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ASPARAGINASE PRODUCTION BY ENDOPHYTIC FUNGI ISOLATED FROM SOME THAI MEDICINAL PLANTS

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ABSTRACT

Asparaginase is an important enzyme used as an anti-cancer compound. Five Thai medicinal plants, *Hiptage benghalensis, Betula alnoides, Adenanthera microsperma, Eupatorium odoratum* and *Houttuynia cordata* were used for fungal isolation by triple surface sterilization method. One hundred and seventeen isolates of endophytic fungi were evaluated for asparaginase production using qualitative and quantitative analyses. Twenty-five isolates demonstrated pink zone around their colonies in the agar plate assays. Fungal isolates from *H. benghalensis* and *E. odoratum* were highly active when tested with the spectrophotometric method. The endophytic fungi with high activity are proposed as a possible asparaginase source to manage cancer cells.

KEYWORDS: endophytic fungi, Thai medicinal plants, asparaginase

1. INTRODUCTION

Asparaginase is an enzyme and it is used as chemotherapeutic agent for a treatment of human cancer and acts as a catalyst in the breakdown of asparagine to aspartic acid and ammonia. Asparagine is a nutritional requirement of both normal and cancer cells. A low level of nonessential amino acid asparagine only affects the viability of abnormal cells; normal cells have enzyme asparagine synthetase for the synthesis of asparagine from aspartic acid, whereas cancer cells have low levels of this enzyme [1]. Asparaginase is produced by a variety of microbial sources including fungi [2-3], yeast [4-5] and bacteria [6-7]. Asparaginase from *Escherichia coli* and *Erwinia* sp. have been used as anti-tumor and anti-leukemia agent. The utilization of asparaginase from the above mentioned sources was initially limited because of its potential toxicity and several side effects [8]. Endophytic fungi from Thai medicinal plants were evaluated for their ability to produce asparaginase.

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2. MATERIALS AND METHODS

2.1 Plant species and sites of collection

Wild medicinal plants; *Hiptage benghalensis*, *Betula alnoides*, *Adenanthera microsperma* and *Houttuynia cordata* were collected from natural forest located at the Queen Sirikit Botanic Garden, Mae-Rim district and *Eupatorium odoratum* from San-Sai district, Chiang Mai province.

2.2 Isolation and culturing of endophytic fungi

The plant materials were washed in running tap water for one hour. Fifty segments of leaves from each plant were cut into 5 mm^2 including a vein (25 samples) and intervein (25 samples), and twenty-five segments of branches were then cut randomly to small pieces (5 mm long). Perennial plants including *H. benghalensis*, *B. alnoides* and *A. microsperma* were used to isolate endophytic fungi from their bark. Twenty five segments (5 mm long) were cut from stem and root of *H. cordata*. All plant materials were treated by triple surface sterilization technique as described by Taylor *et al*. [9]. Each tissue part was placed on malt extract agar [malt extract (20 g/l), rose bengal (0.033 g/l), chloramphenicol (50 mg/l), agar (15 g/l)] [10]. All plates were incubated at 30 ^{o}C until fungal mycelium developed. The hyphal tips were cut and transferred to potato dextrose agar (PDA). Half strength PDA was used for subculture and stock culture.

2.3 Assay for asparaginase

2.3.1 Qualitative analysis

All isolates of endophytic fungi were cultured on PDA for 7 days. The 5 mm disc of mycelium was transferred to the tested agar media. The agar plate assay was routinely used for the screening of asparaginase production. Modified Czapex Dox's (MCD) agar [glucose (2.0 g/l), L-asparagine (10.0 g/l), KH_2PO_4 (1.52 g/l), KCl (0.52 g/l), $MgSO_4.7H_2O$ (0.52 g/l), $CuNO_3.3\ H_2O$ (0.001 g/l), $ZnSO_4.7H_2O$ (0.001 g/l), $FeSO_4.7H_2O$ (0.001 g/l)] supplemented with phenol red (0.009% final concentration) as indicator was used. Control plates were MCD agar without asparagine. All the plates were incubated at 30 ^{o}C. Pink zone radius and colony diameter were measured from positive isolates after incubation for five days.

2.3.2 Quantitative analysis

A 5 mm disc of mycelium from agar plate culture was used as inoculum in each tube. All cultures were incubated at 30°C at 120 rpm for 5 days. The activity of enzyme was determined in MCD culture filtrates by Nesslerization as described by Imada *et al.* [11]. The substrate was prepared in a 0.05M tris (hydroxymethyl)aminomethane (tris-HCl) (pH 7.2), giving final concentration of 0.04 M. The reaction tubes contain 200µl of 0.04M asparagine in 0.05M tris-HCl buffer, 100µl of 0.05M tris-HCl buffer (pH 7.2), 100µl distilled water and 100µl of crude enzyme obtained from the supernatant from which the fungal mycelium was removed by centrifugation. The samples were incubated at 37 ^{o}C for 60 minutes and then stopped with 100µl of 1.5M trichloroacetic acid (TCA). One hundred microlites of mixture was mixed with 750µl distilled water. Then, the Nessler's reagent was added and incubated at 20 ^{o}C for 20 minutes. All reaction mixtures were measured by microplate spectrophotometer reader at 450 nm. One unit of asparaginase is the amount of enzyme which catalysed the formation of 1 µmol of ammonia per min at 37 ^{o}C. The reaction mixture from each isolate was assayed in triplicate.

3. RESULTS AND DISCUSSION

3.1 Isolation and culture of endophytic fungi

Five species of Thai medicinal plants were processed from 2 sites, and 117 fungal isolates were recovered (Table 1).

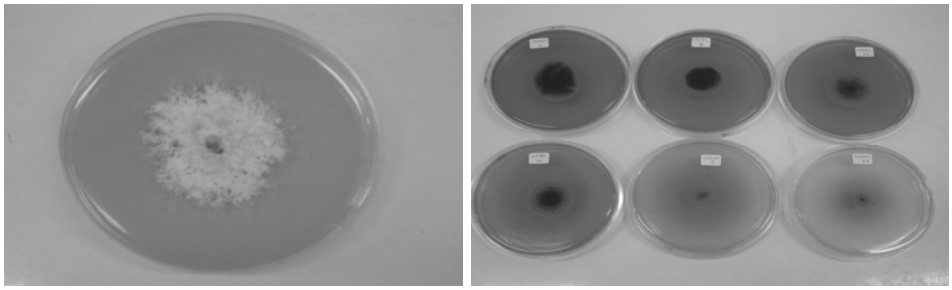
3.2 Assay for asparaginase

3.2.1 Qualitative analysis

All endophytic fungi could grow on MCD agar with phenol red, a dye indicator that changes from yellow (acidic condition) to pink (alkaline condition). The pink zone around fungal colony indicate the pH alteration which originated from ammonia accumulation in the medium (Figure 1). For agar plate assay, forty-one isolates gave positive test; twenty-five isolates demonstrated pink zone around colonies and sixteen isolates demonstrated pink zone within colonies. Isolate H1BA10 from *H. benghalensis* (Figure 2a) and isolates H5BR6 and H5BR7 from *E. odoratum* (Figure 2b) exhibited the large zone redii when compared with their colony diameter. These three isolates are non-sporulating fungi. This method is not complicated and asparaginase production can be examined by plate inspection. It is easy to screen for asparaginase micro-organisms for further evaluation by spectrophotometric method.

Table 1 Number of endophytic fungi isolated from five Thai medicinal plant species on malt extract agar at 30 ^{o}C.

Thai medicinal plant species	Number of endophytic fungi						Total
	Intervein	vein	branch	bark	stem	root	
H. benghalensis	14	7	22	23	-	-	66
B. alnoides	1	2	23	0	-	-	26
A. microsperma	0	0	1	10	-	-	11
E. odoratum	0	0	8	-	-	-	8
H. cordata	0	1	-	-	3	2	6
	15	10	54	33	3	2	**117**



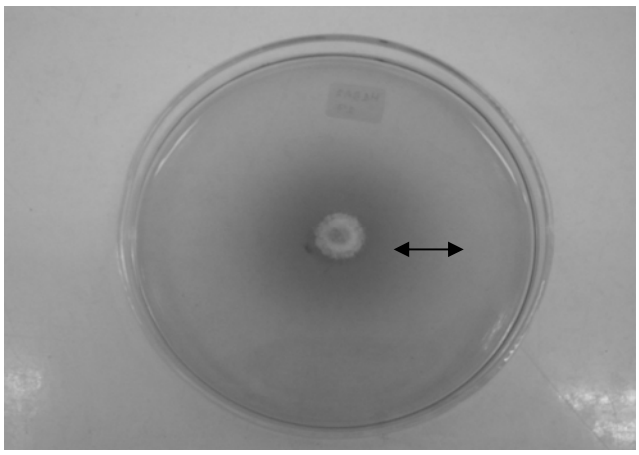
(a) **(b)**

Figure 1 Endophytic fungi on MCD agar. (a) control plate without asparaginase (b) pink zone around positive isolates of endophytic fungi on MCD agar at 30 ^{o}C for 5 days.

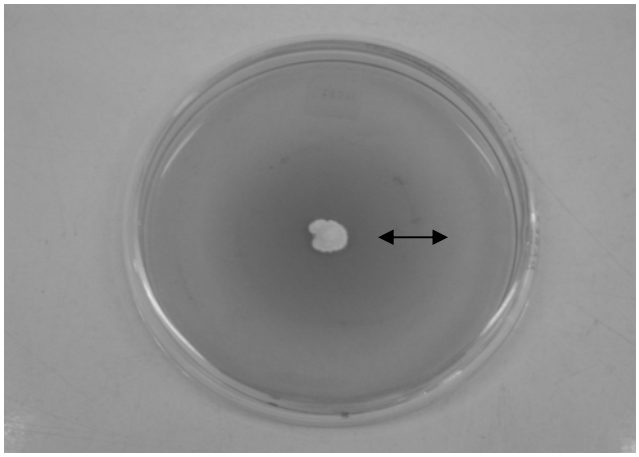
Table 2 Colony diameters, zone radii and asparaginase activities of forty-one positive isolates of endophytic fungi.

Thai medicinal plant Species	Isolated	C olony diameter (cm)	Zone radius (cm)	Enzyme unit (unit/ml)
H. benghalensis	H1IV19	0.8	WC	0.359
	H1BR4 (1)	2.1	0.2	0.014
	H1BR7 (1)	3.5	WC	0
	H1BR7 (2)	3.3	WC	0
	H1BR9 (1)	2.3	0.2	0
	H1BR9 (2)	1.8	0.4	0
	H1BA1	3.1	1.1	0.122
	H1BA2	1.5	0.8	0.014
	H1BA3 (1)	3.1	1.2	0.481
	H1BA5	1.7	0.5	0.086
	H1BA6	3.4	1.2	0
	H1BA8	1.3	0.4	0.194
	H1BA10	1.3	0.8	1.523
	H1BA11 (1)	8.2	WC	0.445
	H1BA12	1.3	0.3	0.050
	H1BA14	1.3	0.4	0
	H1BA16	1.4	0.3	0
	H1BA17	1.1	0.2	0
	H1BA20	2.9	0.9	0
	H1BA21	3.0	1.0	0
	H1BA22	1.1	0.6	0.165
	H1BA24	2.4	0.6	0
B. alnoides	H2V1 (1)	4.9	WC	0.489
	H2BR1 (2)	0.8	0.4	0.022
	H2BR2	2.8	WC	0
	H2BR8 (2)	F	WC	0.047
	H2BR9 (1)	4.0	WC	0.072
	H2BR9 (2)	4.7	WC	0.309
	H2BR9 (3)	4.6	WC	0
	H2BR10	2.5	WC	0.050
	H2BR12 (1)	3.0	WC	0
	H2BR15	2.4	WC	0
H. cordata	H3V1	3.5	0.5	0
	H3PS16	4.7	WC	0
E. odoratum	H5BR5 (1)	1.0	0.3	0.086
	H5BR5 (2)	1.5	0.4	0.093
	H5BR6	1.2	1.0	0.826
	H5BR7	0.9	0.8	1.530
A. microsperma	H6BR2 (6)	2.9	1.1	0.022
	H6BA1	5.0	WC	0
	H6BA2	4.2	WC	0.040

WC = zone of colony



(a)



(b)

Figure 2 The zone (↔) produced by (a) isolate H1BA10 (sterile mycelium) from *H. benghalensis* (b) isolate H5BR6 (sterile mycelium) from *E. odoratum* on MCD agar at 30^{o}C for 5 days.

3.2.2 Quantitative analysis

The fungal isolates that exhibited pink zone around colonies (25 isolates) and pink zone within colonies (16 isolates) were tested for asparginase production in liquid condition by Nesslerization. Twenty-three isolates exhibited asparaginase activity between 0.014 to 1.530 unit/ml (Table 2). Isolates H5BR6, H5BR7 and H1BA10 demonstrated high asparaginase activities of 0.826, 1.530 and 1.523 unit/ml, respectively. When the concordance between agar plate assay and spectrophotometric method was examined, some isolates had no enzyme activity despite the presence of large zone radii. This result was similar to earlier work of Hölker *et al*. [12] and Lee *et al*. [13] who suggested that the ability of fungi to produce enzyme was different in solid and liquid state conditions. For future work, *in vitro* test in 96-wells microplate will be used to observed the sensitivity of cancer and normal cells to asparaginase. Fungal isolate which inhibit the growth of cancer cells properly exhibit low cytotoxicity to normal cells will be selected for further purification of asparaginase.

4. CONCLUSIONS

The one-hundred and seventeen isolates of endophytic fungi were isolated from five wild medicinal plant species. Twenty-five isolates demonstrated pink zones around colonies while sixteen isolates had a zone within their colonies on MCD agar plates. When asparaginase activity in MCD broth was examined, twenty-three isoates exhibited enzyme activity. H1BA10 from *H. benghalensis* and H5BR6 and H5BR7 from *E. odoratum* showed high enzyme activity.

5. ACKNOWLEDGEMENTS

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