EVALUATION AND OPTIMIZATION OF PECTINASE PRODUCTION BY ENDOPHYTIC FUNGI ISOLATED FROM THAI ORCHIDS USING AGROWASTE MEDIUM

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ABSTRACT

Currently, there is an increasing demand for new and more potent sources of industrial enzymes. Among various industrial enzymes, fungal pectinase dominates the global enzyme market due to its wide range of applications in bioindustries. Thai orchids have been shown to be a valuable source of bioactive compounds, including various enzymes. Therefore, endophytic fungi were isolated from 11 different species of epiphytic Thai orchids and screened for pectinase production and the ability of the selected fungal endophytes to produce pectinase enzyme on different agrowaste substrates. In total, 83 endophytic isolates were obtained from the orchid samples, with most (58%) from leaf segments followed by the stem (42%). All the endophytic isolates from orchids were assessed for their ability to produce pectinase enzyme based on an enzymatic test, which resulted in 29% of the isolates exhibiting pectinase. Out of the 83 endophytic fungi, the highest production of pectinase enzyme was from DaS01, GsS01, DcS05 and DcrS02, which were obtained from the stems of *Dendrobium aphyllum*, *Grammatophyllum speciosum*, *Dendrobium cuspidatum* and *Dendrobium cruentum*, respectively. The selected endophytic fungi DaS01 and GsS01 were identified as *Aspergillus niger* and *Aspergillus unguis*, respectively, and the other two isolates (DcS 05 and DcrS 02) were both classified as *Xylaria feejeensis* using a molecular technique combined with morphological characters. All selected fungal endophytes were evaluated for pectinase production in substrates containing different types of agricultural waste (soybean meal, sawdust, pineapple peel, orange peel or lemon peel). The highest pectinase activity was produced by *Aspergillus niger*. Among the various sole carbon sources, the soybean meal was most suitable for pectinase production (2.412±0.131 IU/mL). The optimum conditions for *Aspergillus niger* producing the highest pectinase activity (3.424±0.02 IU/mL) were 72 h incubation at 25°C with an initial pH of 3. As a result, soybean meal could be considered as a cost-effective and easily available source providing an ecofriendly procedure for hyper production of pectinase that could be utilized in different industrial applications.

Key words: agrowaste, endophytic fungi, orchids, pectinase, soybean meal

INTRODUCTION

Endophytic fungi have been proven to be a rich source of bioactive compounds which have important agricultural, medicinal and industrial potential. They can produce antimicrobial, antiviral and antioxidant substances or compounds that can be utilized industrially such as solvents and enzymes (Pavithra *et al.*2012). Currently, pectinase is one of the upcoming enzymes in the commercial sector. It has been reported that pectinase from microbial sources contributes almost 25% of the global trade in

food enzymes (Oumer and Abate 2018). The estimated value of sales of all industrial enzymes in 1995 was $1 billion, of which some $75 million was assessed for pectinases. By 2005, the whole world market for industrial enzymes is expected to be $1. 7±2 billion (Godfrey and West 1996). Additionally, pectinase enzyme comprise a well-established global market projected to reach USD 6.3 billion in 2021 (Sudeep et al. 2020). Pectinase has gained substantial worldwide applications in the food and textile industries, vegetable oil extraction and alcoholic beverage processing (Kumar et al. 2011). Pectinolytic enzyme can also be applied for a specific process such as wastewater treatment, plant tissue maceration, extraction and clarification of juice, degumming of plant webbers, and in coffee and tea fermentations **(**Bhardwaj et al. 2017). The widespread production of this enzyme uses microorganisms such as fungi, bacteria and protozoans. To meet the demand for pectinase and to ensure the benefits of this enzyme for applications in different industrial sectors, several researchers are discovering new sources of industrially useful enzymes.

In addition to being beautiful ornamental plants, orchids like *Dendrobium devonianum* and *Dendrobium thyrsiflorum* harbor a diverse range of endophytic fungi which in turn are a valuable source of bioactive compounds. Fungi and fungal endophytes exhibited antipathogenic activity against phytopathogenic bacteria and fungi (Xing et al. 2011). The endophytic fungi associated with some orchids such as *Cymbidium aloifolium*, *Doritis pulcherrima*, *Dendrobiuma phyllum*, *Dendrobium anosmum, Ascocentrum curvifolium* and *Aerides falcate* have potential to secrete extracellular enzymes, including cellulases, pectinase and lipase (Suryanarayanan et al. 2012; Shubha and Srinivas 2017; Sopalun and Iamtham, 2020). Hence, endophytic fungi from orchids may also represent a new and potential source for obtaining and expanding industrial enzyme production. The production of industrial enzymes by endophytic fungi is greatly affected by the composition of the growth medium as well as the fermentation conditions such as the incubation time, temperature and pH. Therefore, the optimization of culture conditions is very important in obtaining optimum fungal growth and maximum enzyme yield.

The demand for pectinase is increasing but application is limited due to the high cost of enzyme production. Consequently, to meet the growing industrial demands for pectinase, it is most important to explore alternative, cost-effective substrates for pectinase biosynthesis to reduce the costs of enzyme production. Thailand is an agricultural country which produces a large amount of agro waste such as bagasse fiber, rice husk and fruits peels, for which there are disposal problems. The application of agro wastes in bioprocesses has aroused great interest among researchers lately. These residues such as sugarcane bagasse, wheat bran, corn cob and straw, rice straw and husk, soy bran, barley and coffee husk are commonly used as an alternate source for the production of different products like biogas, biofuel, mushroom, and tempeh as the raw material in various researches and industries. In addition, the utilization of such materials as substrates for microbial cultivation intended to produce enzymes, vitamins, antioxidants, animal feed, antibiotics, and other chemicals through solid state fermentation (SSF) has been reported (Sadh et al. 2018). These agricultural wastes can be used more efficiently as a source for biosynthesis of pectinase enzymes by endophytic fungi.

Utilization of these agro wastes as a carbon source is especially applicable due to their low cost and easy availability as well as being renewable and their use minimizes the amount of wastes via an ecofriendly process. All agricultural wastes used in the present study were low cost and easily accessible while also available in abundant quantities throughout the year which allows for their use as substrates for microbial bioconversion of detrimental wastes into a value-added by product such as enzymes due to the provision of some essential nutrients in the agro waste. Without effective and efficient waste management steps, the accumulation of these wastes can result in human health hazards and detrimental pollution to the environment. Discharge of agriculture wastes without any treatments is one of the major sources of air, land resources and especially in water pollution. There are components of agriculture wastes that can be detrimental to humans and animals such as nitrogen, human and animal pathogens, certain heavy metals which can accumulate in lake or river. (Haseena et al. 2017). This study

aimed to transform waste into wealth through the utilization of agro-waste residues to produce profitable pectinase as a practical step to address this problem. Most pectinase enzyme is produced using fermentation from *Aspergillus niger* (Ezike et al. 2014) with less work having been done on pectinase production from submerged fermentation of agro waste using orchid endophytes. The objective of the present research focused on the isolation of endophytic fungi from the leaves and stems of some Thai orchids and assessed the production of pectinase enzyme and once the potential sources was identified, this present study also focused on different types of agricultural wastes and their utilization in the production of pectinase. . The selected endophytic fungi which contained the highest amounts of pectinase were characterized using morphological and molecular characteristics and they were evaluated for optimal cultivation based on the incubation period, pH and temperature for pectinase production under submerged fermentation using various agricultural wastes.

MATERIALS AND METHODS

Collection of orchid samples. Eleven species of orchid (*Ascocentrum miniatum*, *Cymbidium finlaysonianum, Dendrobium aphyllum, Dendrobium cuspidatum, Dendrobium cruentum, Dendrobium farmeri* Paxton, *Dendrobium lindleyi, Dendrobium secundum, Flickingeria ritaeana, Grammatophyllum speciosum* and *Staurochilus fasciatus)* were collected from Pa Khlok Sub-District, Tha Lang District, Phuket Province, Thailand. Each species was morphologically identified following Orchids of Thailand (Thaithong 1999). The healthy leaves, stems and branches of the orchids were sampled and then washed using tap water for 10-15 min and the samples left to air dry. All plant samples were cut using a sterile surgical knife into small fragments of 4 x 4 cm for further study.

Endophyte isolation. Fungal endophytes were isolated using the protocol described by Sunitha et al. (2013) Plant part samples were immersed in 75% ethanol for 30 s, followed by 5% NaOCl solution for 30 s, then immersed in 75% ethanol again for 30 s and washed in distilled water three times (Sopalun 2003). The sterile samples were cut into 1 x 1 cm pieces and placed on water agar and incubated at 25°C for 1-7 days until the fungal mycelia started growing out of the plant part samples; then, the single hypha tip was cut and inoculated into potato dextrose agar (PDA, Difco) to obtain the pure isolated colonies. The growth of the fungus was monitored for 3-5 days, then stored at 4°C and -80°C in 15% (v/v) sterile glycerol for further study.

Screened endophytic fungi for pectinase enzyme production. The enzymatic activity of the fungi was determined using the method of Sunitha et al. (2003). Pectinase enzyme activity was assessed by growing the tips of fungal mycelia which were punched aseptically using a sterile cork borer to a diameter of 5 mm and placed on pectin agar medium (pectin 5 g, yeast extract 1g, peptone 0.5 g, agar 15 g, distilled water 1,000 mL and pH 5.0). After 5 days of incubation, the plates with a fungal colony were flooded with 1% hexadecyl trimethylammonium bromide for 15 mins. The appearance of a clear zone surrounding the colony indicated the presence of pectinase enzyme (Fig. 1). The enzymatic index (EI) was recorded as the ratio between the average diameter of the degradation halo and the average diameter of the fungal colony (Hankin and Anagnostakis 1975), which was used to calculate the percentage of the total number of endophytic fungi that could produce this enzyme.

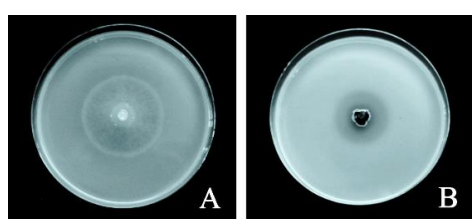

Fig. 1. Production of pectinase enzyme by fungal endophytes on 5-day-old cultures: (A) DfL-06 -01 (negative); and (B) isolate GsS-01 (positive).

Identification of endophytic fungi. Characterization of endophytic fungi was ba sed on the morphological characteristics of the colony and the spores, and on a molecular technique. The isolated fungal mycelia were placed on a glass slide and then stained with lactophenol cotton blue (1% v/v) and examined under a light microscope (Olympus, Tokyo, Japan).The endophytic fungi were identified on the basis of colony color and spore morphology compared with previous descriptions (Klich 2002; Samson et al. 2002). In addition, endophytic fungi were identified using a molecular technique according to the method of White et al. (1990). Mycelia of the fungal endophytes were frozen at -20°C and were scraped and disrupted by grinding to a fine powder using a sterile mortar and pestle. DNA was extracted using the CTAB method (Doyle and Doyle, 1987; Cullings, 1992). The internal transcribed spacer (ITS) regions of the extracted DNA were amplified using the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). Amplification of the ITS region was conducted with 50 µL of polymerase chain reaction (PCR) containing 1 µL DNA templates (10 ng/µL), 5 µL 10X PCR buffer, 5 µL of 25 mM $MgCl_2$, 1 µL 10 mMdNTP, 2 µL 10 µM primer and 0.4 µL 5 U/µL Tag DNA polymerase (Ampli Taq Gold, Perkin-Elmer) PCR was performed in a thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 95 °C for 10 min, 34 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.30 min and final extension at 72 °C for 10 min. The PCR products were analyzed in 1.5% agarose gel in Tris-borate-EDTA buffer, stained with ethidium bromide and visualized under UV light. The PCR products were purified and sequenced by Marcogen™ (Seoul, Korea). The sequences were compared with the sequences available in GenBank via BLAST searches and the Barcode of Life Data (BOLD) Systems.

Pectinolytic activity with different agricultural waste. The endophyte isolates with high pectinase activity were selected to access the pectinase production in different agricultural wastes, consisting of soybean meal, sawdust, pineapple peel, orange peel and lemon peel as the substrates. The agro wastes were obtained from a local market in Kamphaeng Saen district, Nakhon Pathom, Thailand in October 2019. Pectinase production used the submerged fermentation process. The different agro wastes (10 g/L) were added in modified Czapek-Dox agar and 100 mL of each sterile substrate medium was inoculated with mycelial discs (5 mm diameter) that had been punched from five-day-old culture and incubated at 30 °C in an incubator shaker. After 24 h, the culture was harvested using centrifugation at 4,000×*g* for 10 min and the supernatant was used in subsequent steps. The dinitrosalicylic acid (DNS) method was used to quantify the total amount of sugar in the fungal culture supernatants (James 1995).

Enzyme assay. For pectinase assay, 3 mL of each reaction culture was mixed with 1 mL of 3,5-DNS and then kept in a boiling water bath for 5 min. After cooling and adding 5 mL of distilled water, the mixture was measured as a colorimetric readout at 540 nm (Gusakov et al. 2011). Enzyme activity was measured using pectin (Sigma, USA) as the substrate and adding 0.5 mL of crude enzyme sample or standard solution (galacturonic acid) to 0.5 mL of 1% pectin (in 0.05 M sodium acetate buffer, pH 5.5). The mixture was incubated at 50 °C in a water bath for 30 min and the reaction was stopped with 1 mL DNS; then, the mixture was boiled for 10 min and cooled. The enzymatic activity was expressed as the amount of enzyme to liberate 1µmol of D-galacturonic acids per minute milligram of protein under the assay conditions.

Optimization of the cultural conditions for pectinase production. The maximum enzyme production was observed in fermented broth supplemented with soybean meal. Therefore, soybean meal was selected as an appropriate agricultural waste for further study. Enhanced production of pectinase using soybean meal as substrate was investigated for a range of parameters: incubation period (24-120 h), temperature (25-40°C) and pH (3-7).

Statistical analysis. All experiments were performed in triplicate, which were represented in respective graphs with standard deviation error bars and the statistical analysis was performed using the SPSS version 17.0 (SPSS Inc., Chicago, USA) software package. Comparisons were conducted using one-way analysis of variance, followed by Tukey's LSD test with significance set at 0.05.

RESULTS AND DISCUSSION

Endophytic fungal isolated. Endophytic fungi were isolated from 11 species of Thai orchids. In total, 83 fungal endophytes were isolated from the leaves and stems, except for *Ascocentrum miniatum*, where no endophytic fungal isolates were obtained from the stem. A greater number of fungal endophytes were obtained from the leaf tissue (58%) than from the stem tissue (42%) as shown in Table 1. A greater number of the endophytic isolates were obtained from leaves than stems, perhaps because the plant leaves appeared to have fewer infection barriers such as a lignin layer, cuticular wax or outer bark compared to the stem or sapwood (Arnold and Lutzoni 2007). Similarly, Bungtongdee et al. (2018) reported that the highest numbers of endophytic fungi were found in leaves, followed by stems and flowers for 20 orchid samples collected in northern Thailand, because the plant leaves are nutrient-rich and have a greater surface area as well as being thin-walled to allow endophytic fungi distribution. This corroborated Yuan et al. (2009), who reported that greater colonization rates of endophytes were found in foliar tissues of epiphytic orchids than in other organs. Sudheep and Sridhar (2012) also reported that the diversity of non-mycorrhizal endophytic fungi in orchids was higher in leaves than roots, as it is possible that the organ texture provided distinct ecological habitats (air or below ground) with varying physiology and chemistry for the taxa. Thus, the existence of endophytic fungi is influenced by the type of host tissue and environmental parameters (Mangunwardoyo et al. 2012)

Table 1 Isolation of endophytic fungal from Thai wild orchids on PDA medium.

No.	Orchid species	Location		Plant part		Total
		Latitude	Longitude	Leaf	Stem	
1	*Ascocentrum miniatum*	8.05294	98.40657	5	-	5
2	*Cymbidium finlaysonianum*.	8.04101	98.39440	5	3	8
3	*Dendrobium aphyllum*	8.05173	98.40750	5	4	9
4	*Dendrobium cuspidatum*	8.05278	98.40731	4	5	9
5	*Dendrobium cruentum*	8.04083	98.40783	3	2	5
6	*Dendrobium farmeri*	8.05248	98.40652	5	4	9
7	*Dendrobium lindleyi*	8.05186	98.40733	4	5	9
8	*Dendrobium secundum*	8.03906	98.39153	5	3	8
9	*Flickingeria ritaeana*	8.03933	98.39130	4	4	8
10	*Grammatophyllum speciosum*	8.04122	98.40836	3	2	5
11	*Staurochilus fasciatus*	8.03659	98.38541	5	3	8
	Total (isolates)			48	35	83

Screening of endophytic fungi for pectinase enzyme production. All 83 fungal isolates were tested and screened for pectinase enzyme production and 24 (29%) of the endophytic fungi could produce pectinase enzyme (Table 2). The fungal endophytes, GsS01, DaS01, DcS05 and DcrS02, which were isolated from stems of *Grammatophyllum speciosum Dendrobium aphyllum, , Dendrobium cuspidatum* and *Dendrobium cruentum*, respectively, had the highest production of pectinase enzyme with enzymatic index (EI) values ranging from 1.21 to 2.77. Therefore, those isolates were chosen for characterization and further study.

Table 2. Pectinase activity (enzymatic index)*of endophytic fungi isolated from Thai orchids**.**

Isolate code	Pectinase	Isolate code	Pectinase
CfL-03	1.07±0.00^{b}	**DfS-03**	1.07±0.01^{b}
DaL-03	1.15±0.01^{b}	**DlL-01**	1.04±0.00^{b}
DaL-05	1.07±0.00^{b}	**DlS-02**	1.09±0.00^{b}
DaS-01	1.84±0.29^{c}	**DlS-06**	1.11±0.02^{b}
DaS-02	1.04±0.00^{b}	**DlS-07**	1.07±0.00^{b}
DaS-03	1.05±0.00^{b}	**DsL-03**	1.15±0.01^{b}
DaS-04	1.04±0.00^{b}	**DsL-04**	1.05±0.01^{b}
DcL-02	1.05±0.00^{b}	**FrS-02**	1.06±0.01^{b}
DcS-01	1.05±0.00^{b}	**GsS-01**	2.77±0.03^{d}
DcS-05	1.24±0.03^{b}	**SfL-01**	1.21±0.05^{b}
DcrS-02	1.21±0.02^{b}	**SfL-06**	1.05±0.01^{b}
DfL-05	1.12±0.01^{b}	**SfS-03**	1.07±0.02^{b}
No. of isolates producing pectinase (%)	24 Isolates (29 %)		

Cf, *Cymbidium finlaysonianum* Lindl.; Da, *Dendrobium aphyllum*; Dc, *Dendrobium cuspidatum*; Dcr, *Dendrobium cruentum*; Df, *Dendrobium farmeri* Paxton; Dl, *Dendrobium lindleyi*; Ds, *Dendrobium secundum*; Fr, *Flickingeria ritaeana*; Gs, *Grammatophyllum speciosum*; and Sf, *Staurochilus fasciatus*. L, Leaf; S, Stem; and R, Root

*The enzymatic index was measured using degradation halo diameter/fungal colony diameter. Results represent means of three replicates for each isolate ± SD. Values not sharing a common lowercase superscript differ significantly at $P<0.05$ (Tukey's test).

In the current study, the pectinase enzyme was produced by 29% of the endophytic fungi and the enzymatic (EI) index was 2.77 for *Aspergillus unguis* (GsS-01). If the EI is greater than 1, the production of the enzyme of interest is better (Fungaro and Maccheroni 2002). The present findings are consistent with the findings of Sopalun and Iamtham (2020) who reported substantial pectinase production (EI = 1.5) by endophytic fungi of *Dendrobium aphyllum*. Other previous studies showed lower pectinase enzyme production levels than those obtained in the present study (Shishupala 2014; Elkhateeb et al. 2019; Shubha and Srinivas 2017).

Identification of fungal endophyte. The four selected isolates were identified based on their morphological characters and molecular analysis of the ITS regions (ITS1, 5.8S, ITS2) (Table 3). DaS01 could produce spores while the remaining isolates did not produce any spores, although various methods to promote sporulation in culture were used (Fig. 2). DaS01 was morphologically identified as *Aspergillus* sp. based on black colony, biseriate conidial heads and small conidia (2.9-2-9 µm) according to Klich, 2002, and this was confirmed to be *Aspergillus niger* using molecular analysis of the ITS regions, with 89% (Bold systems) 99% (Gen Bank) identity.

A colony of GsS01 was first white in color, becoming greenish in the center and ultimately dark brown and turning black, while both colonies DcS05 and DcrS02 were white in color, with dense aerial mycelia, irregular colony margins plumose, undulate and stromata were finger-like. The isolates GsS01, DcS05 and DcrS02 did not sporulate on the media; since conventional classification of fungi depends on the reproductive structure, these isolates were classified using molecular analysis of the ITS regions. The ITS rDNA sequences of the isolate GsS01 were identified as *Aspergillus unguis*, while isolates DcS05 and DcrS02 were classified as *Xylaria feejeensis*, with 99% identity by comparison with the sequences available in GenBank using BLAST searches and Bold Systems (Table 3).

Table 3. Morphological characteristics and molecular analysis of isolated endophytes.

Isolate	Characteristics (PDA medium, 5 d)	Fungi	% Identity		Accession number in GenBank
			Bold Systems	Gen Bank	
DaS01	- Colony color circular, yellowish initially and then becomes brownish and turns to black - Colony diameter 25-30 mm - Conidiophore slightly granular and colorless - Conidia biseriate conidial heads and small conidia shape globose to subglobose, smooth-walled, uninucleate	*Aspergillus niger*	89	99.26	F318957.1
GsS01	- Colony primary stage-white, mature stage becomes greenish and turns to brownish and black - Colony diameter 20-25 mm - Conidiophore absent - Conidia shape absent	*Aspergillus unguis*	99	99.63	AY373872
DcS05	- Colony white, dense, irregular colony margins plumose, undulate, stromata are finger-like - Colony diameter 15-20 mm - Conidiophore absent - Conidia shape absent	*Xylaria feejeensis*	99	99.62	MH712238
DcrS02	- Colony white, colony margins plumose - Colony diameter 10-15 mm - Conidiophore absent - Conidia shape absent	*Xylaria feejeensis*	99	99.82	KJ767110

Most of the endophytic fungi were not able to sporulate, although various procedures to promote sporulation in culture were tried; therefore, non-sporulating isolates were identified using DNA sequence analyses (Arnold et al. 2000; Guo et al. 2000). Based the ITS rDNA sequences, the isolates DaS01 and GsS01 were related to *Aspergillus niger* and *Aspergillus unguis*, respectively. In another study, endophytic fungi isolated from *Cymbidium aloifolium* (also identified as *Aspergillus japonicus)* had a significant pectinase enzyme index of 1.52± 0.06 (Shubha and Srinivas 2017). In the present study, DcS05 and DcrS02 were classified as *Xylaria feejeensis*. Previous studies have shown that xylariaceous fungi that play an important role in providing enzymes for plant cell wall degradation for litter composition were among the dominant taxa and were associated with all plant organs of epiphytic Orchidaceae plants (Chen et al. 2013; Yuan et al. 2009).

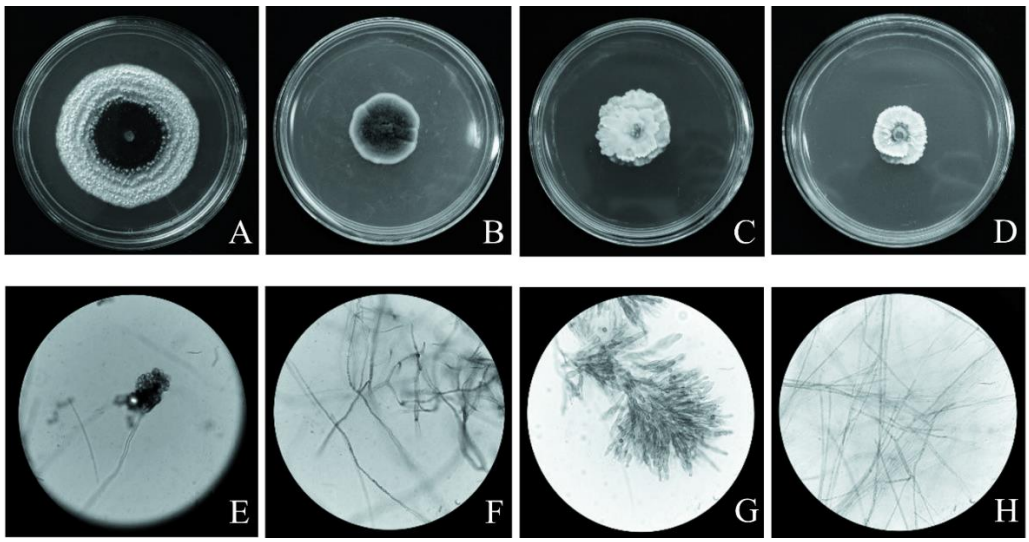

Fig. 2. (A-D) Morphological characteristics of fungal endophyte isolates DaS-01, GsS-01, DcS-05 and DcrS-02 on PDA agar, respectively; (E-H) microscopic examination of fungal endophytes DaS-01, GsS-01, DcS-05 and DcrS-02, respectively, at100× magnification.

Production of pectinase from different agricultural wastes. Soybean meal was the agro waste with the highest pectinase enzyme production (2.4 IU/mL) followed by pineapple peels (1.8 IU/mL) which were significantly different to the other agro waste substrates, suggesting the application of these agro wastes for pectinase production. (Fig. 3). Of the four selected isolates, *Aspergillus niger* produced the significantly highest amount of pectinase in all agro waste substrates compared to the other isolates. Thus, it was used to test the optimization of culture conditions to achieve the highest enzyme activity.

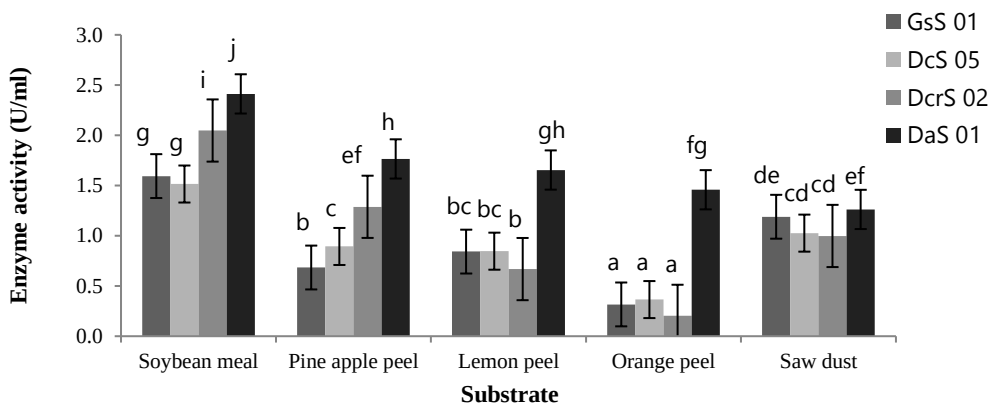

Fig. 3. Pectinase activity with different substrates: DaS 01, *Aspergillus niger*.; DcS 05, *Xylaria feejeensis*; DcrS 02, *Xylaria feejeensis*; and GsS 01, *Aspergillus unguis.* Results represent means of three replicates for each isolate ± SD. Values not sharing a common lowercase superscript differ significantly at $P<0.05$ (Tukey's test).

Pectinase production is expensive. Therefore, the production of pectinase on a large scale uses a submerged fermentation system involving different agricultural wastes as carbon sources. Thailand is an agricultural country, so there are abundant sources of biomass and agricultural wastes left unutilized and these have attracted considerable attention as an alternative carbon and energy source. The current

results revealed that among the five substrates tested, soybean meal had the best pectinase activity with *Aspergillus niger* and was significantly higher than from using other agro wastes. This may be partly due to the presence of the essential nutrients present in soybean meal. Soybean meal (the byproduct after extraction of oil from whole soybeans) is composed of 34.59% cellulose, 18.13% hemicellulose, 9.78% lignin and 43.22 % protein (Vitcosque et al. 2012). Given its protein-rich composition, soybean meal has considerable potential as a substrate for pectinase production by endophytic fungi. These results corroborated Hoa and Hung (2013) who reported that soybean residue was the best substrate for *Aspergillus oryzae*, producing both cellulase and pectinase activity. The combination of wheat bran, corn bran and kinnow peel acted as a good substrate for the production of both cellulase and pectinase by *Aspergillus niger* (Kumar et al. 2011) while wheat bran was the best substrate for *Aspergillus oryzae* CCT3940 to produce pectinase (Malvessi and Silveira 2004).

Optimization of pectinase production from *Aspergillus niger*. Studies on the effect of pH were based on assay of the pectinase enzyme using different media with a pH range from 3 to 7. The results showed that pH 3 was optimum for the production of pectinase; above that (pH5, pH7) there was decrease in the production. An increase in pectinase production was observed at 25°C; above that (30, 35, 40°C) the production decreased. To determine the optimum incubation time for the highest pectinase production *Aspergillus niger* was incubated for up to 120 h. The highest pectinase production was found at 72 h of fungal growth which was significantly different to the other incubation periods Therefore, in submerged state fermentation, the maximum pectinase production was from *Aspergillus niger* at pH 3 and 25°C in soybean meal (3.424±0.02 IU/ml) (Fig. 4 and Fig. 5)

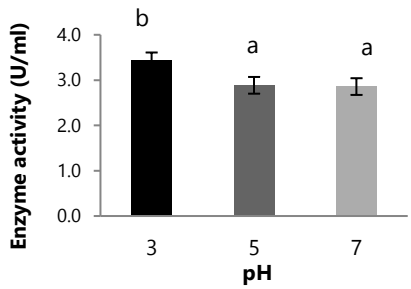

Fig. 4. Effect of pH value on pectinase activity using soybean meal substrate. Results represent means of three replicates for each isolate ± SD. Values not sharing a common lowercase superscript ([a]) differ significantly at $P<0.05$ (Tukey's test).

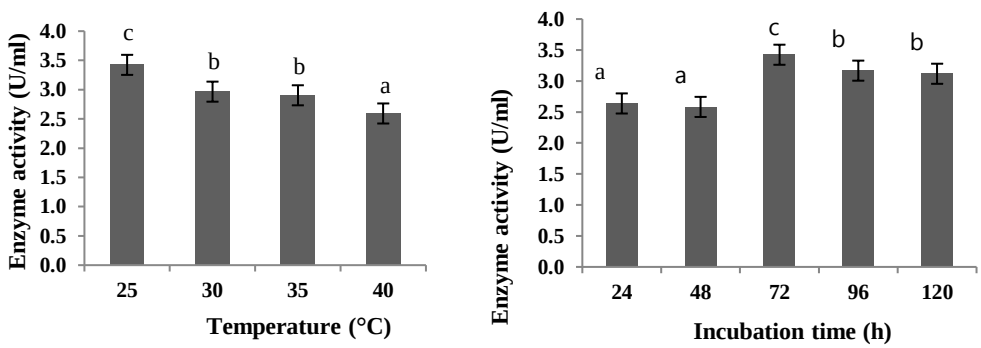

Fig. 5. Effect of incubation temperature and incubation time for pectinase activity using soybean meal as substrate. Results represent means of three replicates for each isolate ± SD. Values not sharing a common lowercase superscript ([a, b, c]) differ significantly at $P<0.05$ (Tukey's test).

To obtain the optimum fungal growth and maximum enzyme production, various culture parameters were investigated. Temperature is an important parameter in fungal growth and enzyme production by increasing kinetic energy which can lead to increased collisions between the enzyme and substrates to form a complex, and thus can increase the end product. Optimization studies revealed that 25°C increased pectinase activity the most, while productivity decreased when the temperature increased above 25°C, which may have been due to enzyme denaturation at the higher temperatures. Thus, 25°C was the optimal temperature for this enzyme activity. In a study by Joshi *et al*. (2011), the highest production of pectinase from apple pomace by *Aspergillus niger* was observed in the temperature range 30-50°C which supported the present finding, as above 50°C, the pectinase activity eventually declined. Similarly, Ezugwu et al. (2012), reported that pectinase activity from mango peels by *Aspergillus fumigatus* increased from 25°C to 40°C, after which the pectinase activity decreased steadily. It has been reported that most fungi examined for pectinase production had an optimum growth temperature in the range 15°C to 35°C (Thangaratham and Manimegalai 2014).

The incubation period is another important parameter that greatly affects fungal growth and enzyme productivity. In the present study, fungal culture was examined using different incubation periods (24, 48, 72, 96 and 120 h) and the maximum pectinase enzyme was produced at 72 h of incubation; beyond that, enzyme productivity decreased gradually, partly due to the depletion of nutrients in the medium. A similar finding was reported by Thangaratham and Manimegalai (2014), where high pectinase production by *Aspergillus flavus* was observed in pineapple residue at 72 h of incubation. Similarly, Abdullah et al. (2014), reported that the highest pectinase production (15.5 U/ml) was obtained from *Aspergillus niger* ABT-5 at 72 h of incubation. Another study reported that 48 h was the optimum incubation time for pectinase production from fermented pineapple residue using a mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii*. Therefore, variation in the incubation period influences the fungal growth rate and the enzyme production pattern.

The pH of the culture also plays a critical role in enzyme production by regulating the growth, and it also reflects the substrate consumption of the fungal culture (Sethi et al. 2016). Optimal pectinase production was found at pH 3. These results were comparable with Dhembare et al. (2015) where production of pectinase by *Aspergillus niger* increased when the pH increased from 3 to 4, but declined beyond pH 5. This finding revealed that a lower pH condition was preferable for the growth and metabolism of *Aspergillus niger*. Likewise, Joshi et al. (2011), reported that the maximum activity of pectinase produced by *Aspergillus niger* in solid state fermentation of apple pomace was at pH 3.5, and above this pH, the pectinase activity declined. It has been reported that the optimal pH for different pectinases varies from 3 to 9 depending on the type of enzyme and the source (Joshi et al. 2011).

CONCLUSIONS

Of the endophytic fungi screened from Thai orchids, 29% were able to produce pectinase. Maximum pectinase activity was observed by *A. niger*, the endophyte derived from the stem of *Dendrobium aphyllum* which was successfully produced from all agro wastes tested; however, soybean meal was found to be the best suited for maximum pectinase production from *A. niger* under the optimum culture conditions of 72 h incubation time at 25°C with an initial pH of 3. The present study revealed that endophytic fungi derived from the orchids have potential to produce pectinase by utilizing different, cheap substrates that can be easily accessible during their harvest season. This study also explores agricultural wastes as a veritable resource for wealth creation by using agro wastes as substrates for the pectinase production via submerged fermentation process which can solve waste disposal problem and also produce less waste water. Among these agro- wastes, soy bean meal not only proved to be the best source for hyper production of pectinase but as its availability is not seasonal, it remains available in large amounts throughout the year, providing a better alternative approach to reduce the environmental pollution caused by the current dumping of such agricultural waste. Therefore, the utilization of soybean meal will increase the production of enzyme while utilizing agro waste for a

beneficial purpose. Furthermore, the method is and eco-friendly for pectinase production on an industrial scale.

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