

Endophytes: A Prospective Source of Enzyme Production

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Endophytic microorganisms mostly comprising of bacteria and fungi are a diverse group of microbes, existing within a unique biological niche which is the intracellular spaces of higher plants. In this study, 17 endophytic fungi and 14 endophytic bacteria were isolated from the medicinal plants. These endophytes displayed the ability to produce some important enzymes namely amylase, protease and cellulase when screened using starch, casein and CMC as substrates respectively. The endophytic fungi *Guignardia mangifera* (CosA) isolated from *Costus igneus* was having maximum amylase activity (8.9 IU/ml) compared to others while the chief producer of protease was identified as Ne1(not yet identified) isolated from *Nerium odoratum* with the enzyme activity of 1.5 IU/ml. The endophytic bacteria *Bacillus cereus* (Adab) was showing maximum amylase activity of 0.58 IU/ml. The isolate *Bacillus thuringiensis* (Ci2b) isolated from *Calophyllum inophyllum* was having the maximum protease activity (3.5 IU/ml) compared to the other endophytes. In an attempt to optimize the conditions for maximum production, pH of the medium has been varied to study its influence on the enzyme production.

Key Words: Endophytes, Enzymes, Amylase, Protease.

The myriad applications of enzymes in various fields ranging from medical to industrial, makes the process of identifying novel sources of enzyme production a much researched area. Non-microbial sources like plants and animals have been major contributors to the production of enzymes on a large scale. But the inherent advantages of microbes like their ability to produce abundant quantities of enzymes under suitable growth conditions, need of inexpensive media for cultivation, production of the enzyme in a short period, ease in genetically manipulating them for

increased production, recovery, isolation and purification processes needing little effort, makes them a much sought after source for enzyme production compared to that of the non-microbial sources. In fact most enzymes of industrial application have been successfully produced by microorganisms. Hence, isolation and characterization of new promising strains, in an attempt to enhance the production of enzymes, is a continuous process (Kumar *et al.*, 2002).

Endophytes are one such class of microbes comprising bacteria and fungi whose potential for the production of enzymes on a large scale has not been tapped completely. Endophytic microorganisms are those that inhabit the interior of virtually all plants especially leaves, branches and stems, showing no apparent harm to the host as stated by Azevedo (1998, 2000). Bacon *et al.* (2000) defined endophytes as "microbes that colonize living internal tissues of plants without

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causing any immediate, overt negative effects” whereas Strobel and colleagues (1999) suggest that the relationship can range from mutualistic to bordering on pathogenic. The most frequently encountered endophytes are fungi and bacteria that co-exist with each other (Strobel 2003).

Endophytes have been a source of various natural products like antibiotics, (Strobel 1999; Walsh 1992; Miller 1998; Ballio 1994; Harrison 1991) antiviral compounds (Guo 2000), volatile antibiotics (Strobel 2001), anticancer agents (Strobel 1996), antioxidants (Strobel 2002), antidiabetic agents (Zhang 1999) and immunosuppressive compounds (Lee 1995). They were also shown to possess the ability to produce various kinds of industrially important enzymes like protease, amylase, cellulase, tannase, asparaginase, xylanase etc. (Maria G L 2005; Subhadip Mahapatra 2009; Thirunavukkarasu 2011; Manabu Suto 2001).

In the present study, endophytic fungi and bacteria were isolated from medicinal plants and screened for the activity of enzymes that include amylase, protease and cellulase both qualitatively and quantitatively.

MATERIALS AND METHODS

The following medicinal plants obtained from Siddha Institute, Chennai were used to isolate the endophytic bacteria and fungi. They include *Nerium odoratum*, *Bryophyllum pinnatum*, *Klenia grandiflora*, *Costus igneus*, *Calophyllum inophyllum*, *Crataeva magna*, *Syzygium cumini*, *Garcinia xanthochymus*, *Wrightia tinctoria*, *Aravae lanata* and *Adathoda beddomei*.

Isolation of the Endophytes

Healthy leaves from each plant were collected and cleaned under running tap water to remove debris and then air dried and processed within 5 hrs of collection. The leaf samples are cut into 4 segments of 1 cm length and treated as replicates. Surface sterilization was carried out by submerging them in 75% ethanol for 2 mins. The explants were further sterilized sequentially in 5.3% sodium hypochlorite (NaOCl) solution for 5 min and 75% ethanol for 0.5 min (Ravi Raja N S 2006).

Samples were allowed to dry on paper towel in a laminar air flow chamber. Four segments per plant were placed horizontally on separate Petri

dishes containing Potato Dextrose Agar supplemented with antibiotic streptomycin and Nutrient Agar for the growth of endophytic fungi and bacteria respectively. After incubation, individual fungi and bacteria were collected and placed onto potato dextrose agar and nutrient agar and regularly checked for culture purity. Eventually, pure cultures were transferred to PDA and nutrient agar slant tubes and subcultured regularly.

Culture Conditions of the Endophytes

Endophytic Bacteria

The endophytic bacterial culture was maintained on nutrient agar slants by subculturing at monthly intervals. 100 mL of Nutrient Broth medium was prepared, sterilized and inoculated with 12 hr old cultures of the endophytic bacterium. The culture flasks were incubated for 36 hrs at 37°C with shaking at 150 rpm. After incubation period, the broth was centrifuged at 10,000 rpm for 10 min and filtered to obtain the supernatant that was used for screening the enzyme activity.

Endophytic Fungi

The fresh mycelia (grown on PDA) of the endophytic fungi were transferred to 250ml Erlenmeyer flask containing 100 ml of the PDB. The flasks were incubated at 25±1°C for 6 to 7 days with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile mesh cloth to separate the mycelia from the culture broth. The latter was used to study the enzyme activity.

Enzyme assays

The activity of the enzymes namely amylase, protease and cellulase were screened by inoculating the endophytic fungi and bacteria on a growth media supplemented with starch, casein and CMC as substrates respectively. The activity was observed by the formation of clear zones around the colonies.

Amylase Assay

Amylase assay was performed using Dinitrosalicylic acid method. The reaction mixture contained 1 ml of standard starch solution (1% soluble starch in 50mM phosphate buffer, pH 6.9) and incubated for 1 hr at 45° C. The reaction was stopped by adding 3 ml of dinitrosalicylic acid and boiled for 5 min and cooled. Absorbance of the resulting solution was determined at 550 nm. The amount of sugars produced was read from

standard curve using glucose as standard. Enzyme activity was expressed in units where 1 unit/ml is the amount of enzyme which releases 1 μ l mole of glucose under the assay condition (Mishra and Behera 2007).

Protease assay

Protease production was assayed by a modified method of Kunitz (1947). Samples containing 400 μ l of 0.5 % (w/v) casein in 50 mM Tris-HCl buffer, pH 10, with 100 ml enzyme solution were incubated in a water bath at 50 °C for 20 min. The enzyme reaction was terminated by addition of 500 μ l of 10 % (w/v) trichloroacetic acid and was kept at room temperature for 10 min. The reaction mixture was centrifuged at 10,000 g for 10 min at 4 °C and the absorbance was measured against a blank at 280 nm. One unit of proteases was defined as the amount of the enzyme releasing an equivalent of one mmol of tyrosine per minute under the defined assay conditions (Abdelnasser 2007).

Cellulase Assay

The reaction mixture for the cellulase assay contained 1 ml of culture supernatants and CMC in 1 ml of 0.1 M sodium phosphate buffer, pH 6.5. After incubation at 60°C for 10 min the reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent. The tubes were placed in a boiling- water bath for 50 min and then cooled to room temperature and filtered. The optical densities of the filtrates were measured at 570 nm and converted to glucose equivalents (Lee 1975).

Effect of pH on enzyme production

The effect of pH on the production of enzymes was studied by varying the pH of the medium at 3, 5, 7, 9 and 11. The media with varying

pH were inoculated with the endophytic fungal and bacterial cultures and incubated. The optimum pH for the production of the enzymes was observed by performing the assays.

RESULTS AND DISCUSSION

Endophytic microorganisms are found to be in virtually every plant on earth. These organisms reside in the living tissues of the host plant sharing a plethora of relationships from symbiotic to slightly pathogenic. Their contribution to the host plant makes it a storehouse of multitude of potential substances used in agriculture, modern medicine and industry. Fungal endophytes occur in all major lineages of land plants and in natural and anthropogenic communities ranging from the arctic to the tropics (Arnold 2007). Bacteria that are common inhabitants in the interior of plants without causing diseases to their hosts are called endophytic bacteria (Petrini *et al.*, 1989; Azevedo *et al.*, 2000).

Endophytic microorganisms have been isolated from different types of vegetation such as grass (Naffaa *et al.*, 1998), corn (Araújo *et al.*, 2000; McInroy and Kloepper, 1995), *Eriaceae* (Petrini, 1984), cotton (McInroy and Kloepper, 1995), bryophytes (Costa *et al.*, 2001), *Solanum lycocarpum* (Maitan, 1998) and agricultural crops (Halmann *et al.*, 1997) which indicated their ubiquity and biodiversity.

In the current study, a total of 17 endophytic fungi and 14 endophytic bacteria were isolated from the surface sterilized leaves and stems of the medicinal plants (Table 1). These results

Table 1. Endophytic fungal and bacterial Isolates

Name of the Plant	Fungi Isolates	Number	Bacteria Isolates	Number
<i>Nerium odorum</i>	NE1, NE2	2	NE1b	1
<i>Bryophyllum pinnatum</i>	Bp1, Bp2	2	BpSb, BpLb	2
<i>Klenia grandiflora</i>	-	-	P3b	1
<i>Costus igneus</i>	CosA, CosB	2	Cos1b, Cos2b	2
<i>Calophyllum inophyllum</i>	Ci 1	1	Ci1b, Ci2b	2
<i>Crataeva magna</i>	Cm1	1	Cm1b, Cm2b, Cm3b	3
<i>Syzygium cumini</i>	Syz1, Syz2	2	--	
<i>Garcinia xanthochymus</i>	Gx1, Gx2, Gx3, Gx4, Gx5	5	Gx1b, Gx2b	2
<i>Wrightia tinctoria</i>	Wt1	1	--	
<i>Aravae lanata</i>	Ara1	1	--	
<i>Adathoda</i>	-	-	Ada1	1

obtained, showed these common phenomenon of association between the microorganisms and plants (Azevedo, 1998).

The isolated endophytic fungi and bacteria were tested for their ability to produce extracellular amylase, protease and cellulase on solid media by providing the appropriate substrates namely starch, casein and CMC respectively. It was observed that all the isolates were able to produce a clear zone for the enzymes under study indicating the ability of the endophytes to produce enzymes. In general, it has been reported that the hydrolytic enzymes of endophytes appear to be important for the colonization of plant roots (Quadt-Hallmann *et al.*, 1997; Reinhold-Hurek and Hurek, 1998).

Quantification of Enzyme Production

The positive result shown by the endophytic fungi and bacteria when screened for enzyme activity is followed by the quantification of enzyme production using the standard assay procedures (Fig.1 and Fig.2).

Enzyme Production by Endophytic fungi

All the endophytic fungi, when quantified for enzyme activity, were showing considerable amylase and protease production where as the cellulase production was observed to be negligible which is evident in Fig. 1.

It was observed that the endophytic fungi CosA isolated from *Costus igneus* which was identified as *Guignardia mangifera* by ITS analysis was having maximum amylase activity (8.9 IU/ml)

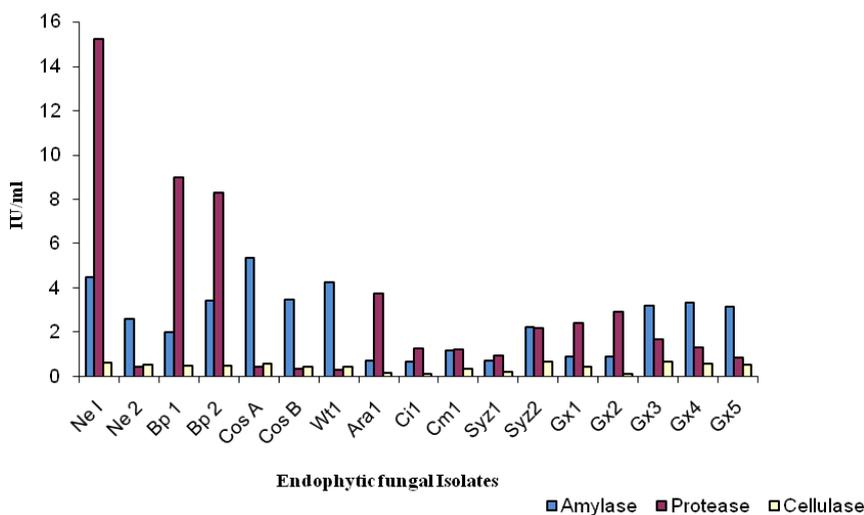


Fig. 1. Enzyme Activity of endophytic fungi

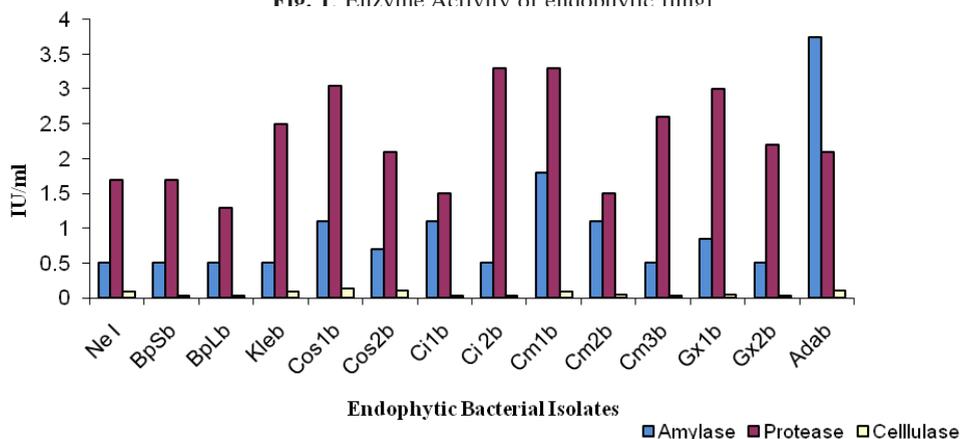


Fig. 2. Enzyme Activity of endophytic bacteria

compared to all other endophytic fungi while the chief producer of protease was identified as Ne1 (1.5 IU/ml) isolated from *Nerium odoratum* that has not yet been identified.

Though the production of cellulase was considerably low by all the endophytic fungi compared to the other enzymes, the isolate Syz2 isolated from *Syzygium cumini* was shown to produce maximum cellulase (0.52 IU/ml). This is in corroboration with the results obtained earlier where the production of cellulase by the endophytic fungi was observed to be low or insignificant and only very few of them were found to have significant cellulase production (Ravindran 2010).

Enzyme Production by Endophytic Bacteria

The pattern of enzyme production by the endophytic bacteria cultured in nutrient broth is shown in Fig 2.

Amylase activity was observed in all the endophytic bacteria to a considerable extent. The isolate showing maximum activity was Adab (0.58 IU/ml) isolated from *Adathoda beddomei* and was identified as *Bacillus cereus* by 16s rRNA analysis. It has been earlier reported that enzyme activity was observed in mangrove associated endophytic bacteria (Gayatri et al 2010).

Protease production was observed to be high from the isolate Ci2b (3.5 IU/ml) isolated from *Calophyllum inophyllum*. The endophytic bacterium was identified as *Bacillus thuringiensis*. The use of nutrient broth as growth media was considered suitable for the production of protease (E.M. Upton 1977). The cellulase production was comparatively low by the endophytic bacteria which are in agreement with the reports by Elbeltagy et al., (2000) and Aysha (2006).

Effect of pH on enzyme activity

The physical parameter, pH of the growth medium, plays an important role by inducing morphological changes in microbes and in enzyme secretion (Kathiresan 2006) and influences the stability in the medium (Rani Gupta et al., 2003). The production of cellulase was significantly low by the endophytic fungi and bacteria and hence an attempt has been made to identify the optimum pH for the production of amylase and protease.

Effect of pH on Amylase Activity

The influence of pH on the production of

amylase by the endophytic fungi *Guignardia mangifera* (CosA) isolated from *Costus igneus* is clearly evident from Fig. 3. Maximum amylase activity of 12.4 IU/ml was observed at pH 7.0 (Fig. 3) after which a considerable reduction in the activity was observed. These reports are in agreement with the findings obtained by Patel et al., (2005), Yabuki et al., (1977), Vincent W. Ogundero (1979) and Hegde (2011) who found out that the maximum amylase activity was observed at pH 7.0.

The effect of pH on the production of amylase by endophytic bacterium *Bacillus cereus* (Adab) isolated from *Adathoda beddomei* (Fig. 4) was shown to have maximum amylase activity of 0.065 IU/ml at pH 5 which is in accordance with the reports of Petrov (2008). This study stated that the bacteria *Lactococcus* sp. was also displaying maximum amylase activity at pH 5.2.

Effect of pH on Protease Activity

The influence of pH on the production of protease was studied by varying the pH of the medium from 3 to 11 for the endophytic fungi NE1 that showed maximum protease activity (Fig.5).

It has been observed that the isolate NE1 was showing a higher enzyme activity of 1.5 IU/ml at pH 5 and 11 (Carolina 2007). Fungal proteases are active over a wide range of pH with an optimum between pH 4 to 11 (Duo-Chuan Li 1997; Coral et al.2002). Similar studies on the influence of pH on protease production states that the strain *A. terreus* was having an optimum pH between 5.5 and 9.5 (Chakrabarti et al., 1999).

Endophytic bacterium *Bacillus thuringiensis* (Cib2) was subjected to varying pH (Fig.6) to study its influence on protease activity.

The bacteria showed activity over the entire range of pH from 3-11 but maximum activity of 1.5 IU/ml was reported at pH 5 and 7. It was earlier reported that medium with a neutral pH was optimum for alkaline protease production by *Bacillus pumilus* c172 transformant (Yaoyu et al 1997). There were a few other studies which revealed that the media with slightly acidic was optimum for the protease activity in *Bacillus coagulans* (Gajju et al., 1996), *Pseudomonas maltophilia* (Kobayashi et al., 1985) and *Streptomyces moderatus* (Chandrasekhar and Dhar 1983).

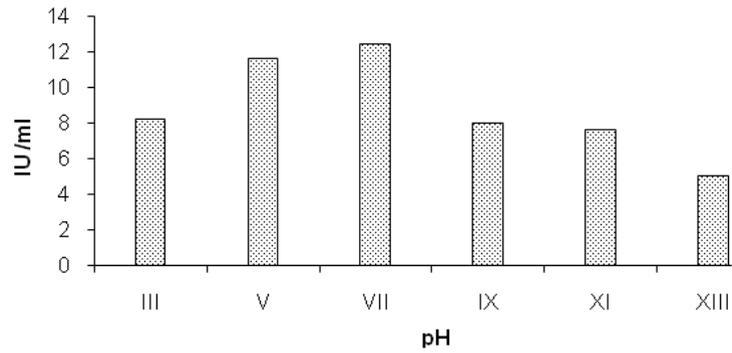


Fig. 3. Varying pH and its effect on amylase activity of the endophytic fungus *CosA*

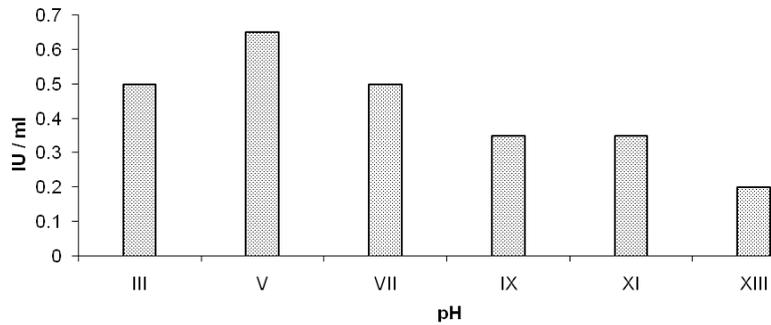


Fig. 4. Varying pH and its effect on amylase activity of the endophytic bacteria *Adab*

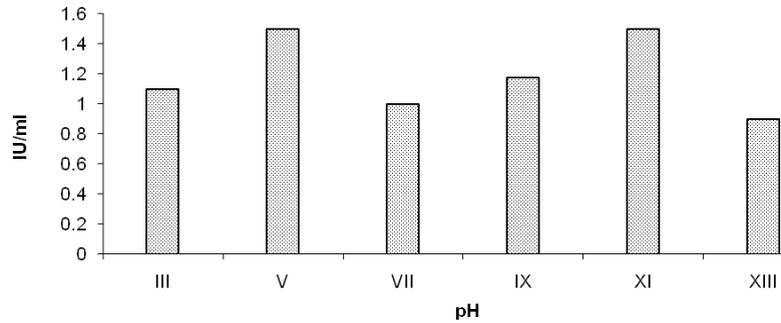


Fig.5. Varying pH and its effect on protease activity of the endophytic fungi *NE1*

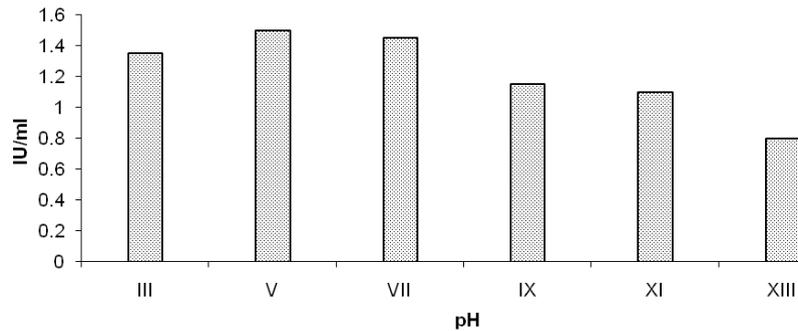


Fig. 6. Varying pH and its effect on protease activity of the endophytic bacteria *Cib2*

CONCLUSION

Endophytes are one of the varied classes of microbes which offer great potential in the quest for different biological products, the applications of which range from industry to medicine. Using endophytes for the production of enzymes on a large scale opens a new avenue of research in this field. This study primarily reports the production of enzymes on a quantitative basis by the isolated endophytic fungi and bacteria. It has been observed that there has been significant production of enzymes especially amylase and protease than cellulase. Further, the influence of pH on the enzyme activity has been studied as an initial step to optimize the parameters for enhanced production.

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