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Evaluation of hydrolytic enzyme activities of endophytes from some indigenous medicinal plants

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The evaluation of some endophytes was carried out for their possible hydrolytic enzymatic activity from various parts of medicinal plants belonging to Jalgaon Maharashtra, India. A total of fourteen bacterial endophytes and twenty four fungal endophytes were isolated from the aerial and underground parts of seven medicinal plants. Six bacterial isolates and fifteen fungal isolates expressed the amylolytic activity. Cellulolytic activity was observed in seven bacterial isolates and three fungal isolates. Nine bacteria and ten fungal isolates were able to produce esterase and lipase enzymes. Amylase, cellulase, esterase and lipase activity occurred in low amount in the endophytic bacteria coded HB1 of rhizomes of *Curcuma longa* L. in comparison with other endophyte isolates code NB1*, Lactobacillus fermentum* and *Escherichia coli*. Proteolytic activity was found in nine bacterial and twelve fungal endophytes, highest potential to hydrolyse of casein and gelatine that was recorded in bacterial isolate coded SB1 from leaves of *Vinca rosea* (L.) G. Don. Asparaginase activity was noted only by fungal isolates coded NF1 andNF2 and bacterial isolates, coded NMB2 from stem of *Azadirachta indica* A. Juss.

Key words: endophytic microorganisms, fungi and bacteria, medicinal plants, enzymes activity

Introduction

Microbes that inhabit plant tissues for their life without causing any harm to their host plant are endophytes. Endophytes lives symbiotically within the plants (Azevedo *et al*., 2000). Bary mentioned that endophytes for first time in 19th century. Recently endophytes are viewed as outstanding source of secondary metabolites bioactive natural products and industrial important enzymes. Enzymes of microbial origin have great biotechnological interest such as textile industries, food and pharmaceutical products (Falch *et al*., 1991; Rao *et al*., 1998). Present study was carried out on some indigenous medicinal plants inhabiting of Jalgaon District of Maharashtra state viz *Aloe vera* (L.) Burn. F*, Curcuma longa* L., *Eucalyptus globules* Dehnh*, Musa paradiasa* L.

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and *Pongamia glabra* Vent*., Vinca rosea* (L.)G. Don and *Withania somnifera* (L.) Dunal.

The aim of this study was to confirm ability of endophytic microorganisms which isolated to screen for production extracellular enzymes with economical importance. After the screening, the maximum potential of enzyme biosynthesis was recorded endophytic microorganisms involving in proteases and amylases activities.

Materials and methods

Microorganism isolation

Mature healthy plant materials were collected and identified by experts from Department of Botany Moolji Jaitha College Jalgaon. By sampling different parts of the selected medicinal plants, fifteen samples were taken from each tree five each from roots, inner bark and leaves. From each sample ten subsamples were separated further for isolation of endophytes. Samples were immediately brought to laboratory and were used within 24 hrs .Samples were washed in running tap water for 10 minutes to remove soil particles and adhered debris, and finally washed with distilled water. Surface sterilization was done using method described by Petrini (Petrini *et al*., 1992). Samples were immersed in 70% ethanol for 1-3 minutes and 4% aqueous solution of sodium hypochlorite 1.5 minutes, again 1 minute with 70% ethanol and finally rinsed with sterile distilled water. Samples were selected by aseptic cutting using sterile knife and inner tissues were excised. Samples were inoculated on to the water agar media and incubation allowed for 2-5 days, at 25°C, to isolate endophytic bacteria. For isolation of endophytic fungi, samples were inoculated on potato dextrose agar, and cultivated in Petri dishes for 20-25 days, at 27^{0} C. Isolates were further purified and maintained nutrient on agar and potato dextrose agar for bacterial and fungal endophytes.

Bacterial endophytes presenting strong enzyme activity were identified using Bergeys Manual of Systematic Bacteriology. Also fungal endophytes were morphologically identified by experts from Banaras Hindu University, Varanasi, India.

Enzyme activity evaluation

Amylolytic activity

The isolates were inoculated in point on nutrient agar supplemented with 1% starch, pH 6.0 as stated by Hankin and Anagnostakis (1975). After

incubation period, culture plates were treated with iodine which allowed to observed clear zone around active colonies.

Proteolytic activity

The casinolytic activity was determined by cultivation endophytes in medium supplemented with 1% milk. Milk was separately autoclaved and added in 1% (v/v) pH 7.0-7.4.The microorganism was inoculated in point on agar medium in Petri dishes and incubated for 28- 48 h, at 37^0C. For active strain, a clear zone of proteolysis around colony was observed. Another method used was to screened the capacity to gelatin hydrolysis by using nutrient agar with 1% bacterial gelatin (gl^{-1}) and acidic $HgCl_2$ solution (HCl 20.0 ml and $HgCl_2$ 15.0gm), clear zone indicated as a positive gelatinase activity.

Esterasic activity

For esterasic activity media was used as suggested by Sierra (1957) which contain (gl^{-1}): peptone 10.0, NaCl 5.0, $CaCl_{2.}2H_20$ 0.1, agar 18.0, pH 7.0. Previously sterilized Tween 80 was added separately after autoclaving in concentration of 1 %(v/v). Inoculation of microorganisms was done and allowed for incubation to observed clear zones around the colonies.

Lipase activity

Isolates were grown on to the medium containing (gl^{-1}): peptone 10, NaCl 5, $CaCl_2$ H_2O 0.1, agar 16, pH 6.0 with 1 %(v/v). Tween 20 separately added after sterilization in .A clear zone formed around the active colony indicates lipase activity (Hankin and Anagnostakis, 1975).

Cellulolytic activity

For microorganisms cultivation was used a medium containing (gl^{-1}): yeast extract 1, peptone 0.5, agar 16, 0.5 Na carboxy methyl cellulose according to method described by Samanta and Sem (1989). After incubation, plates were flooded with 0.2 aqueous congo red and destained with 1M NaCl for 15 minutes. Clear zone surrounding the active colony indicated cellulase activity.

Asparaginase

Isolate was inoculated on to the modified Czapek dox agar medium which phenol red as an indicator and incubated for 2-4 days at 27^{0}C and observed for pinkish red colour zone indicating positive asparaginase activity (Theantana *et al.,* 2007). Enzyme activity = diameter of hydrolysis zone (cm)/ diameter of colony (cm). All chemicals of practical grade were purchased from Himedia Laboratory Ltd., Mumbai.

Table 1. Endophyte isolates of selected seven indigenous medicinal plants

Host plant	Family	Bacteria	Fungi
Azadirachta indica	Meliaceae	3	2
Curcuma longa	Zingiberaceae	1	4
Musa paradiasiaca	Musaceae	4	1
Eucalyptus globules	Myrtaceae	0	4
Pongamia glabra	Leguminosae	4	0
Vinca rosea	Apocynaceae	2	12
Withania somnifera	Solanaceae	0	1
	Total	14	24

Table 2. Enzyme activities of endophytic bacteria isolated from indigenous medicinal plants

Strain code	Host	Tissue*	Enzyme activity*						
			A	C	E	L	CS	G	AS
HB1	*C.longa*	Rz	0.6	0.9	1.0	1	0	0	0
NB1	*E.golobules*	Rt	1.5	1	0	0	3.5	1.2	0
NB2		Rt	2	0	0	0	0	0	0
NB3		Ls	2	0	0	1	0	1.5	0
NB4		Ls	0	0	0.7	2	1	0	0
KB1	*P.glabra*	St	0	0	1	0	0	0	0
KB2		St	0	1.3	0	0	2	0	0
KB3		St	0	1	0	1	2	0	0
KB5		Rt	0	1.5	0	0	0	0	0
L. fermentum	*V.rosea*	Ls	1.5	1.5	0	0	4.5	2.5	0
SB2		Ls	1.0	0	0	0	1	1	0
NMB1	*A.indica*	Ls	0	0	0	0	0	0	0
NMB2		St	0	0	0	0	0	0	1.5
E.coli		St	2.1	2.0	1.9	2	0	0	0

* Rz: Rhizomes, Rt: Roots, Ls: Leaves, St: Stem,
*A:Amylase; C:Cellulase; E:Esterase; L:Lipase; CS:Casinase; G:Gelatinase; AS:Asparaginase
(0) No enzyme activity

Table 3. Enzyme activities by endophytic fungi isolated from indigenous medicinal plants

Fungal strains code	Host	Tissue*	Enzyme activity**						
			A	C	E	L	CS	G	AS
HF2	*C.longa*	Rz	0	0	0	0	1	0.5	0
HF3			0	0	0	1.5	1.7	0	0
HF5			1.4	0	0	1.3	0	0.6	0
HF6			1.0	0	0	0	1.6	0	0
NF1	*E.globules*	Rt	0	0	1	0.5	0	0	0
SF1	*V.rosea*	Ls	1.2	0	0	0.4	0	0	0
SF2		Ls	1.3	0	1.2	0.2	0	0	0
SF12		Rt	1	1	0	0	0	0	0
SF13		Rt	1.1	0	1	0	1.6	0	0
SF14		Rt	1.8	0	0	0	1.8	1	0
SF15		Rt	0	0	0	0	0	2	0.4
M. sterilia		Rt	1	2	1.4	0	0	1.8	0
SF17		Rt	0.4	1	0	0	0	0	0
SF20		St	0	0	0	0.5	0	0	0
SF21		St	0.3	0	0	0	0	0	0
SF24		St	1.5	0	0	0	0	0	0
SF25		St	0	0	0	0	1.8	0	0
BBF1	*M.paradiasa*	Ls	0.5	0	0	0	0	0	0
BBF2		Ls	0.9	0	0	0	0	0	0
BBF3		Ls	1.0	0	0	0	0	0	0
BBF4		Ls	1.4	0	0	0	0	0	0
NMF1	*A.indica*	St	0	0	0	0	0	0	1.5
NMF2		St	0	0	0	0	0	0	2
ASHF2	*W.somnifera*	Rt	0	0	0	1	0.6	0	0

* Rz: Rhizomes, Rt: Roots, Ls: Leaves, St: Stem,
**A:Amylase; C:Cellulase; E:Esterase; L:Lipase; CS:Casinase; G:Gelatinase; AS:Asparaginase
(0) No enzyme activity

Table 4. Qualitative proteolytic activity evaluation by using different culture media

Medium	Zone of hydrolysis of casein (mm)	
	L. fermentum	*Escherichia coli*
Peptone cysteine agar	30	24
Yeast extract peptone agar	22	22
Peptone agar	12	18
Tryptone glucose yeast extract agar	12	14
Yeast extract malt agar	24	20
Tryptone dextrose agar	28	26
Nutrient agar	20	22
Soybean meal yeast extract agar	20	24

Table 5. Quantitative proteolytic activity evaluation of isolates by using different culture media

Medium	Optical density at 280nm	
	L. fermentum	*Escherichia coli*
Peptone cysteine broth	0.325	0.304
Yeast extract peptone broth	0.189	0.169
Peptone broth	0.208	0.108
Tryptone glucose yeast extract broth	0.156	0.126
Yeast extract malt broth	0.134	0.130
Tryptone dextrose broth	0.302	0.298
Nutrient broth	0.104	0.106
Soybean meal yeast extract broth	0.269	0.241

Table 6. Morphological and biochemical characteristics of enzymes active endophytic bacteria selected strain

Morphological characteristics	***L. fermentum***	***E.coli***
Gram stain	Positive	Negative
Capsule staining	Capsulated	Capsulated
Motility	Non motile	Non motile
Cell shape	Bacillus	Bacillus
Endospore forming	Absent	Absent
PHB granules	Absent	Absent
Catalase	Negative	Negative
Urease	Negative	Positive
Biochemical properties		
Indole	Negative	Positive
Methyl red	Negative	Positive
Voges Prosker	Positive	Negative
Citrate utilization	Positive	Negative
H_2S production	Negative	Negative
Sugar fermentation		
Glucose	Positive	Positive
Sucrose	Positive	Positive
Lactose	Positive	Positive
Fructose	Positive	Positive
Mannose	Positive	Positive
Rhamnose	Positive	Positive

Results and discussions

Different parts of seven indigenous plants belonging to various families were screened for isolation of bacterial and fungal endophytes using water agar and potato dextrose agar at specified condition. The result showed that presence of fourteen bacterial and twenty four fungal isolates indicating prevalence of

more fungal isolates in plant tissues under taken for investigation. The bacterial and fungal endophytes isolated from seven indigenous medicinal plants were summarized in Table 1. The root tissues of *Vinca rosea* is rich source of endophytic fungi for various hydrolytic enzyme. Equal number of bacterial endophytes occurred in the *P. glabra* and *M. paradiasiaca*. About thirty nine total endophytic microorganisms were screened for production of extracellular enzymes.

The method of radial diffusion in solid media indicated the activity in qualitative form, directly correlating the diameter of the zone of substrate hydrolysis and colony growth which is practical tool that facilitates and speeds the selection and comparison of the enzymatic production of different isolates. The enzyme biosynthesis capacity of isolated bacterial and fungal endophytes was shown in Tables 2 and 3. The rhizomes of *C. longa*, the roots and the leaves of *E.globules,* stems and roots of *P. glabra,* leaves of *V.rosea,* and leaves and stems of *A.indica,* gave ascending number of positive bacterial isolates. About four positive fungi isolated from rhizomes *C. longa,* single isolate found in the roots of *E. globules* and *W. somnifera,* twelve from leaves, roots and stem of *V. rosea,* four from leaves of *M. paradiasiaca,* two from stem *of A. indica*. Bacterial isolates *Lactobacillus fermentum* NB1 and *Escherichia coli* showed a potent as far as enzyme activity, followed by KB2, NB4, KB3, and SB2. The highest casinolytic and gelatinolytic activity was recorded in bacteria *L. fermentum* from leaves of *Vinca rosea* of values for substrate hydrolysis index of 4.5 and 2.5 respectively, other isolates were less potent. Fungal endophytes *Mycelia sterilia* expressed greater gelatinolytic activity than other isolates. Strains coded NF1and NF2 gave a asparaginase activity and strains coded HF5, SF2 and SF14 showed greater amylolytic activity. In general, the isolated bacterial strains showed greater enzyme activity as compared to fungal isolates. Among extracellular enzyme activities proteolytic activity found to be superior. The positive proteolytic activity using different culture media maximum enzyme activity was found when the *L. fermentum* cultivation which realized in peptone cysteine broth (Table 4). It was found that bacterial isolate *L. fermentum* was able to produce protease enzyme in solid as well as liquid media (Tables 4 &5). Others culture medium where also showed competent to produce high quality of enzyme in liquid and solid media such as tryptone dextrose broth (SHI=28 mm), and soybean meal yeast extract broth (SHI=27mm) by endophytic bacteria strain coded NB1.

Enzymes from microbial sources are now realized importance in agriculture, industry and human health, as they are more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals sources. Fungal enzymes are largely required in food industries,

beverages, confectioneries, textile and leather processing. Also some wood inhabiting fungi serve as a potential source of exoenzymes (Rohrmann and Molitoris, 1993; Raghukumar *et al*., 1994; Raghukumar *et al*., 1999; Pointing *et al.*, 1998; Pointing *et al.,* 1999). The obtained result showed the common phenomenon of association between the microorganisms and plants as stated by McInory and Kloepper (1995), Costa *et al.* (2001) and Hallmann *et al.* (1995). Enzymatic activity of endophytic bacteria isolated from *Jacaranda decurrens* Cham. Carrim *et al*. (2006) was also studied in Brazil. Production of asparaginase by endophytes isolated from Thai medicinal plant was studied by Theantana *et al*. (2007). Studies on isolation and enzymes production potential of endophytic microorganisms from indigenous plants are rare. This is the first report of isolation the endophytic microorganisms of the medicinal plants for their enzyme activities from Jalgaon in India. The highest proteolytic activity was recorded in bacteria *L. fermentum* from leaves of *Vinca rosea*, as per our knowledge, this activity is superior to non endophytic protease positive bacteria. Endophytic bacteria are more potent than fungal isolates as per enzyme activity concerned.

Conclusion

The order of enzyme activities for isolated microorganisms is proteolytic > amylolytic > cellulolytic > esterolytic >lipolytic > asparaginase. Bacterial isolates showed a greater enzyme activity as compared to fungal isolates. The occurrence of endophytes showed more in aerial parts than the underground parts. The diversity of endophytes obtained from healthy plant tissues suggested that an even broader flora of endophytes might be found across diverse plant species. It is clearly understood that endophytes isolated form medicinal plants may be beneficial to the host.

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