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Research article

Asparaginase and amylase activity of thyme endophytic fungi

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Abstract: Asparaginase and amylase are widely used enzymes in various industries, which can be produced by endophytic fungi. In this study, the ability of producing these two enzymes in endophytic fungi isolated from six species of *Thymus* has been reported for the first time in the world. Among 89 isolates of the test, 34 isolates produced asparaginase among which M24 (*Fusarium subglutinans*) displayed the greatest enzyme activity. Thirty three isolates showed the ability to produce amylase while the greatest enzyme activity belonged to M53 (*Curvularia akaii*). This study can be regarded as a preliminary work and endophytic fungi of high activity are proposed as possible resources for control of cancer in humans and for industrial applications.

Keywords: Amylase, Asparaginase, Endophyte, Fungi, *Thymus*

Introduction

Endophytes (fungi or bacteria) are microorganisms which live in higher plant tissues at least in a part of life cycle, without any symptoms, with intracellular or intercellular growth (Pimentel *et al.,* 2011, Jain *et al.,* 2012). The studies carried out show that these microorganisms are rich sources of bioactive compounds (Pimentel *et al*., 2011). Medicinal plants are known to harbor fungal endophytes and it is believed that these microorganisms are related to the production of medicinal drugs (Khan *et al*., 2010) and are able to produce bioactive compounds similar to their hosts (Strobel *et al*., 1996, Caruse *et al*., 2000). Therefore, due to the possibility of production of useful medicinal compounds by endophytic microorganism, they have biotechnological potentials such as antitumor agents (*Pestalotiopsis microspore*, taxol), anti-fungal compounds (*Cryptoriopsis criptocandina*, quercine), plant growth promoting factors, toxins and enzymes (Stierle, *et al.,* 1993, Strobel, 2002, Jain *et al*., 2012) which provide numerous opportunities to discover new products of economic importance. Enzyme production is one of the important abilities of endophytes. During fungal infection, a range of hydrolytic enzymes are excreted which help them to promote host colonization. Depending on ecological niches occupied by each fungus, a set of specific enzymes mostly composed of proteases and carbohydrases, are secreted (Maccheroni *et al*., 2004). Wide range of bacteria, fungi, yeasts, actinomycetes, algae and plants are found to be producers of enzymes. Yet, microbes are regarded as better resources of producing enzymes due to their easer of culturing, extraction and purification of enzymes (Patro *et al*., 2011). Among the widely used and important enzymes, we can

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mention asparaginase which is the first enzyme with antitumor activity studied comprehensively in human diseases. This enzyme is used as a factor in chemotherapy to treat cancer (Theantana *et al*., 2007). After Broome (1961) showed that asparaginase is involved in antitumor activity of guinea-pig serum, it was paid specific attention. Mashburn and Wriston (1964) showed that asparaginase obtained from *Escherichia coli* bacterium has an effect similar to that in guinea-pig serum and is effective against tested tumors. The following clinical assessments revealed the high value of the enzyme for treatment of acute leukemia. This enzyme affects asparagine and hydrolyzes it into L-aspartic acid and ammonia (Shah *et al*., 2010, Kamble *et al.,* 2012). At present, the main source of asparaginase for clinical tests is *E. coli* bacterium (Mazini, 2007). Fungal asparaginase has less adverse effects compared with bacterial asparaginase which brings about allergic reactions such as skin rash, respiratory and breathing difficulty, hypotension, sweating or loss of consciousness (Patro *et al*., 2011).

Amylase is another widely used enzyme in industry, able to break starch molecules, with a wide application in food industry, fermentation and pharmaceutical industry. Therefore, fungal and bacterial amylase can be useful in food and pharmaceutical industries (de Souza *et al*., 2010). The studies on producing fungal amylase, especially in developing countries, are increasing due to ubiquity and simple food needs of these microorganisms (Jain *et al.,* 2012).

Among medicinal plants, *Thymus* is a member of lamiaceae family with a long history in traditional and modern medicine (Naghdibadi and Makkizadeh Tafti, 2003). This plant is disinfectant and has antimicrobial properties (Falahatgar Lysh, 2003) and the presence of endophytic fungi has been verified in it (Fisher *et al.*, 1992, Masumi *et al*., 2015). As mentioned, endophytes are of great ability to produce enzymes and are likely to produce enzymes similar to produced by their hosts. The present research aims to investigate production of asparaginase and amylase enzymes in endophytic fungi of *Thymus* species, carried out in Hamadan Province to obtain microbial sources of these enzymes for industrial applications.

Materials and Methods

Fungal strains and their sources

Ninety five endophytic fungi were isolated from six species of *Thymus, T. eriocallyx, T. lancifolius, T. fallax, T. kotschyanus, T. vulgaris* and *T. daenensis*, in Hamedan province (Western Iran) (Masumi *et al*., 2015). Out of 95 fungal isolates, 6 isolates were identified as yeast; therefore they were excluded from further experiment.

Qualitative assay of asparaginase activity

To investigate the enzyme activity in endophytic fungi, endophyte isolates were cultured on potato dextrose agar (PDA). Then, a five millimeter plug of mycelium was transferred to Modified Czapex Dox (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), $MgSO_4$.7H2O (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)) containing phenol red (0.009 % final concentration), as indicator which turned from yellow (acidic condition) to pink (alkaline condition) due to acidic condition change of medium. Therefore, the pink zone around colony of each isolates showed a change in pH resulted from accumulation of ammonia in cultivation environment and the activity of this enzyme (Theantana *et al.*, 2007).

Control plates were MCD agar without asparagine. The experiment was done in a completely randomized design in three replications and the whole experiment was done twice. The plates were incubated for 5 days at 30 ºC. The enzyme activity was measured by means of the formula presented by Maccheroni *et al*. (2004) as follows:

Potential of enzyme activity = halo diameter of color change / fungal colony diameter.

In this method, the production of asparaginase in Petri dishes can be examined qualitatively which is a simple way of screening microorganisms that produce asparaginase for later assessment by means of spectrophotometry (Theantana *et al.*, 2007).

Qualitative assay of amylase activity

In the present study, endophyte isolates were cultured on PDA. Then, a five millimeter plug of mycelia was transferred to glucose yeast extract peptone (GYP) agar (glucose (1 g/l), yeast extract (0.1 g/l), peptone (0.5 g/l), agar (16 g/l)) along with 2% of soluble starch. The pH of media was set to 6 and the fungus of consideration was cultured on it. The experiment was done in a completely randomized design in three replications and the whole experiment was done twice. After 5 days of incubation at 30 ºC, plates were flooded with iodine solution (0.2% iodine and 0.4% potassium iodide in 100 ml of distilled water). Appearance of yellow areas around the fungal colony in an otherwise purple medium indicated amylase activity (Sun *et al.*, 2011, Suganthi *et al.*, 2011). The activity of this enzyme was assessed by the formula presented in qualitative assay of asparaginase activity.

Results

Qualitative assay of asparaginase activity

34 out of 89 endophyte isolates produced asparaginase. There was a pink zone around colony of 15 isolates showing the enzyme production extracellularly (Fig. 1, a). In 19 isolates the pink color was seen inside the colony indicating intercellular production of enzyme (Fig. 1, b). In control plates, pink color was not observed (Fig. 1, c and d). It was found that there is a significant difference among the isolates at the level of 1% and M24 (*Fusarium subglutinans*) having the greatest enzyme activity (2.73) located in statistical group A, followed by M56 (*Fusarium reticulatum)* with enzyme activity of 2.5 located in the next group (table 1).

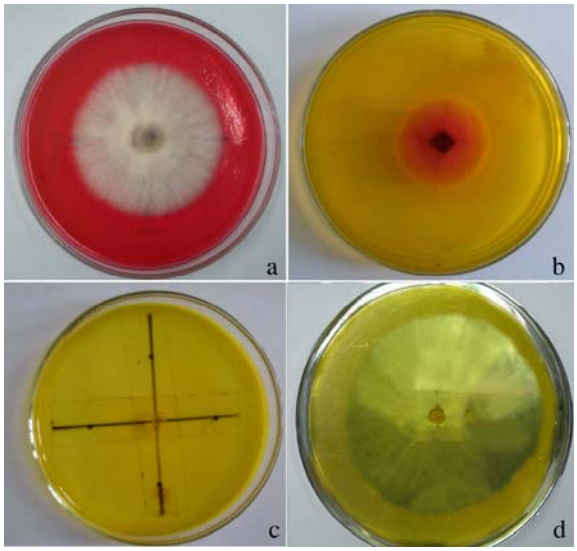

Figure 1 Asparaginase activity in endophytic fungi isolated from *Thymus* species, a) extracellular production of enzyme, b) intracellular production of enzyme, c) control: agar plug, d) control: fungus growth in MCD agar without asparagine.

Qualitative assay of amylase activity

In the present study, we examined the amylase production in endophytic fungi isolated from *Thymus* species. Amylolytic activity was confirmed by means of iodine solution in petri dishes. The starch was not decomposed in free-enzyme plates which remained blue after iodine solution was used (Fig. 2, a), while in enzyme containing plates with starch decomposition, the media turned yellow (Fig. 2, b). Thirty three isolates showed the ability to produce amylase enzyme with a significant difference at 1% level. The greatest enzyme activity belonged to M53 (*Curvularia akaii*) located in statistical group A (Table 2).

Discussion

During the development and relationship between endophytic fungi and host plants, the genetic recombination becomes possible (Tan and Zou, 2001). Medicinal plants can produce compounds with pharmacological properties therefore the endophytic fungi isolated from these plants may also produce bioactive compounds such as the host plants do. This

issue was put forth after the discovery of endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* and it became clear that this fungus can produce anti-cancer drugs of Taxol (Stierle *et al*., 1993, Stierle *et al*., 1995). In the past, the information about endophytic fungi was limited to identification and classification (Theantana *et al.,* 2009). During the recent years, medicinal plants have been studied to investigate the presence of endophytic fungi capable of producing enzymes.

Data of tables 1 and 2 show the mass production of asparaginase and amylase by endophytes isolated from *Thymus* spp. In qualitative assay of asparaginase production, it was shown that all species of *Fusarium* can produce this enzyme as confirmed by Theantana *et al.* (2009), who proposed *Fusarium* as one of the fungi with ability to produce asparaginase enzyme and used it as an anti-cancer agent in their experiments. It is, therefore, suggested that the enzymes produced by some species of this fungus with great potential of producing enzymes could be extracted and purified for use in cancer prevention and pharmaceutical tests.

Table 1 Asparaginase activity in endophytic fungi isolated from *Thymus* species.

Isolates number	Scientific Name of isolates	Scientific Name of host	Enzyme activity (X)[1, 2]	Isolates number	Scientific name of isolates	Scientific name of host	Enzyme activity (X)[1, 2]
M24	*Fusarium subglotinans*	*T. eriocalyx*	2.73a	M53	*Curvularia akaii*	*T. eriocalyx*	1.00i
M56	*Fusarium reticulatum*	*T. eriocalyx*	2.50b	M43	*Curvularia akaii*	*T. eriocalyx*	1.00i
M77	*Fusarium oxysporum*	*T. kotschyanus*	2.03c	M16	*Alternaria sp. 2*	*T. eriocalyx*	1.00i
M61	*Colletothericum* sp.	*T. kotschyanus*	2.01c	M83	*Aspergillus sp.*	*T. fallax*	1.00i
M29	*Fusarium* sp.	*T. kotschyanus*	1.97c	M67	*Fusarium equiseti*	*T. eriocalyx*	1.00i
M30	*Fusarium oxysporum*	*T. kotschyanus*	1.83d	M45	*Alternaria franaseriae*	*T. eriocalyx*	1.00i
M22	*Fusarium oxysporum*	*T. daenensis*	1.71e	M85	*Cladosporium cladosporioides*	*T. lancifolius*	1.00i
M15	*Fusarium javanicum*	*T. fallax*	1.70e	M54	*Phoma pimpinellae*	*T. eriocalyx*	0.89u
M9	*Colletothericum* sp.	*T. kotschyanus*	1.63ef	M13	*Alternaria alternata*	*T. lancifolius*	0.87j
M12	*Fusarium equiseti*	*T. eriocalyx*	1.59f	M8	*Alternaria tangelonis*	*T. kotschyanus*	0.86j
M73	*Fusarium* sp.	*T. eriocalyx*	1.50g	M48	*Phoma valerianae*	*T. eriocalyx*	0.65j
M34	*Fusarium reticulatum*	*T. eriocalyx*	1.41g	M69	*Alternaria alternata*	*T. eriocalyx*	0.61k
M70	*Alternaria tangelonis*	*T. lancifolius*	1.40g	M10	*Alternaria alternata*	*T. fallax*	0.58k
M38	*Fusarium acuminatum*	*T. eriocalyx*	1.27h	M27	*Phoma pereupyrena*	*T. kotschyanus*	0.43l
M89	*Fusarium lateritium*	*T. eriocalyx*	1.24h	M23	*Alternaria franaseriae*	*T. kotschyanus*	0.42l
M63	*Curvularia akaii*	*T. kotschyanus*	1.00i	M72	*Alternaria alternata*	*T. eriocalyx*	0.15m
M31	*Cladosporium cladosporioides*	*T. eriocalyx*	1.00i	Control	-	-	0n
M2	*Alternaria tangelonis*	*T. eriocalyx*	1.00i				

[1] X > 1 indicates extracellular production of enzyme, X ≤ 1 indicates intracellular production of enzyme.
[2] Means followed by the same letters are not significantly different (Duncan's multiple range test at 5% level).

Figure 2 The activity of amylase in endophytic fungi isolated from *Thymus* species a) control, b) production of amylase enzyme in M53.

In the qualitative assay of amylase production, endophytic fungi were known to produce this enzyme. Pandey *et al.* (2000) mentioned that *Aspergillus* species produce a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. In present study *Aspergillus* (M83) produced this enzyme too.

Table 2 Amylase activity in endophytic fungi isolated from *Thymus* species.

Isolates number	Scientific name of isolates	Scientific name of host	Enzyme activity (X)[1, 2]	Isolates number	Scientific name of isolates	Scientific name of host	Enzyme activity (X)[1, 2]
M53	*Curvularia akaii*	*T. eriocalyx*	2.00a	M9	*Colletothericum sp.*	*T. kotschyanus*	1.00b
M83	*Aspergillus* sp.	*T. fallax*	1.00b	M30	*Fusarium oxysporum*	*T. kotschyanus*	0.50c
M64	*infertile mycelium*	*T. daenensis*	1.00b	M1	*Infertile mycelium*	*T. eriocalyx*	0.50c
M72	*Alternaria alternata*	*T. eriocalyx*	1.00b	M71	*Infertile mycelium*	*T. eriocalyx*	0.50c
M10	*Alternaria alternata*	*T. fallax*	1.00b	M77	*Fusarium oxysporum*	*T. kotschyanus*	0.46d
M42	*Phoma* sp.	*T. eriocalyx*	1.00b	M5	*Phoma selaginellicola*	*T. eriocalyx*	0.34e
M43	*Curvularia akaii*	*T. eriocalyx*	1.00b	M36	*Infertile mycelium*	*T. kotschyanus*	0.20f
M52	*Drecheslera tetrarrhenae*	*T. eriocalyx*	1.00b	M60	*Phoma capitulum*	*T. eriocalyx*	0.20f
M31	*Cladosporium cladosporioides*	*T. eriocalyx*	1.00b	M28	*T. kotschyanus*	*Infertile mycelium*	0.17g
M61	*Colletothericum* sp.	*T. kotschyanus*	1.00b	M63	*Curvularia akaii*	*T. kotschyanus*	0.15h
M8	*Alternaria tangelonis*	*T. kotschyanus*	1.00b	M37	*Phoma pimpinellae*	*T. eriocalyx*	0.14i
M45	*Alternaria franaseriae*	*T. eriocalyx*	1.00b	M85	*Cladosporium cladosporioides*	*T. lancifolius*	0.12j
M81	*Alternaria* sp. *1*	*T. lancifolius*	1.00b	M70	*Alternaria tangelonis*	*T. lancifolius*	0.10k
M82	*Alternaria alternata*	*T. eriocalyx*	1.00b	M16	*Alternaria* sp. *2*	*T. eriocalyx*	0.06l
M40	*Alternaria alternata*	*T. eriocalyx*	1.00b	M49	*Ulocladium atrum*	*T. vulgaris*	0.06l
M6	*Alternaria franaseriae*	*T. lancifolius*	1.00b	M7	*Alternaria alternata*	*T. eriocalyx*	0.05m
M25	*Alternaria alternata*	*T. kotschyanus*	1.00b	control	-	-	0n

[1] X > 1 indicates extracellular production of enzyme, X ≤ 1 indicates intracellular production of enzyme.
[2] Means followed by the same letters are not significantly different (Duncan's multiple range test at 5% level).

The precise identification of relationship between endophyte and host can help to obtain suitable methods to isolate endophytic fungi producing bioactive compounds (Hyde and Soytong, 2008). It is worthwhile that the plants in unique environments with pharmaceutical history are considered as promising sources of endophytes producing new bioactive compounds and necessary provisions are carried out to extract and purify these enzymes.

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فعالیت آنزیم‌های آسپاراژیناز و آمیلاز در قارچ‌های اندوفیت جدا شده از آویشن

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چکیده: آسپاراژیناز و آمیلاز از آنزیم‌های مهم و پرکاربرد در صنایع مختلف می‌باشند و توانایی تولید این آنزیم‌ها در قارچ‌های اندوفیت به اثبات رسیده است. در این بررسی برای اولین بار در دنیا توانایی تولید این دو آنزیم در قارچ‌های اندوفیت جداسازی شده از شش گونه از گیاه دارویی آویشن مورد ارزیابی قرار گرفته است. در بین ۸۹ جدایه‌ی مورد آزمایش، ۳۴ جدایه آسپاراژیناز تولید کردند که جدایه M24 (*Fusarium subglutinans*) بیشترین فعالیت آنزیمی را در مقایسه با سایر جدایه‌ها از خود نشان داد. ۳۳ جدایه نیز قابلیت تولید آنزیم آمیلاز را نشان دادند که بین آن‌ها در سطح یک درصد اختلاف معنی‌دار مشاهده شد و بیشترین فعالیت این آنزیم نیز مربوط به جدایه M53 (*Curvularia akaii*) بود. این بررسی به‌عنوان مطالعه‌ای مقدماتی بوده و قارچ‌های اندوفیت با فعالیت بالا به‌عنوان منابع احتمالی برای بررسی کنترل سرطان در بشر و سایر کاربردهای صنعتی پیشنهاد می‌شوند.

واژگان کلیدی: آسپاراژیناز، آمیلاز، اندوفیت، آویشن، قارچ