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# Fungal Flora of Hot Springs of Taiwan (1): Wu-Rai

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**ABSTRACT :** Five species of 25 isolates of thermophilic and thermotolerant fungi were isolated from hot springs of Wu-Rai of Northern Taiwan and they are identified as following five species: i.e., *Aspergillus fumigatus*, *Thermomyces lanuginosus* (*Humicola lanuginosa*), *Humicola insolens*, *Penicillium dupontii* and *Rhizoctonia sp.* Each species shows special characteristics in physiology and enzymatic activity. In physiological characteristics, all isolates can grow at 55°C and 4 strains of *T. lanuginosus* can grow over 60°C even reach to 64°C. It may be the maximium growth temperature for fungi so far recorded. In primary screening test, 80 % of isolates have amylase and 60% have proteinase activities and 20% have lipase activities. This is the first report to show fungal flora and the rich microbes potential in hot springs of Taiwan.

**KEY WORDS: Taiwan, Hot springs, Fungi, Microbes potential.**

## INTRODUCTION

Fungi which are so called thermophiles (heat-lovers) are those with high optimum temperature for growth, and unable to grow below 20°C (Cooney and Emerson, 1964). Thermotolerant and thermophilic fungi are common in the large, well insulated, dump heaps of plant materials where the waste heat from microbial metabolism becomes trapped, causing the internal temperatures of the heap to rise in a self-heating process. If compost heaps are well made and large enough, temperatures at peak heating can easily reach 60°C and providing ideal conditions for the growth of heat-tolerant fungi. Heat-tolerant fungi have also been found in more exotic warm habitats such as birds' nests (Apinis and Pugh, 1967), coal tips (Evans, 1971), volcanic hot springs (Tansey and Brock, 1971; Hedger, 1975), power plant cooling pipes and effluents (Ellis, 1980) and geothermal soils (Redman *et. al.*, 1999). Until 1997, more than 50 species of thermophilic fungi have been described (Mouchacca, 1997). Heat-tolerant fungi show a whole spectrum of physiological behavior with regard to their enzyme activity such as decompose plant polymers. Consequently thermostable enzymes from those fungi were useful on several fields such as food and chemical industry (Seal and Eggins, 1972).

Thermotolerant and thermophilic fungi have a great potential in application such as bioconversion of lignocellulosic crop and industrial wastes. Besides agricultural and poultry wastes treatments, thermostable enzymes can play an important role in organic synthesis and food production. Not only in agricultural applications, all information from thermophiles will provide scientist the new view of life. Parameters include organelle composition and organization that may yield explantations of unusual effects of temperature extremes (Wildeman and Nazar, 1982). Further study of these interesting isolates from hot springs should be useful in promoting understanding the effects of temperature, especially temperature extremes, on the growth of fungi.

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In general, there is an inverse relationship between biological diversity and the amount of adaption required to survive in a specific habitat (Whitaker, 1975). Thermophiles are no exception to this rule. Monitoring hot springs fungal communities and understanding their responses to environmental changes are prime importance to observe the biodiversity of fungal community at the extreme environment such as hot springs.

Although there are about 128 different hot springs have been discovered in Taiwan island (Chung, 1995), there is no information available for numbers and kinds of thermophiles existed in these hot springs. The objectives of this study were (i) to enhance the isolation efficiency of thermophiles, (ii) to determine the diversity of culturable thermotolerant and/or thermophilic fungal species, (iii) to determine the optimal in vitro growth conditions of these isolates, (iv) to estimate the potential of application of hot springs fungi.

## MATERIALS AND METHODS

### Sampling

Wu-Rai hot springs area located in southeastern vicinity of Taipei, Taiwan (Fig. 1). It composed of eight springs distributed along the bank of the Nai-Shing River. Two hundred milliliter water sample including litter layer sediments were collected from eight individual hot springs sources of this area. After sampling, all samples were treated and screening within 12 hours to preserve microbial activity (Bills, 1995).

### Water analysis

Temperature and pH of sample were determined immediately after collection by a portable pH-meter (AP-10, Denver Instrument Company, USA). Several measurements were determined and a mean value was calculated for each sample.

### Fungal isolation and identification

To determine the efficiency of different isolating methods of hot springs sample, three methods including traditional directed pour-plate method, serial dilution method and alternative membrane filter technique (Mulvany, 1969) were applied in this study.

In directed poured-plated method, 5 ml of sample were mixed with 15 ml sterilized and melted potato dextrose agar (PDA, Difco). In dilution method, sample were diluted to 10 folds by dd. $H_2O$. Five milliliter diluted samples were mixed with 15 ml sterilized and melted PDA and poured into Petri dish. Besides the traditional methods,

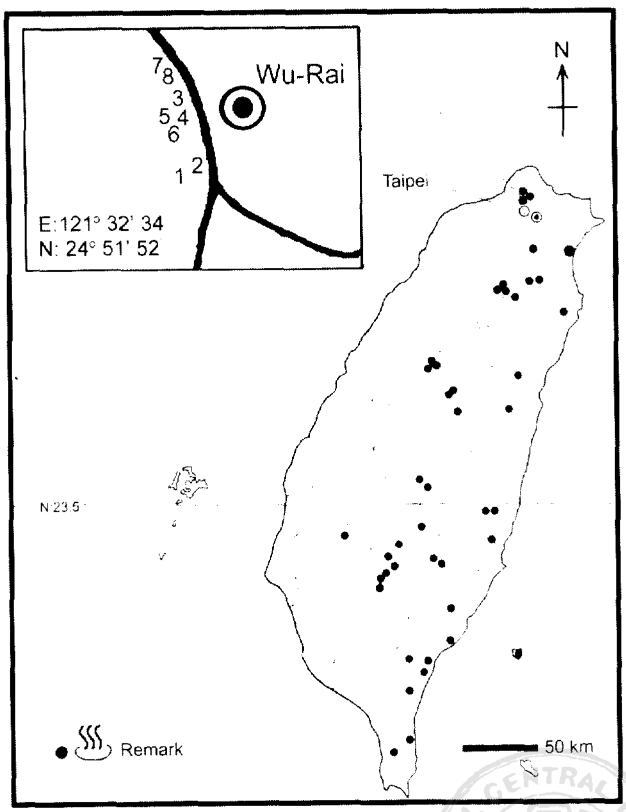


Fig. 1. The Wu-Rai hot springs and distribution of other hot springs of Taiwan. Numbers appeared at the cornor show the related position of sample sites from this study.

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alternative membrane filter technique was applied to concentrate all samples. One hundred milliliter of hot springs sample was filtered through 0.45-$\mu$m PVDF filter membrane (Minipore). Then 20-ml dd. $H_2O$ was used to flush this membrane and finally concentrate all the samples in 5 folds. Five milliliters of concentrated sample with 15 ml sterilized and melted PDA were mixed and poured into a Petri dish. All samples were incubated at 50°C for 72-120 hours and isolates were identified by morphological and physiological characteristics.

**Temperature assay**

All isolates were incubated in PDA between 15-70°C at 10°C interval to obtain their temperature profiles. Growth was measured as growth zone radius. Each temperature interval were measured every 12 hours for 7 days and this experiment was repeated three times.

**Screening of enzymatic activities**

Substrates described below were added into normal growth medium to determine the enzyme activity for the isolates. Proteinase activity was assayed by using 1% skimmed milk as substrate (Fergus, 1969). Amylase activity was determined by the hydrolysis of soluble starch and stained by iodine solution (Frich *et al.*, 1985). One percent emulsified tributyrin were used to determine lipase activity (Jorhi *et al.*, 1985). Mycelia disc with radius of 0.5 cm were put onto the test media and incubated between 20°C to 75°C at 10°C interval for 72 hours. After incubation, diameter of clear zone were measured to show the enzymatic activity.

## RESULTS

**Fungal isolating efficiency**

Eight hot springs samples, with pH range 6.8-7.2 and temperature range 45-82°C, were collected from individual hot springs sources. A total of 25 isolates were isolated from three methods. Three isolates were obtained from directed pour-plate method, only 1 from serial dilution method, and membrane filter technique had 21 isolates (Fig. 2). We monitored and calculated the number of fungal isolates from each method and each sample site. Membrane filter technique that based on concentrating samples showed the highest isolating efficiency among three methods. Not all sample sites were suitable to isolate thermophilic or thermotolerant fungi but over 80% of isolates were from the membrane filter technique.

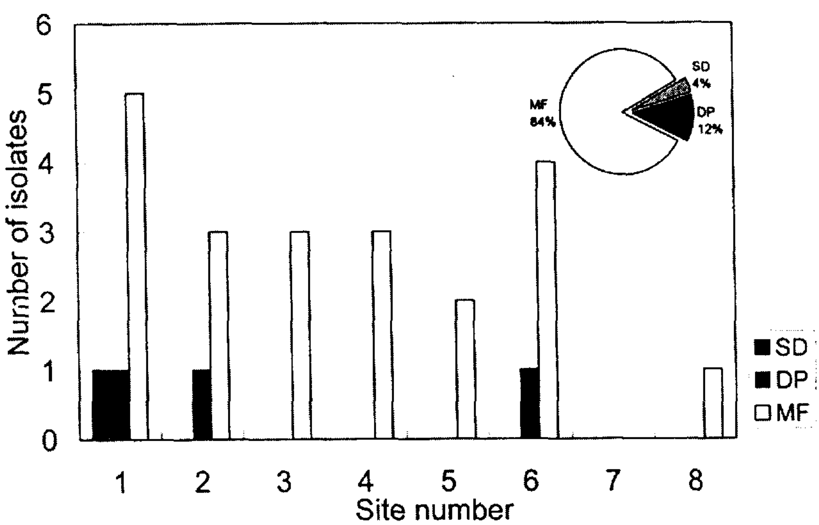


Fig. 2. Numbers of isolates that successfully recovered from each isolating method. DP: direct-pour method, MF: membrane filter technique, SD: serial-dilution method. Pie chart located on the upper right showed the relative numbers of each isolating method.

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**Fungal isolates**

A total of 25 isolates were identified from preliminary screens in solid culture as thermotolerant and thermophilic fungi. According to slide-culture techniques and microscopic obervation (Grams *et al.*, 1987), all isolates were identified by morphological characteristics described by Chen (Chen, 1992), Cooney and Emerson (Cooney and Emerson, 1964). Twenty isolates were identified in four species: *Aspergillus fumigatus*, *Thermomyces lanuginosus*, *Humicola insolens*, *Penicillium dupontii* and one genus: *Rhizoctonia sp*. Characteristics of isolates were listed below:

1. *Aspergillus fumigatus* Fres. (1863). Colonies are Smoky Gray-Green with a Slight Yellow reverse. Old colonies (greater than a month) turn slate gray. Hyphae septate, hyaline. Conidial heads strongly columnar. Conidiophores smooth-walled, uncolored, up to 300 $\mu m$ long but can quite short, vesicles dome-shaped (20-30 $\mu m$ in diameter), uniseriate with closely compacted phialides (4-10 x 2-3 $\mu m$) occuring only on the upper portion of the vesicle. Conidia smooth to finely roughened. Subglobose, 2-3.5 $\mu m$ in diameter. Two different identified temperatures isolates: thermotolerant isolate: E09 (Fig. 3c); thermophilic isolate: E07 (Fig. 3e).

2. *Thermomyces lanuginosus* Tsiklinskaya (1899). The color of colonies changed by spore maturation, giving White, Hydrangea Pink to Red Dark Green. Conidial structure as aleuriospores borne on conidiophores. Conidiophores 2.6-20.5 x 1.3-3.8 $\mu m$, more or less perpendicularly from the vegetative hyphae, unbranched or rarely branched near the base. Slightly curved, swollen at the upper part of conidiophore adjacent to the conidia, giving the conidiophores a flask-shaped appearance, the diameter of swollen part, 1.3-3.8 $\mu m$. Spores born single at the tip of conidiophore, smooth, globose, subglobose, pale green at young stage, turned brown to dark brown when mature and formed thick-walled structure with surface irregularly sculptured as warts, verrucose or irregularly reticulate on the surface, 8-12 $\mu m$ in diameter. Identified isolate: E06 (Fig. 3b).

3. *Humicola insolens* Cooney & Emerson (1982). The color of colonies changed from White to Mouse Gray. Conidiogenous cells are aleuriospores, single or a short chain of 2-3 spores, with stalk or without. Stalk hyaline, unbranched, 1.6-4.2 x 3.4-18.0 $\mu m$. Spores smooth, mostly globose or fusiform (8.0-12 x 11.2-18 $\mu m$), pale yellow at first, becoming brown then turning to dark brown with age. The falling spores posses apiculum on the area of connection with stalk. Identified isolate: E08 (Fig. 3a).

4. *Penicillium dupontii* Griffin & Maugbl (1911). The colonies color changed from White to Pale Green. Conidiophores: Stipes hyaline, smooth, some contain pale yellow fatty bodies, septate, bearing penicilli irregularly at the apex, 10.2-62 x 1.3-3.2 $\mu m$. Penicilli monoverticillate or symmetrical-biverticillate; Phialides 2-4, 1.3-3.2 x 6-10 $\mu M$. Conidia, smooth, elliptical, pale yellowish green, 1.9-3.7 x 2.5-5.6 $\mu m$. Identified isolate: E03 (Fig. 3f).

5. *Rhizoctonia sp.* were identified by morphological characteristics described by Sneh *et al.* (Sneh *et al.*, 1991): Color of mycelium is hyaline to yellow on PDA. Cell of mycelium usually long, with lateral right or 45° angle branches. The septa of branches usually set off from the main hypae with special constriction. Identified isolate: E11 (Fig. 3d).

The other 5 isolates from different sample sites: i. e., sample site No. 4 (1 isolate), No. 5 (3 isolates) and No. 8 (1 isolate) was regarded as unidentified species, since there was no available spores formation and proper morphological characteristics for the identification.

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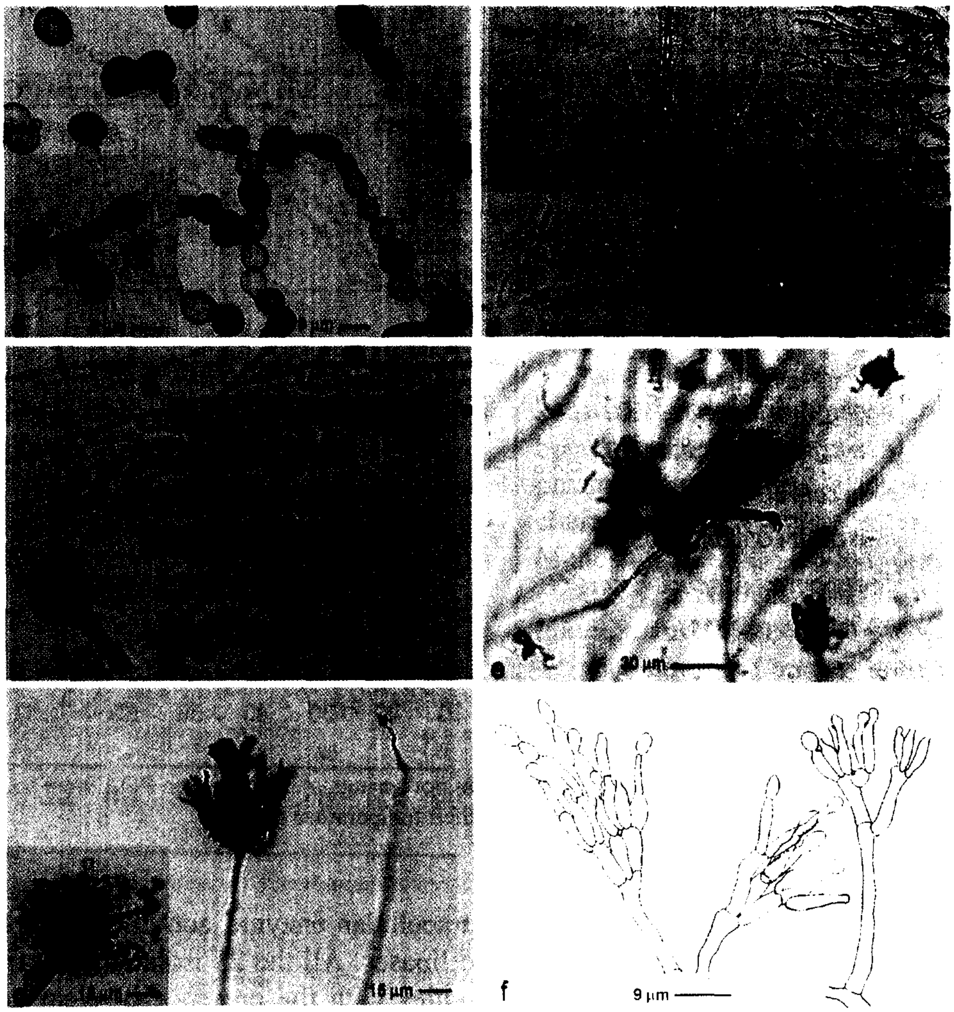


Fig. 3. Microscopic view of isolates of hot springs. a: *Humicola insolens* E08; b: *Thermomyces lanuginosus* E06; c: *Aspergillus fumigatus* E09; d: *Rhizoctonia sp.* E11. e. *Aspergillus fumigatus* E07; f: *Penicillium dupontii* E03.

## Temperature profile of isolates

Six identified strains from those 25 isolates were selected as the test isloates (Table 1). They are *A. fumigatus* (E09, E07), *H. insolens* (E08), *P. dupontii* (E03), *T. lanuginosus* (E06) and *Rhizotonia sp.* (E11). Each isolate was tested for growth temperature in liquid and solid media between 20 and 70°C at 10°C interval. The six tested isolates showed different temperature response (Fig. 4). Only the isolate of *A. fumigatus* E09 was thermotolerant since it grow below 20°C, while other isolates that could not grow below 20°C were regarded as thermophilic fungi.

All the thermophilic isolates showed the similiar resopnses to temperature: growth in high temperature and can not grow below low temperature (20°C). Those are the characteristics of thermophilic fungi. But thermophilic fungi isolated from hot springs samples showed different temperature responses to other isolates of the same species, i.e., *T. lanuginosus* (E05, E06) isolated in this study showed a new tempeature profile that this isolated could grow up to 64°C. This appeared to be a new records that the highest growth upper limit of fungi so far reported.

Table 1. Test isolates of fungi from hot springs samples and related environmental factors. All the sampling sites are shown in Fig 1.

| Isolates (strain number) | Characteristics | Site No/ Site temperature (°C) | Site pH |
|---|---|---|---|
| *Aspergillus fumigatus* (E09) | Thermotolerant | 1/68 | 7.0 |
| *Aspergillus fumigatus* (E07) | Thermophilic | 1/68 | 7.0 |
| *Humicola insolens* (E08) | Thermophilic | 3/72 | 7.1 |
| *Penicillium dupontii* (E03) | Thermophilic | 2/70 | 7.2 |
| *Thermomyces lanuginosus* (E06) | Thermophilic | 4/80 | 7.2 |
| *Rhizoctonia sp.* (E11) | Thermophilic | 6/67 | 6.9 |

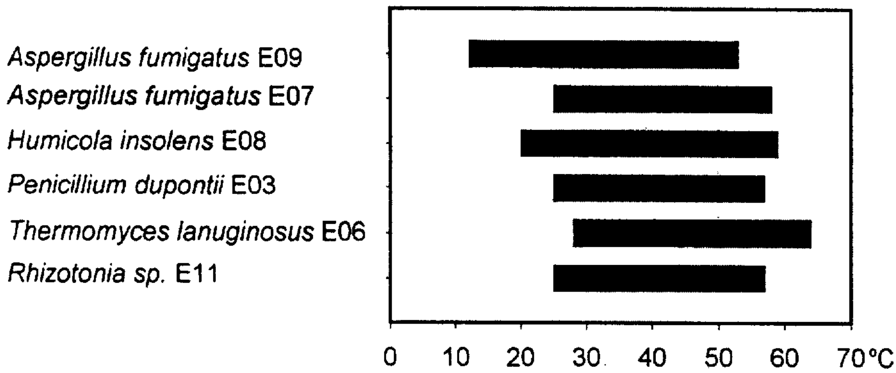


Fig. 4. Temperature profile of test isolates from Wu-Rai hot springs. All isolates on PDA were incubated in PDA between 15-70°C at 10°C interval for 7 days to obtain their temperature profiles.

### Screening of enzymatic activities

The isolates were screened for their extracellular enzyme activities in normal growth condition including amylases, proteinases and lipases. All the 25 isolates were tested at 50°C (isolation temperature) to show enzyme activities with the presence of clear zone by plate assay. After staining with iodine solution , 80% (16/ total 25 isolates) of isolates showed amylase activities. There were 60% (12/ total 25 isolates) of isolates showed proteinase activities and 20% (5/ total 25 isolates) of isolates showed lipase activities. Six test isolates including *A. fumigatus* E09, *A. fumigatus* E07, *H. insolens* E08, P. *dupontii* E03, *T. lanuginosus* E06 and *Rhizotonia sp.* E11 were selected to test enzymatic activity for each enzyme at different temperature and the result is shown in Fig. 5.

## CONCLUSION AND DISSCUSSION

Hot springs are recognized as an extreme environment for living species including microbes. Before studying hot springs microbes, we suspect that some fungal species could be presented in the geothermal environment, but they were not detected by traditional methods (Hawksworth and Rossman, 1997). For example, species present in very low population were not detected by traditional serial dilution method. In the present investigation, seeking of alternative methods were attempted and membrane filter technique that can concentrate the sample was proved to be the best for isolating the low density of microbes of hot springs samples.

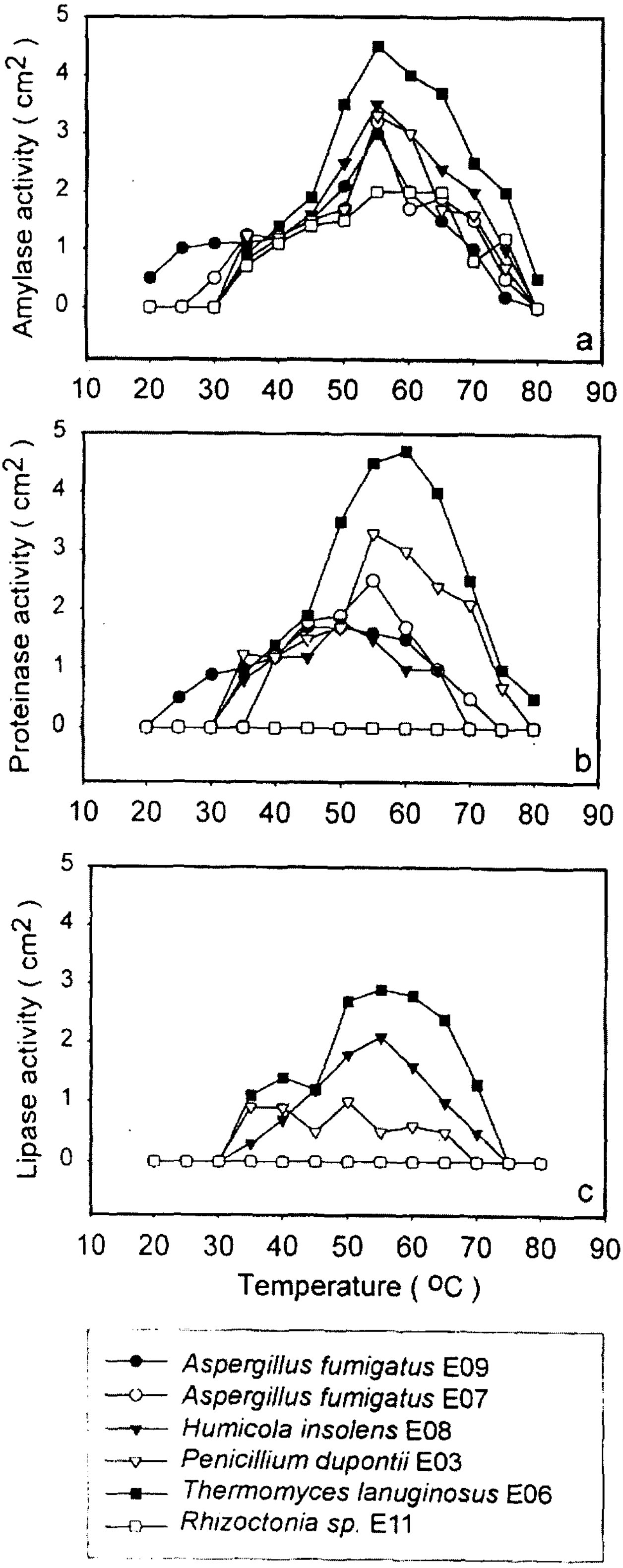


Fig. 5. Diagrams of enzymatic activity assay of different enzymes and different temperature by plate assay method. 5a. amylase; 5b. proteinase; 5c. lipase. All tests were made by test isolates list on the bottom box.

The main aim of this study was to isolate and study thermotolerant and thermophilic fungi of hot springs of Taiwan. As the result, five species, i.e., *Aspergillus fumigatus*, *Humicola insolens*, *Thermomyces lanuginosus*, *Penicillium dupontii* and *Rhizctonia sp.* and five isolate of sterile mycelia were recognized. Some isolates showed special physiological responses to high temperature, i.e. *Thermomyces lanuginosus* is a well known thermophilic fungi and has been studied extensively in its physiological and morphiological characteristics (Trent *et al.*, 1994; Maheshwari and Balasubramanyam, 1988). The isolates (E05, E06) of *T. lanuginosus* from hot springs of Taiwan have the same characteristics and higher growth temperature limit of 64°C which represents the highest upper growth temperature of all fungi so far recognized (Tansey and Brock, 1972).

Studies on the stability of macromolecules of microorganisms growing at high temperature indicate that many proteins are produced which are thermostable. Thermophilic bacteria, for example, produce numerous enzymes and structural proteins with demonstrably high degrees of thermostability (Crisan, 1973). Like thermophilic bacteria, thermophilic and thermotolerant fungi, upon induction by nutrientional factors, can secrete enzymes into its extracellular environment. There are several reports discuss about the thermophilic fungi and the thermostable enzymes produced by them. The most famous thermophilic fungi which can produce many thermostable enzymes including proteinases is *T. lanuginosus* (Cowan and Daniel, 1996; Hasnain *et al.*, 1992). In our study, specific strains of *T. lanuginosus* with higher growth temperature and other thermophilic fungi were isolated from hot springs. Primary screening work has been done on basic thermostable fungal enzymes and high enzymatic activities were present among hot springs isolates. It would be a great potential to screening more thermostable enzymes such as xylanase and chitinase in further study.

Hot springs is one kind of geothermal environments with rich thermophiles such as algae, fungi and bacteria. In this study, not only the fungi could be isolated from hot springs but also some thermophilic bacteria were isolated at the same time. We have isolated over 80 strains of thermophilic and hyperthermophilic bacteria from the same sample sites of Wu-Rai hot srpings (un-published data).

Taiwan is a small island located in the circum-Pacific vocanic zone with many geothermal environment. Until 1996, there are over 128 hot springs of different types were reported (Chung, 1995) but few biological studies were implemented. From summer 1998, we started to investigate the microorganisms in hot springs of northern Taiwan. Over 16 hot springs with 50 sample sites were investigated systematically. Over 100 strains of fungi and over 120 bacterial strains were isolated from those sample sites (un-published data).

Wu-Rai is the first hot springs we studied and there are more hot springs like Wu-Rai are located in Taiwan island. In this preliminary investigation, thermophilic isolates showed the great potential of hot springs microbes and biodiversity in extreme environments of Taiwan. This is a good example and more studies are necessary to be implemented in the future to protect and preserve extreme microbe species in Taiwan.

## ACKNOWLEDGMENTS

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# 台灣溫泉區真菌相之研究(一)：烏來溫泉

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## 摘　　要

本研究以台灣北部烏來溫泉為採樣區，共分離出 25 株嗜熱性與耐熱性真菌，分屬下列五種：*Aspergillus fumigatus*, *Humicola insolens*, *Penicillium dupontii*, *Thermomyces lanuginosus* 及 *Rhizoctonia sp.*。由溫泉所分離出的真菌在生理及酵素反應上有許多的特色。在生理方面，所有由溫泉所分離出的真菌皆可生存於攝氏 55 度的高溫，而其中更有菌株 *Thermomyces lanuginosus* (E05，E06) 可以高於攝氏 60 度（目前已知真菌生存的最高溫度），達到攝氏 64 度的生長極限，這是一個新的生長極限紀錄。在酵素活性方面，80%分離株有 amylase 活性及 60% 菌株有 proteinase 活性，至於具 lipase 活性菌株則為 20%。本研究報告是首次以台灣溫泉為研究對象，分離並分析其中所蘊藏的真菌，可說明台灣溫泉區含極豐富的微生物資源。

關鍵詞: 台灣，溫泉，真菌，微生物資源。

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