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L-Asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soils

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Abstract
The actinomycetes (445 strains), isolated from 16 Thai medicinal plant rhizosphere soils were examined for their L-asparaginase activity. It was found that thirty strains showed enzyme activity. The range of enzyme production was 0.03-1.50 µmol ammonia/ml/hour. Strain CMU-H002 isolated from lemongrass (*Cymbopogon citratus*) rhizosphere soil showed highest enzyme activity. Based on the 16S rDNA sequence analysis, this strain was most closely related to *Amycolatopsis kerataniphila* subsp. *kerataniphila* DSM 44409^T (98.6% similarity) and *Amycolatopsis keratiniphila* subsp. *nogabecina* DMS 44586^T (98.7% similarity). The L-asparaginase activity was maximums (3.05 µmol ammonia/ml/hour) when strain CMU-H002 was cultivated in asparagine dextrose salts broth amended with soluble starch (0.2%) and yeast extract (1.5%), pH 7.0 and incubated at 30°C with shaking at 125 rpm for 7 days.

Keywords: actinomycetes, L-asparaginase activity, medicinal plant rhizosphere soil, *Amycolatopsis* CMU-H002.

INTRODUCTION

Enzymes produced by microorganisms have been used in various industries in the world. Microbial L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia (Gallagher *et al.*, 1989; Verma *et al.*, 2007). The production of L-asparaginase has been studied in *Serratia marcescens (*Khan *et al.*, 1970), *Erwinia carotovora* (Maladkar *et al.*, 1993), *Enterobacter aerogenes* (Mukherjee *et al.*, 2000), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Bacillus subtilis* (Fisher and Wray, 2002) and *Saccharomyces cerevisiae* (Ferrara *et al.*, 2006). Actinomycetes have been shown to be a good source for L-asparaginase too. Various actinomycetes, especially those isolated from soils such as *Streptomyces griseus, S. karnatakensis, S. albidoflavus* and *Nocardia* sp. have abilities to produce this enzyme (DeJong 1972; Narayana *et al.*, 2007; Mostafa and Salama, 1979). The roots and rhizomes of some Thai medicinal plants such as lemongrass (*Cymbopogon citratus*), ginger *(Zingiber officinale*) have long been used in Thai traditional medicine for stomachache and asthma treatment (Wutthithamavet 1997). Rhizosphere soil of these plants may be an attractive source of actinomycetes, capable of producing novel secondary metabolites.

The aims of this study was to investigate L-asparaginase production of actinomycetes isolated from Thai medicinal plant rhizosphere soils and to study the optimization of L-asparaginase production by an active isolate, *Amycolatopsis* CMU-H002.

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MATERIALS AND METHODS

Isolation of actinomycetes

Soil samples were collected from the rhizosphere of sixteen medicinal plants in Lumphun Province, Thailand during 2003-2006. The actinomycetes were isolated by plating serially diluted samples on to humic acid vitamin (HV) agar, pH 7.0 supplemented with 100 µg /ml nystatin, 100 µg /ml cycloheximide and 50 µg /ml nalidixic acid (Taechowisan *et al.*, 2003) and incubated at 30°C for 4 weeks. Individual colonies were re-grown on the International *Streptomyces* Project

medium 2 (ISP-2) agar (Shirling and Gottlieb, 1966) at 28°C for obtaining pure cultures. The isolated colonies were sub-cultured onto Hickey-Tresner (HT) slants and kept in 20% glycerol at -20ºC as stock cultures.

Morphological identification and chemotaxonomic analysis

The pure culture isolates were identified to genus level. Spore chain and spore surface ornamentation were examined by light and electron microscope (Tresner *et al*., 1961). Cell wall diamino pimelic acid (A_2pm) and sugar isomer were analyzed as described by Hasegawa *et al.* (1983). Biochemical characterization such as pigment production, starch hydrolysis, nitrate reduction and gelatin hydrolysis were studied.

Screening for L-asparaginase production

All actinomycete strains were evaluated for their ability to produce L-asparaginase. Actinomycete discs (8 mm), grown on yeast malt extract (YM) agar incubated at 28°C for 5 days, were inoculated on asparagine dextrose salts (ADS) agar (Saxena and Sinha, 1981), pH was adjusted to 7.0 and supplemented with phenol red (0.009% final concentration) and incubated at 28°C for 7 days. Colonies with pink zones were considered as L-asparaginase-producing strains.

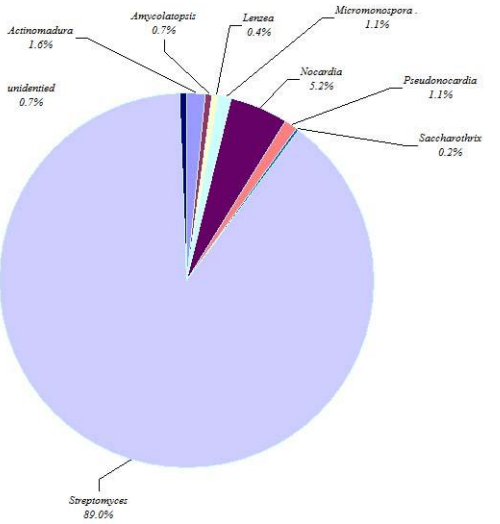

Figure 1: Occurrence and distribution of actinomycete isolates from 16 medicinal plant rhizosphere soils.

Determination of L-asparaginase activity

The active strains were cultured on ADS broth, pH 7.0 and incubated at 28°C with shaking at 125 rpm for 7 days. The growth of actinomycetes was measured and expressed as dry weight. L-asparaginase activity was measured following method of Imada *et al.* (1973). The cultures were centrifuged at 11,000 x g for 15 min. This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml supernatant into 0.5 ml 0.04 M L-asparagine and 0.5 ml 0.05 M tris (hydroxymethyl) aminomethane (tris–HCl) buffer, pH 7.2 and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined colorimetrically by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min, and absorbance of the supernatant was read using a UV–visible spectrophotometer at wavelength of 450 nm. One unit of asparaginase is the amount of enzyme which catalyzed the formation of 1 µmol of ammonia per min at 37°C. The reaction mixture from each strain was assayed in triplicate.

Effect of incubation time, pH, temperature, carbon and nitrogen sources on L-asparaginase production by actinomycete strain CMU-H002

The strain CMU-H002, which showed highest activity, was studied optimal condition for L-asparaginase production. Enzyme activity was measured at different parameters by taking one parameter at one time.

Effect of incubation time

Strain CMU-H002 was grown in YM agar and incubated at 30°C for 5 days. Discs (8 mm diameter) were taken from the test strain and cultivated in 50 ml ADS broth, pH 7.0 and incubated at 30ºC with shaking at 125 rpm for 10 days. A sample was withdrawn every 24 h and L-asparaginase activities were measured.

Effect of pH

Strain CMU-H002 was cultivated in 50 ml ADS broth in different pH level ranging from 6.0- 9.0 at 30ºC with shaking at 125 rpm for 7 days.

Effect of temperature

Strain CMU-H002 was cultivated in 50 ml ADS broth and incubated at various temperatures (10, 25, 30, 37 and 45ºC) with shaking at 125 rpm for 7 days.

Effect of carbon sources

The effect of different carbon sources on cell growth and L-asparaginase production was studied in ADS broth. Carbon compounds (0.2%), including glucose, lactose, fructose, sucrose and soluble starch, were added to ADS medium supplemented with L-asparagine (1%) as nitrogen source. The strain CMU-H002 was inoculated in the medium and incubated at 30°C with shaking at 125 rpm for 7 days.

Effect of nitrogen sources

The effect of different concentrations of nitrogen sources was studied by adding a nitrogen source, including asparagine, glutamine, proline and yeast extract, to ADS medium containing soluble starch (0.2%) as carbon source.

Molecular identification

Genomic DNA of strain CMU-H002 was prepared according to a modified CTAB method (Murray and Thompson, 1980). PCR amplification of 16S rDNA was carried out with a set of universal primers 27F and 1525R. The PCR products were sequenced using universal primers 27F, MG3F and MG5F in an ABI PRISM DYE Terminator cycle sequencer (1st Base, Malaysia). The nucleotide sequences of the 16S rDNA obtained were subjected to BLAST analysis with the NCBI database. Sequences were aligned with representative actinomycete 16S rDNA sequences and phylogenetic tree was constructed using the Molecular Evolution Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Isolation of actinomycete and L-asparaginase production

From sixteen Thai medicinal plant rhizosphere soils, 445 actinomycete isolates were obtained. The number of actinomycetes isolated from *Curcuma mangga* Valeton and Zijp. rhizosphere were higher than other soils (Table 1 *[Supplementary data]*). According by morphology and chemotaxonomy, about 89% of the isolates were presumed to be in genus *Streptomyces* and 11% were non-*Streptomycetes*, identified to the genera *Actinomadura, Amycolatopsis, Micromonospora, Nocardia, Pseudonocardia, Lentzea* and *Sacharothrix*. Three isolates were unidentified (Fig. 1). *Streptomyces* spp. were present in all rhizosphere soils, regardless of wild or cultivated plant species, suggesting their wide distribution in association with plants in the natural environment. The other actinomycete species were rare and could be isolated from some rhizosphere soils. *Streptomyces* spp. were the dominant actinomycetes isolated from all 16 plant rhizosphere soils, as the same as reported by others (Jayasinghe and Parkinson, 2008; Pandey and Palni, 2007). Previous studies have shown that diversity of actinomycetes in rhizosphere soils is positively correlated to the level of organic matter and depended on the species of plant (Germida *et al.*, 1998). Tewtrakul and Subhadhirasakul (2007) found that the roots of *Curcuma mangga* produce antimicrobial compounds. It is probable that root exudates from this plant might promote the growth of actinomycetes while antimicrobial compounds from root might decrease number of other soil bacteria and fungi, so diversity of actinomycetes from this soil is higher than other soils.

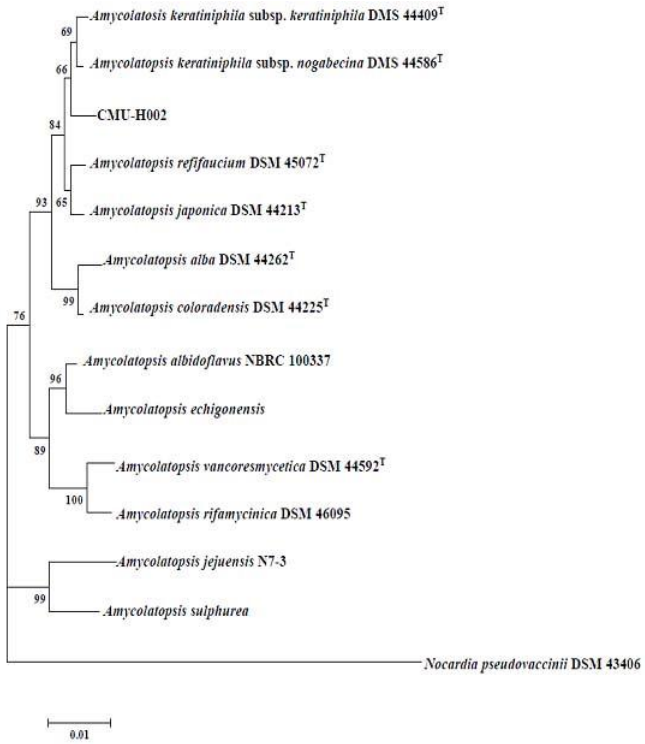

Figure 2: Neighbour-joining tree based on 16S rRNA gene sequences showing the positions of strain CMU-H002 and related strains. The sequence of *Nocardia pseudovaccinii* was used as an outgroup. Bootstrap values were calculated from 1000 re-samplings, and only values greater than 50% are shown. The bar represents 0.01 showed substitution per nucleotide position, only values greater than 50% are shown.

Thirty actinomycete strains showed L-asparaginase activity on ADS agar. All active strains were cultivated in ADS broth. It was found that the range of L-asparaginase production was 0.03-1.50 µmol ammonia/ml/h. Out of 30 strains, *Amycolatopsis* CMU-H002 (accession number FJ455098) isolated from lemongrass (*Cymbopogon citratus*) rhizosphere soil showed highest L-asparaginase activity (Table 2 *[Supplementary data]*). Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain CMU-H002 was grouped into genus *Amycolatopsis* with 66% bootstrap support (Fig. 2). The sequence of this strain was most closely related to *A. keratiniphila* subsp. *keratiniphila* DMS 44409T (98.6% similarity) and *A. keratiniphila* subsp. *nogabecina* DMS 44586T (98.7% similarity). In the rhizosphere soil, root exudates are the natural source of amino acid for rhizosphere microorganisms, which may enhance L-asparaginase biosynthesis. It is possible that amino acids are present in root exudates of *Cymbopogon citratus* and enhance L-asparaginase biosynthesis in strain CMU-H002. Actinomycetes, especially *Streptomyces* spp. from soil, marine sediment and fish gut have been reported to produce L-asparaginase (Koshy *et al.*, 1997; Dhevagi

and Poorani, 2006; Narayana *et al.*, 2007) but no research has been reported about L-asparaginase produced by *Amycolatopsis* spp. isolated from rhizosphere soils.

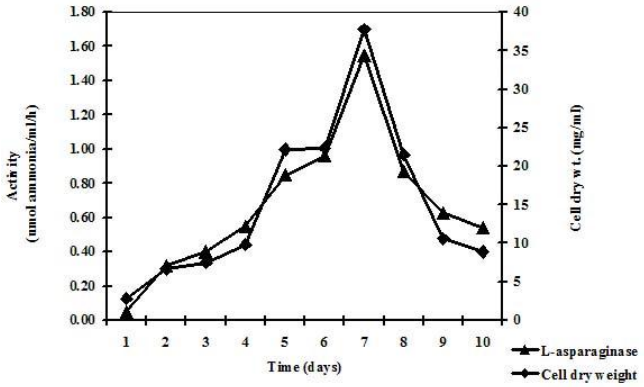

Figure 3: Effect of incubation period on L-asparaginase production by *Amycolatopsis* CMU-H002

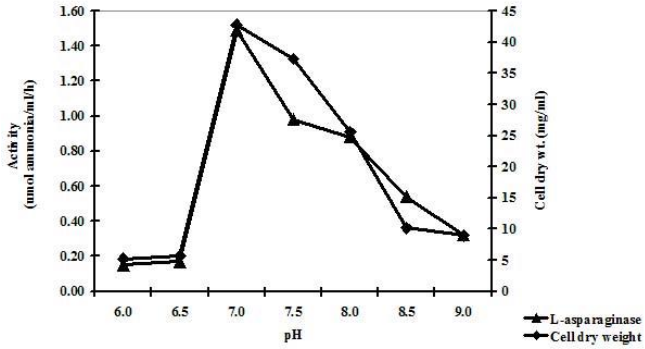

Figure 4: Effect of pH on L-asparaginase production by *Amycolatopsis* CMU-H002

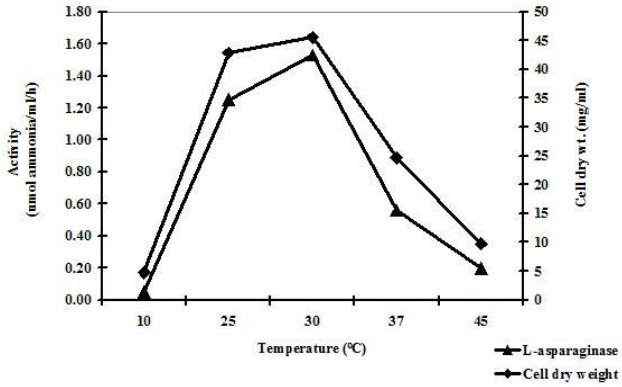

Figure 5: Effect of temperature on L-aspaginase production by *Amycolatopsis* CMU-H002

Effect of incubation time, pH, temperature, carbon and nitrogen sources on L-asparaginase production by *Amycolatopsis* CMU-H002

Production of L-asparaginase started after 24 h and reached a maximum after 178 h (7 days) and then decreased (Fig. 3). Maximum biomass production obtained with 178 h old culture could also be correlated with maximum L-asparaginase production. L-asparaginase formation has been shown to have a firm link to active cell growth (Savitri and Azmi, 2003). The enzyme activity was optimum at pH 7.0 (Fig. 4). Koshy *et al.* (1997) found maximum L-asparaginase activity of *Streptomyces plicatus* at pH 7.0 while Narayana *et al.* (2007) reported maximum L-asparaginase production of *S. albidoflavus* at pH 7.5. It was observed that maximum activity and growth of *Amycolatopsis* CMU-H002 was at 30°C (Fig. 5). Extreme temperature did not favored cell growth or L-asparaginase production in this strain. *S. collinus* produced high amount of L-asparaginase when grown at 28–30°C and *S. albidoflavus* produced maximum amount of enzyme when cultured at 35°C (Mostafa and Salama, 1979; Narayana *et al.*, 2007). The effect of carbon sources on L-asparaginase production by *Amycolatopsis* CMU-H002 is presented in Table 3 *[Supplementary data]*. The L-asparaginase activity and cell growth were highest when strain CMU-H002 was cultivated in ADS broth with 0.2% soluble starch. It is possible that soluble starch enhance growth and L-asparaginase biosynthesis in strain CMU-H002. The final pH of the fermentation broths containing soluble starch, lactose and fructose became alkaline (7.7–8.5) while final pH of the fermentation broths containing sucrose and glucose became acidic (6.5-6.7). The acidic nature of fermentation medium could inhibit L-asparaginase biosynthesis and growth of strain CMU-H002. Geckil *et al.* (2006) reported that glucose was a repressor of asparaginase synthesis due to acid production. Among different nitrogen sources used, strain CMU-H002 showed maximum enzyme activity when cultivated in 1.5% yeast extract and minimum in 1% proline (Table 4 *[Supplementary data]*). Verma *et al.* (2007) reported that yeast extract is important for cell growth and L-asparaginase synthesis, but in high concentration L-asparaginase production was inhibited. In the present study, 3.05 µmol ammonia/ml/h was recorded when *Amycolatopsis* CMU-H002 was cultivated in the ADS broth amended with soluble starch (0.2%) and yeast extract (1.5%), pH 7 and incubated at 30°C with shaking at 125 rpm for 7 days.

CONCLUSION

From this study, it is clearly indicated that medicinal plant rhizosphere soils can provide a rich source of L-asparaginase-producing actinomycetes. *Amycolatopsis* CMU-H002 isolated from lemongrass (*Cymbopogon citratus*) rhizosphere soil has the ability to produce a significant amount of L-asparaginase enzyme. This is the first report about *Amycolatopsis* spp. from rhizosphere soil to produce L-asparaginase. However, more detail investigation is required to characterize this microbial enzyme, which may be effectively used in the

large scale production for commercial and pharmaceutical purposes in the future.

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