabouraud's Dextrose Broth (SDB) and Malt Extract Broth (MEB). Our past report revealed a promising mycelia biomass generation in five different media at different time intervals and finally FC2AP produced maximum mycelium biomass in SDB till 15th day of incubation and FC8ABr produced highest in MEB till 21st day of incubation. FC2AP produced highest amount of mycelia biomass when compared to FC8ABr and both of the strains produced pink and brown colored crude metabolites respectively. These crude metabolites are found to have antioxidant and antimicrobial potentiality in our research.These crude metabolites produced by the two fungal strains were taken in different solvents for the determination of phytochemical constituents qualitatively and quantitatively. The hexane extract for the strain FC2AP showed positive activity for flavonoids, phenols and tannins; petroleum ether extract exhibited activity for tannins and steroids; ethyl acetate extract showed positive activity for flavonoids, tannins, cardiac glycosides and steroids whereas methanol extract showed activity only for tannins (Table 2). Similarily, FC8ABr endophytic fungus also revealed their potency in phytochemical constituents and crude metabolite extract in hexane was found to possess flavonoids, phenols and saponins; petroleum ether metabolite extract contained flavonids and steroids whereas methanol extract possessed alkaloids, flavonoids and saponins. Both the strains exhibited the presence for flavonoids and phenols in hexane, petroleum ether and methanol extract. So the extracts were assessed for quantification of these two phytochemicals flavonoids and phenols with the standards rutin and gallic acid respectively.

Table 1
Qualitative extracellular enzyme analysis of FC2AP and FC8ABr

S.NO	ENDOPHYTIC FUNGAL STRAIN	MORPHOLOGICAL IDENTITIY	EXTRACELLULAR ENZYME PRODUCTION RATIO					
			AMYLASE	PROTEASE	LACCASE	CELLULASE	LIPASE	XYLANASE
1	FC2AP	Curvulariaaustraliensis	0.6	1.7	0	2.2	1.8	0
2	FC8ABr	Alternariacitrimacularis	0.8	1.4	0	2.1	0	1.6

Table 2
Analysis of phytochemicals in different crude extracts of two potential endophytic fungal strains

S. No.	Phytochemical constituents	QUALITATIVE ANALYSIS OF PHYTOCHEMICALS						
		Strain FC2AP				Strain FC8ABr		
		Hexane	Pet. Ether	Ethyl acetate	Methanol	Hexane	Pet. Ether	Methanol
1.	Alkaloids	-	-	-	-	-	-	+
2.	Flavonoids	+	-	+	-	+	+	+
3.	Phenols	+	-	-	-	+	-	-
4.	Tannins	+	+	+	+	-	-	-
5.	Cardiac glycosides	-	-	+	-	+	-	-
6.	Steroids	-	+	+	-	-	+	-
7.	Saponins	-	-	-	-	+	-	+
		QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS						
		Strain FC2AP				Strain FC8ABr		
1.	Phenols (µg gallic acid/ mg of the extract)	15.5	-	-	-	20.0	-	-
2.	Flavonoids (µg rutin/ mg of the extract)	22.0	-	89.5	-	64.0	21.6	42.5

'+' denotes presence of phytochemicals; '-' denotes absence of phytochemicals.

On comparing the two endophytic fungal strains FC8ABr exhibited highest value of 20.0 µg GAE/ mg of the extract while the obtained value for the strain FC2AP was 15.5 µg GAE/ mg of the extract. Phenolics are the well known compounds, owing to the potent antioxidant activities and bioactivities, are also known to diffuse the free radicals.[11]This unveiled the potency of antioxidant capacity was higher in FC8ABr than FC2AP and in several investigations the phenolic compounds are found to have an important role in stabilizing the lipid oxidation process and also association with the antioxidant assessment.[12] However the content of the phenolics were dependent on the solvents used for the extraction process. In a study of Liu et al.[13], higher amount of phenolics were present in the polar solvents but in our study there is a new finding of highest phenolics were present in non polar solvent extraction; this is a controversial part to the research of Liu et al.[13] Report of Huang et al.[14] stated the analysis of 292 endophytes from Chinese traditional plants showed the antioxidant capacities which were significantly correlated with the total phenolic content. The total flavonoids was present in hexane and ethyl acetate extract of FC2AP with the values of 22.2 and 89.5 µg rutin/ mg of the extract respectively. This obtained value was higher when compared to the extract of FC8ABr (hexane, petroleum ether and methanol – 64.0, 21.6 and 42.5 µg rutin/ mg of the extract). Hence, flavonoids are considered to be the strong scavengers of ROS.[15]However, the phenol content was highest in FC8ABr and flavonoid content was highest in FC2AP. From the results of this research, it is evident the phytochemicals and enzymes of these two strains FC2AP and FC8ABr can be utilized for the industrial purposes.

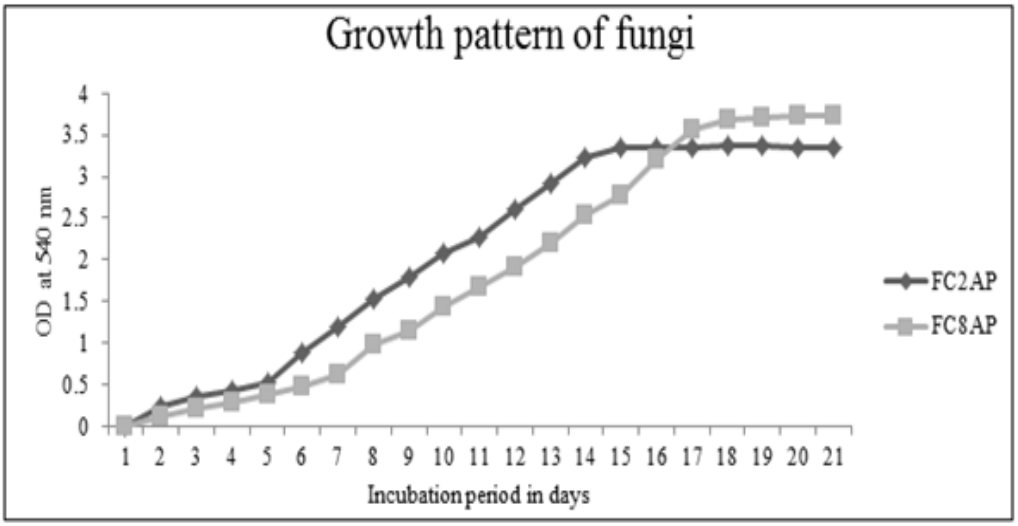

Figure 1
Growth pattern of fungal strains FC2AP and FC8ABr

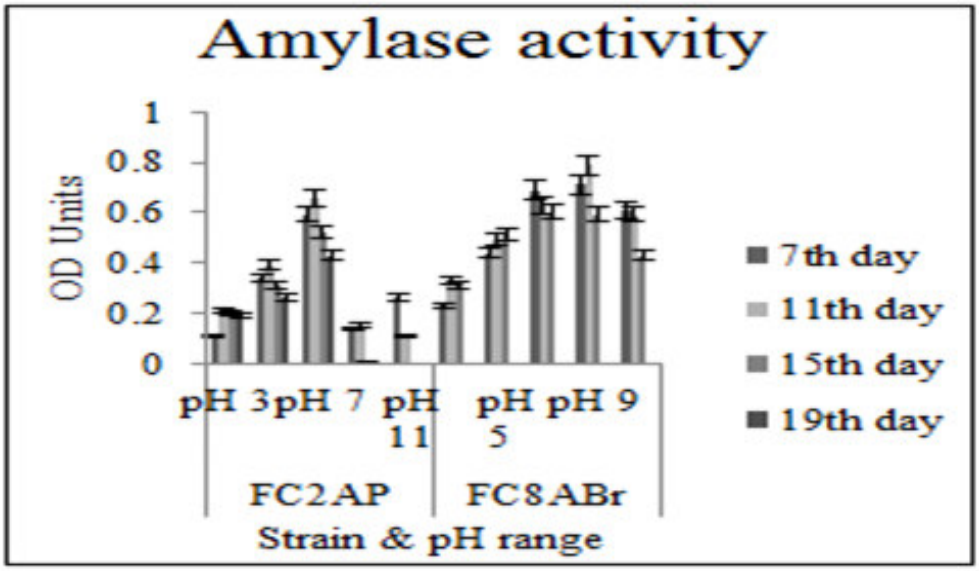

Figure 2
Production of amylases

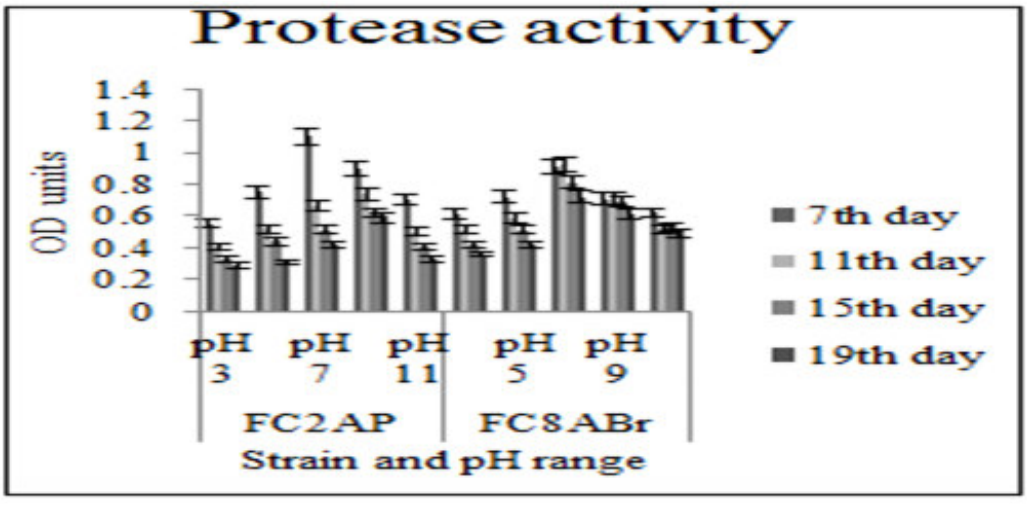

Figure 3
Production of proteases

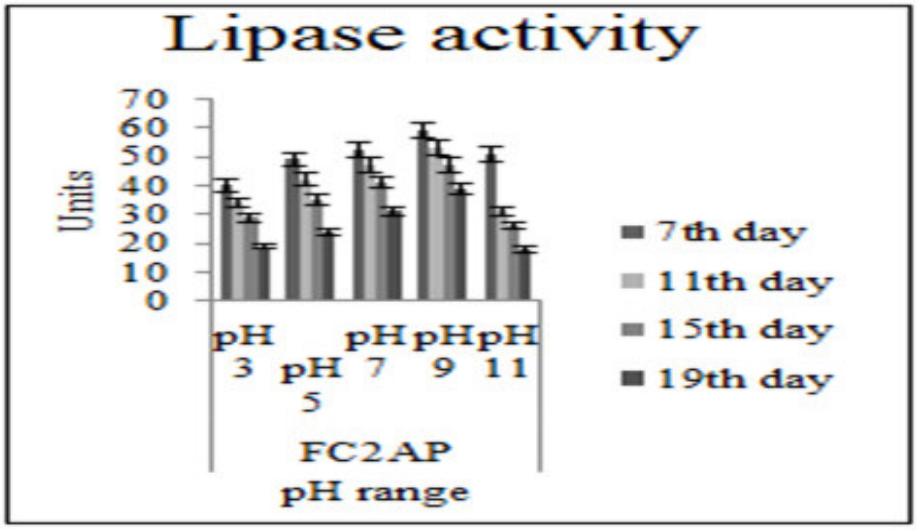

Figure 4
Production of lipases

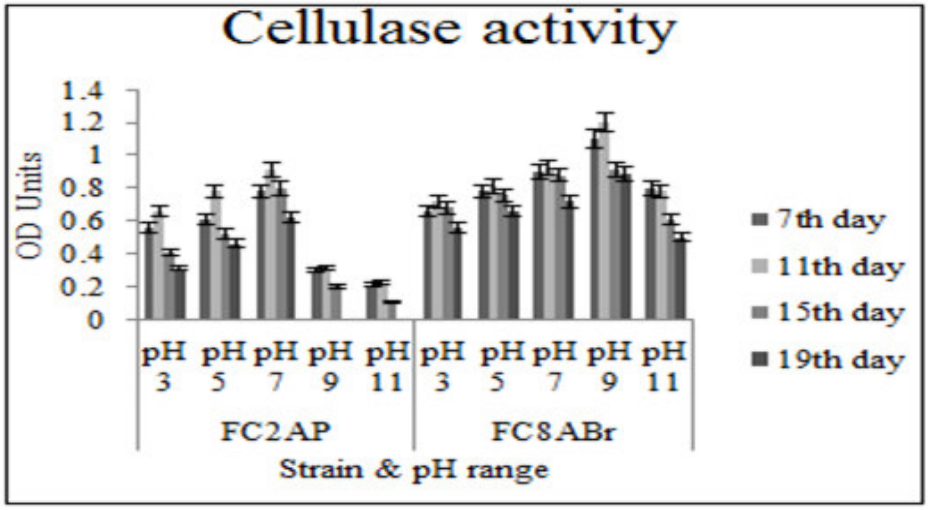

Figure 5
Production of cellulases

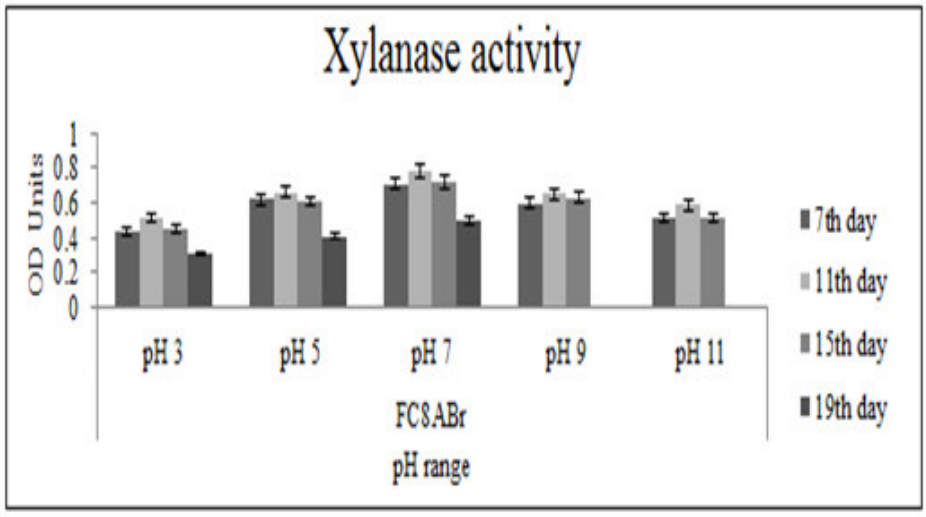

Figure 6
Production of xylanases

CONCLUSION

The endophytes are very useful to the industries by the production of enzymes and metabolites and, this can be also used as biocontrol agents. To the best of our knowledge this is the first report of endophytic fungal extracellular enzyme and phytochemicals production from ethno medicinal tree A. marmelosof Western Ghats (Nilgiris cluster) of Tamil Nadu state, India. The presence of phenols and flavonoids represents the strong antioxidant activities which can reduce the cell damage responses. Further studies will be carried out to purify the enzymes and produce through bioprocess and biotechnological process, and applied for medicinal purposes.

CONFLICT OF INTEREST

Conflict of interest declared none.

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