Alkaline-tolerant fungi from Thailand

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A collection of 490 alkaline-tolerant fungi was made by isolating fungi from natural habitats using Petri-dishes with Potato Dextrose Agar medium buffered at pH 11.0. Alkaline-tolerant fungi were isolated from 51 out of 71 samples collected from different habitats in Thailand. Twenty-eight samples were taken from tree-holes with different pH. The remaining were samples of soil and sand, wood, seeds, rock holes, roots, leaf material or various other substrates. A total of 324 strains (66%) were screened for enzymes which were active at alkaline pH (alkaline enzymes). Arabinanase, amylase, potato-galactanase and protease activity were assayed. Alkaline-tolerant fungi isolated from tree-holes in alkaline and acidic habitats were good sources for alkaline enzyme production. This screening demonstrates that there exists a population of fungi able to tolerate high pH. Importantly, alkaline-tolerant fungi were isolated from acidic environments. Freshwater habitats appear to be a good source of fungi with alkaline enzyme production capability.

Key words: alkaline enzymes, extremophiles, screening

Introduction

The biodiversity of fungi in Thailand is poorly understood as compared to that of many other countries in the region (Hyde, 2001), although recent publications have advanced the knowledge of fungal diversity in Thailand (e.g. McKenzie *et al*., 2002; Sivichai *et al*., 2002; Somrithipol *et al*., 2002). However, it is increasingly realised that fungi may be good sources of new compounds, beneficial to mankind. For example, in modern detergents there is a need for enzymes that actively work in alkaline conditions (i.e. pH >8.0). Therefore, alkaline–tolerant fungi are considered to be a potential source for alkaline-tolerant enzymes (Horikoshi, 1996).

In industrial biotechnology, more than 30 different types of fungal enzymes are used commercially; e.g., α-amylase from *Aspergillus niger*, *A. oryzae*; cellulase from *Humicola insolens*, *Penicillium sunicalo*; glucoamylases

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from *A. phoenicis*, *Rhizopus delemar*; glucose oxidase from *A. niger*; laccase from *Coriolus versicolar*; pectinase from *A. niger, A. oryzae* and protease from *A. melleus* (Neilsen and Oxenbøll, 1998).

An ideal industrial enzyme should possess high stability and high activity over a wide range of reaction conditions. Such enzymes are increasingly being sought from microorganisms existing in extreme environments (Hamlyn, 1998; Nagai *et al*., 1998; Dalbøge and Heldt-Hansen, 1994. Extremophiles are isolated from harsh environments such as hot springs (thermophiles), Arctic/Antarctic sea-water (psychrophiles), deep-sea hydrothermal vents (barophiles), alkaline lakes (alkalophiles), hot sulphurous springs (acidophiles) and natural or artificial salt lakes (halophiles). Extremophiles are considered to be an excellent source of extremozymes. Consequently, many extremozymes await discovery.

This principle has led us to study alkaliphilic enzymes which can function at pH ≥ 9.0. Extensive screening programmes for alkaline fungi may lead to the discovery of novel extremozymes and could be useful in detergents, where alkaline tolerant protease is an important component (Horikoshi, 1996; Ito *et al*., 1998; Igarashi *et al*., 1998; Maheshwari *et al*., 2000). Alkaline-tolerant fungi (fungi capable of growth at pH 11.0) were isolated from natural forest microhabitats in Thailand and screened for enzyme activity against arabinan, amylose, potato-galactan, and skimmed milk at pH 7.0 and 9.0.

Materials and methods

Sampling and isolation

Samples were collected from various habitats in limestone areas of northern, central and southern parts of Thailand. Collected samples included material from microhabitats such as tree-holes, roots, leaf litter, wood and soil. Wherever fractionally possible the pH of each sample was measured. Each sample was washed with sodium bicarbonate buffer (pH 11.0). The solution, which contained the fungal spores, was spread over a Potato Dextrose Agar plate buffered to pH 11.0 (PDA11). PDA11 was prepared by using 39 g^{-1} of Difco PDA in 20 mM of sodium bicarbonate buffer (at pH 11), with 1 ppm of streptomycin added. After the spores were spread on PDA11, the plates were incubated at room temperature (25°C) for 4-5 days or longer until colonies developed. Germinating conidia were picked off using a dissection microscope and transferred to fresh plates of PDA11. When pure cultures were established, these were maintained on PDA11 and PDA7 slopes. The collection is maintained under cryogenic storage (-80°C) at BIOTEC.

Alkaline-tolerant enzymes screening

Azurine dyed and cross-linked (AZCL) substrates were used to detect enzyme activity. Due to the cross-linked nature of the substrate it could be dispersed in the agar plates as granules. If fungi could produce the specific enzyme for the AZCL substrate, the enzyme would degrade and convert the insoluble substrate to a soluble form revealing its activity by the formation of coloured haloes around the colony, due to the release of soluble dyed substrate fragments (Fig. 1; Dalbøge and Heldt-Hansen, 1994).

The test fungi were transferred from PDA7 slopes to 5% Wheat Bran Agar (pH 7.0) which contained 5 g^{-l} of wheat bran in 20 mM of phosphate buffer (at pH 7.0), and incubated at 25°C for 10 days to provide a source of inoculum. Erlenmeyer flasks (250 ml containing 25 ml of media) were inoculated with the test fungi. Two liquid media were used: FG4 (3 g of soybean, 1.5 g of malto dextrin and 0.5 g of bacto peptone in one litre of distilled water) and Mex-1 (2 g of soybean, 1.5 g of wheat flour, 1 g of cellulose avicel, 0.5 g of malto dextrin, 0.3 g of bacto peptone in one litre of distilled water). Cultures were grown at 25°C and samples were harvested at 4, 7, 10 and 14 days. Aliquots of 20 µl of the harvested liquid from each sample was tested on the substrate plates.

AZCL substrate (0.1% w/v) was mixed with 1% (w/v) agar at 60°C. The four substrates used were AZCL-arabinan, amylose, potato-galactan, and skimmed milk; these were prepared both at pH 7.0 and pH 9.0. Wells (5 mm diam.) were cut in the agar and Aliquots of 20 µl of the liquid was then pipetted to the pre-made wells in the substrate plates. These plates were then incubated at 30°C for 6-12 hours. The presence or absence of blue zones (for the AZCL reactions) or clear zones denoted enzyme activity (Fig 1.).

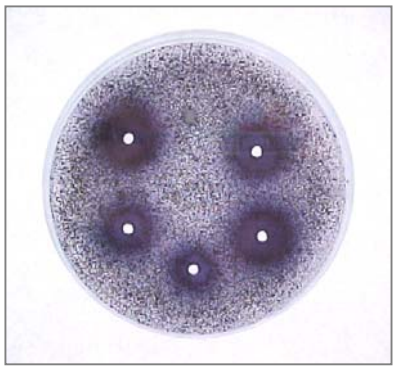

Fig. 1. AZCL-substrate used to detect enzyme activity. A blue diffusion zone around the well indicates a strong enzyme activity.

Results

Isolation and biodiversity of alkaline–tolerant fungi

Seventy-one samples were collected throughout Thailand and examined for the presence of alkaline-tolerant fungi (Table 1). Of these, 51 samples yielded alkaline-tolerant fungi (Table 2). These alkaline-tolerant fungi were divided into four pH-groups: alkaline, neutral, acid and pH-unmeasured habitats. Alkaline habitats yielded 158 isolates of alkaline-tolerant fungi; neutral habitats - 45 isolates; acid habitats - 54 isolates; and pH-unmeasured habitats 233 isolates. The percentage of alkaline-tolerant fungi from measured-habitats was 52% of all alkaline-tolerant fungi in the collection. Of the eight types of samples (habitats) collected (Table 2), tree-holes supplied most (263) of the isolates of alkaline-tolerant fungi.

Alkaline samples (Fig. 2) yielded 3-59 isolates of alkaline-tolerant fungi; neutral samples yielded 3-17 isolates, and acid samples yielded 0-16 isolates. pH-unmeasured samples (Fig. 3) yielded 0-13 isolates. Most isolates came from tree-holes (53.7%), leaf and forest litter (11.4%) and roots (11.2%).

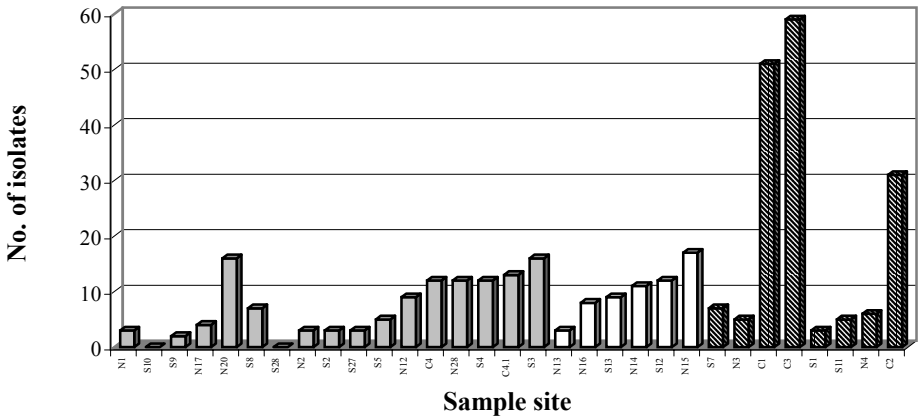

Fig. 2. Number of alkaline-tolerant fungi from habitats where pH was measured;
Acid habitats; Neutral habitats; Alkaline habitat

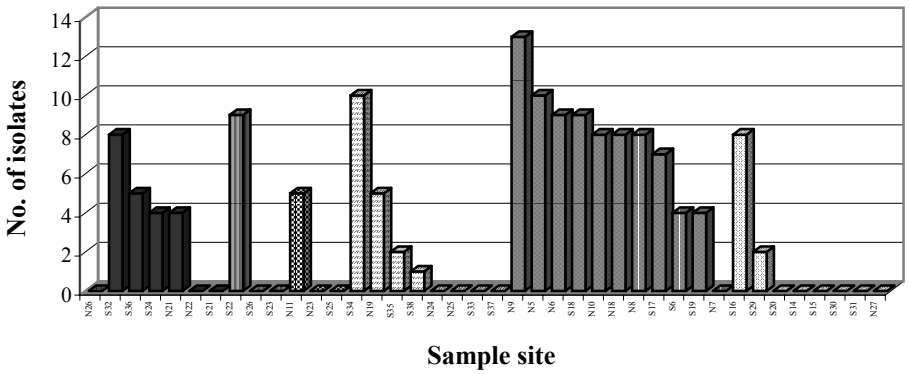

Fig. 3. Number of alkaline-tolerant fungi from various habitats where pH was not measured.

Soil Wood sample Leaf and seed samples
Sand samples Treehole samples
Coconut fibers samples Miscellaneous

Three-hundred and twenty-four of the 490 isolates were screened for four enzymes (arabinanase, amylase, potato-galactanase and protease) which could be active at high pH (9.0). Four soil samples, over a range of pH 5-8 (Table 3.1) yielded 36 isolates of alkaline-tolerant fungi. Of these, 20 isolates were screened yielding five positive isolates. Notably, the sample from the alkaline environment produced no isolates. All four enzymes were present in isolates from soil (Table 3.1).

Two of the three sand samples where it was possible to measure the pH (Table 3.2) were alkaline, but produced no positive results for alkaline tolerant fungi. Conversely, of the three sand samples where it was not possible to measure the pH, two samples produced positive results. Moreover, sample S36 yielded two isolates both of which were positive for all four enzymes assayed (Table 3.2).

Root samples from an alkaline river (C1 from River Kwae Yai; Table 3.3) produced only five positive strains out of 48 strains screened, but all five positive strains were active for all four enzymes assayed. Four rock holes were sampled (Table 3.3) with one, sample S12, producing three positive strains showing activity for all four enzymes. Significantly, the one rock hole with an alkaline pH (S11) produced only alkaline protease activity.

The leaf and forest litter samples (Table 3.4) yielded a single positive strain showing alkaline protease activity from a single sample (C4.1). This

strain was determined to represent a mycelia sterilia. Likewise, wood samples (Table 3.5) yielded only one positive strain determined as a *Fusarium* sp.

Table 1. Samples collected from Thailand (C = central; N = northern; S = southern).

No.	Site	Place	pH of habitat	Type of Sample	No. of alkaline-tolerant fungi
1	C1	Erawan Waterfall, Kanchanaburi	8	Root	51
2	C2	Kong Kaew, Khao Yai NP (KK2)	9	Treehole material	31
3	C3	Kong Kaew, Khao Yai NP (KK3)	8	Treehole material	59
4	C4	Wang Cham Pi, Khao Yai NP	6	Leaf litter in river	12
5	C4.1	Wang Cham Pi, Khao Yai NP	6	Leaf litter in river + rice leaf	13
6	N1	Doi Innthanon/Chiang Mai	4	Tree-hole	3
7	N2	Doi Innthanon/Chiang Mai	6	Tree-hole	3
8	N3	Doi Innthanon/Chiang Mai	8	Tree-hole	5
9	N4	Doi Innthanon/Chiang Mai	9	Tree-hole	6
10	N5	Doi Innthanon/Chiang Mai	-	Dry tree-hole + moss	10
11	N6	Doi Innthanon/Chiang Mai	-	Dry tree-hole	9
12	N7	Doi Innthanon/Chiang Mai	-	Dry tree-hole	0
13	N8	Doi Innthanon/Chiang Mai	-	Dry tree-hole + leaf	8
14	N9	Doi Innthanon/Chiang Mai	-	Dry tree-hole	13
15	N10	Doi Innthanon/Chiang Mai	-	Dry tree-hole	8
16	N11	Doi Innthanon/Chiang Mai	-	Leaf + yellow disease	5
17	N12	8th Mae Sa waterfall/Mae Rim/Chiang Mai	6	Tree-hole	9
18	N13	Mae Sa waterfall/Mae Rim/Chiang Mai	7	Rock hole	3
19	N14	8th Mae Sa waterfall/Mae Rim/Chiang Mai	7	Tree-hole	11
20	N15	8th Mae Sa waterfall/Mae Rim/Chiang Mai	7	Small rock	17
21	N16	8th Mae Sa waterfall/Mae Rim/Chiang Mai	7	Soil	8
22	N17	Doi Suthape Phu Ping 5 km/Chiang Mai	5	Tree-hole	4
23	N18	Doi Suthape Phu Ping 5 km/Chiang Mai	-	Dry tree-hole	8

Table 1 (continued).

No.	Site	Place	pH of habitat	Type of Sample	No. of alkaline-tolerant fungi
24	N19	Doi Suthape Phu Ping 5 km/Chiang Mai	-	Small wood	5
25	N20	Doi Suthape Phu Ping 5 km/Chiang Mai	5	Soil - small rock	16
26	N21	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Sand	4
27	N22	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Sand	0
28	N23	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Leaf	0
29	N24	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Wood	0
30	N25	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Wood	0
31	N26	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Soil	0
32	N27	Hot spring/Ampour Pai/Mae Hong Son	7.5 (45 °C)	Slime	0
33	N28	Pai River/Mae Hong Son	6	Soil	12
34	S1	Ton Nga/Songkla	9	Tree-hole	3
35	S2	Ton Nga/Songkla	6	Tree-hole	3
36	S3	Ton Nga/Songkla	6	Tree-hole	16
37	S4	Ton Nga/Songkla	6	Tree-hole	12
38	S5	Ton Nga/Songkla	6	Tree-hole	5
39	S6	Ton Nga/Songkla	-	Tree-hole	4
40	S7	Boripat WF/Songkla	7.5	Tree-hole	7
41	S8	Boripat WF/Songkla	5.5	Tree-hole	7
42	S9	Boripat WF/Songkla	5	New foam	2
43	S10	Boripat WF/Songkla	5	Old foam	0
44	S11	Khao Pu-Ya/Pattalung	9	Rock hole	5
45	S12	Khao Pu-Yha/Pattalung	7	Rock hole	12
46	S13	Khao Pu-Yha/Pattalung	7	Rock hole	9
47	S14	Ton Nga/Songkla	-	*Cordyceps* on spider	0
48	S15	Ton Nga/Songkla	-	*Cordyceps* on ant	0
49	S16	Thae-Pa/Songkla	-	Shell	8
50	S17	Ton Nga/Songkla	-	Tree-hole	7
51	S18	Ton Nga/Songkla	-	Tree-hole	9

Table 1 (continued).

No.	Site	Place	pH of habitat	Type of Sample	No. of alkaline-tolerant fungi
52	S19	Thae-Pa/Songkla	-	Tree-hole	4
53	S20	Thae-Pa/Songkla	-	Shell	0
54	S21	Thae-Pa/Songkla	-	Sand	0
55	S22	Thae-Pa/Songkla	-	Coconut fibers	9
56	S23	Thae-Pa/Songkla	-	Coconut fibers on tree	0
57	S24	Thae-Pa/Songkla	-	Root in sand	4
58	S25	Thae-Pa/Songkla	-	Seed	0
59	S26	Thae-Pa/Songkla	-	Coconut fibers	0
60	S27	Yha Ring/Pattanii	6	Tree-hole	3
61	S28	Yha Ring/Pattanii	6	Tree-hole	0
62	S29	Mangrove forest/Pattanii	-	Shell	2
63	S30	Mangrove forest/Pattanii	-	Mushroom	0
64	S31	Mangrove forest/Pattanii	-	Seaweed	0
65	S32	Thae-Pa/Songkla	-	Sand	8
66	S33	Thae-Pa/Songkla	-	Marine fungi on wood	0
67	S34	Tad Ta Phu/ KY, Tad 2	-	Wood	10
68	S35	Km.29.9/KY	-	Wood	2
69	S36	Thae-Pa/Songkla	-	*Corollospora maritima* from sand	5
70	S37	Thae-Pa/Songkla	-	Marine fungi on wood (white mycelium)	0
71	S38	Thae-Pa/Songkla	-	Marine wood (white mycelium)	1

Of the other miscellaneous substrates sampled (Table 3.6), only new foam (S9 - resulting from leachates of fallen leaves) and mollusc shell (S16, S29) samples provided alkaline-tolerant fungi. The single screened isolate from new foam, however, showed no positive activity for the enzymes assayed. Of the four-screened alkaline-tolerant fungi isolated from mollusc shells, two strains showed positive activity for all four enzymes.

Table 2. Types of samples and the percentages that yielded alkaline-tolerant fungi (ATF).

Samples	Total number of samples	Number of samples yielding ATF	Percentage of samples yielding ATF	Number of alkaline-tolerant fungi isolated
Tree-holes	28	26	96	263
Soil	4	3	75	36
Sand	6	4	67	34
Roots	2	2	100	55
Leaf, leaf litter and seed	9	5	56	43
Wood	8	4	50	18
Rock holes	4	4	100	29
Miscellaneous	10	3	30	12

Alkaline fungi from tree-holes

Two-hundred and sixty-three alkaline-tolerant fungi were isolated from tree-hole habitats with pH values ranging from 4.0-9.0 (Table 4), 179 strains of which were screened for enzyme activity. Importantly, most of the strains positive for enzyme activity were from alkaline and acid tree-hole samples.

Twenty-eight samples were collected from tree-holes, of which 5 represent alkaline environments (pH 8.0-9.0), 4 neutral (pH 6.5-7.5), 10 acid (pH 4.0-6.0), and 9 were from unmeasured-pH tree-holes (Table 4). All five samples from alkaline tree-holes yielded alkaline-tolerant fungi with positive enzyme activity. The percentage of strains yielding positive results ranged from 14-50%, and two samples (C2, C3) contained strains that showed positive activity for all four enzymes. Positive strains from alkaline tree-hole habitats were identified as *Acremonium*, *Fusarium* and *Paecilomyces* species.

Seventeen of the 21 strains of alkaline-tolerant fungi from neutral pH tree-holes (Table 4) were screened, but none showed positive enzyme activity.

From the ten samples obtained from acid tree-holes (Table 4), 67 alkaline-tolerant strains were isolated and 36 of these were screened for enzyme activity. The percentage of strains yielding positive results ranged from 0-100%. Strains from four samples (S3, S4, S5, N1) showed activity for all four enzymes. Positive strains from acid habitats belong to the genera *Stilbella, Fusarium, Metarhizium* and *Scopulariopsis*.

Seventy-two isolates of alkaline-tolerant fungi were isolated from tree-holes of unknown pH value (Table 4), of which 24 were screened for enzyme activity. Of these strains, only 5 showed positive activity and were determined to belong to the genera *Acremonium* and *Stilbella*. The percentage of all positive strains isolated from tree-holes with known pH values is shown in Fig. 4.

Tables 3.1-3.6. Alkaline-tolerant fungi from different habitats. (+ = Active on substrate, - = Negative on substrate).

Habitat and enzyme activity				**Soil**					
3.1	**N26**			**N16**		**N28**		**N20**	
pH	8.0			7.0		6.0		5.0	
No. of Alkaline- tolerant fungi	0			8		12		16	
No. of screened fungi	–			7		2		11	
No. of positive strains	–			1		0		4	
Percentage positive strains	–			14		0		36	
Arabinanase				+		-		-	
Amylase				-		-		+	
P-galactanase				+		-		-	
Protease				-		-		+	
3.2				**Sand**					
	N21	**N22**	**N15**	**S21**	**S32**	**S36**			
pH	7.5	7.5	7.0	–	–	–			
No. of Alkaline- tolerant fungi	4	0	17	0	8	5			
No. of screened fungi	4	–	3	–	4	2			
No. of positive strains	0	–	0	–	1	2			
Percentage positive strains	0	–	0	–	25	100			
Arabinanase	-		-		+	+			
Amylase	-		-		+	+			
P-galactanase	-		-		-	+			
Protease	-		-		-	+			
3.3	**Root**				**Rock holes**				
	C1	**S24**	**S11**	**S12**	**S13**	**N13**			
pH	8.0	–	9.0	7.0	7.0	7.0			
No. of Alkaline- tolerant fungi	51	4	5	12	9	3			
No. of screened fungi	48	0	5	11	8	3			
No. of positive strains	5	–	2	3	1	0			
Percentage positive strains	10	–	40	27	13	0			
Arabinanase	+		-	+	+	-			
Amylase	+		-	+	-	-			
P-galactanase	+		-	+	-	-			
Protease	+		+	+	-	-			
3.4	**Leaf, leaf litter or seed**								
	N23	**C4**	**C4.1**	**S22**	**S19**	**S23**	**S25**	**S26**	**N11**
pH	7.5	6.0	6.0	–	–	–	–	–	–
No. of Alkaline tolerant fungi	0	12	13	9	4	0	0	0	5
No. of screened fungi	–	2	12	4	4	–	–	–	0
No. of positive strains	–	0	1	0	0	–	–	–	–
Percentage positive strains	–	0	8	0	0	–	–	–	–
Arabinanase		-	-	-	-				
Amylase		-	-	-	-				
P-galactanase		-	-	-	-				
Protease		-	+	-	-				

Tables 3.1-3.6 (continued).

Habitat and enzyme activity	**Wood**							
3.5	**N24**	**N25**	**S33**	**S34**	**S35**	**S37**	**S38**	**N19**
pH	7.5	7.5	-	-	-	-	-	-
No. of Alkaline tolerant fungi	0	0	0	10	2	0	1	5
No. of screened fungi				8	2		ns	ns
No. of positive strains				0	1			
Percentage positive strains				0	50			
Arabinanase				-	-			
Amylase				-	-			
P-galactanase				-	-			
Protease				-	+			

3.6	**Slime, seaweed, foam, shell, mushroom, *Cordyceps***									
	N27	**S31**	**S9**	**S10**	**S14**	**S15**	**S16**	**S20**	**S29**	**S30**
pH	7.5	6.5	5.5	5.5	–	–	–	–	–	–
No. of Alkaline- tolerant fungi	0	0	2	0	0	0	8	0	2	0
No. of screened fungi	–	–	1	–	–	–	3	–	1	–
No. of positive strains	–	–	0	–	–	–	2	–	0	–
Percentage positive strains	–	–	0	–	–	–	67	–	0	–
Arabinanase			-				+		-	
Amylase			-				+		-	
P-galactanase			-				+		-	
Protease			-				+		-	

Sixty-eight isolates showed positive enzyme activity. It was possible to identify 63 of these strains to genus level. Of these 59 belong in the *Hypocreales* (Table 5). Importantly, *Acremonium* and *Stilbella* were the dominant source of alkaline enzymes (Table 5). *Acremonium* spp. (Fig. 5) were isolated from all types of samples while *Stilbella* spp. were found only in rock-holes and tree-holes. Other positive strains were identified as *Paecilomyces*, *Fusarium*, *Metarhizium, Gliomastix*, *Verticillium*, *Phialophora*, *Scopulariopsis* and *Mucor*.

Discussion

There was no significant geographical separation with respect to where the alkaline-tolerant fungi came from; 167 isolates were from the north, 178 from the central region and 145 from the south. Of the samples collected, the pH varied from 4.0 to 9.0. Some of the alkaline habitats were especially good sources of these fungi; e.g. a submerged tree root in an alkaline waterfall (River Kwae Yai, Kanchanaburi) yielded 51 isolates. However, these fungi

Table 4. Percentage of positive strains from tree-holes with various pH values.

Habitat	**Alkaline samples**					**Neutral samples**			
	C2	**S1**	**N4**	**C3**	**N3**	**S7**	**N14**	**S27**	**S28**
pH	9.0	9.0	9.0	8.0	8.0	7.5	7.0	6.5	6.5
No. of Alkaline tolerant fungi	31	3	6	59	4	7	11	3	0
No. of screened fungi	31	3	6	58	4	4	10	3	–
No. of positive strains	8	1	4	8	2	0	0	0	–
Percentage positive strains	26	33	67	14	50	0	0	0	–
Arabinanase	+	+	+	+	+	-	-	-	
Amylase	+	-	+	+	-	-	-	-	
p-Galactanase	+	-	-	+	-	-	-	-	
Protease	+	-	+	+	+	-	-	-	

Habitat	**Acid samples**									
	S2	**S3**	**S4**	**S5**	**S6**	**N2**	**N12**	**S8**	**N17**	**N1**
pH	6.0	6.0	6.0	6.0	6.0	6.0	6.0	5.5	5.0	4.0
No. of Alkaline tolerant fungi	3	16	12	5	4	4	9	7	4	3
No. of screened fungi	1	11	3	1	1	2	3	7	4	3
No. of positive strain	1	7	3	1	1	1	0	0	1	2
Percentage positive strains	100	64	100	100	100	50	0	0	25	67
Arabinanase	+	+	+	+	+	-	-	-	+	+
Amylase	-	+	+	+	+	-	-	-	-	+
P-Galactanase	-	+	+	+	+	-	-	-	-	+
Protease	-	+	+	+	-	+	-	-	+	+

Habitat	**pH unmeasured**								
	S17	**S18**	**N5**	**N6**	**N7**	**N8**	**N9**	**N10**	**N18**
pH	–	–	–	–	–	–	–	–	–
No. of Alkaline tolerant fungi	7	9	10	9	0	8	13	8	8
No. of screened fungi	1	3	8	9	–	1	1	0	1
No.of positive strains	1	3	1	0	–	0	0	0	0
% Positive strains	100	100	13	0	–	0	0	0	0
Arabinanase	+	+	+	-		-	-		-
Amylase	+	+	-	-		-	-		-
P-Galactanase	-	+	-	-		-	-		-
Protease	+	+	-	-		-	-		-

were found in neutral or acidic habitats. A significant observation was that acid environments also proved to be a good source of alkaline-tolerant fungi. This implies that either these fungi can tolerate a wide range of pH in the environment or that some habitats may have a pH that changes over time (Krulwich *et al*., 1996, 1997, 1998; Higashibata *et al*., 1998; Schäfer *et al*., 1996).

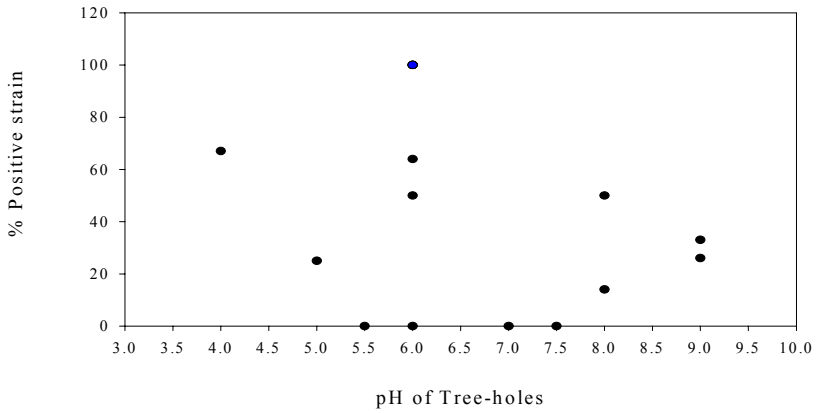

Fig. 4. Percentage of positive strains from tree-holes of known pH value.

Although some habitats were acidic when collected, it is possible that at other times of the year these habitats might have been more alkaline. Therefore, it is feasible that the alkaline-tolerant fungi from such habitats arose because they were at times subjected to alkaline conditions. This hypothesis needs to be studied further.

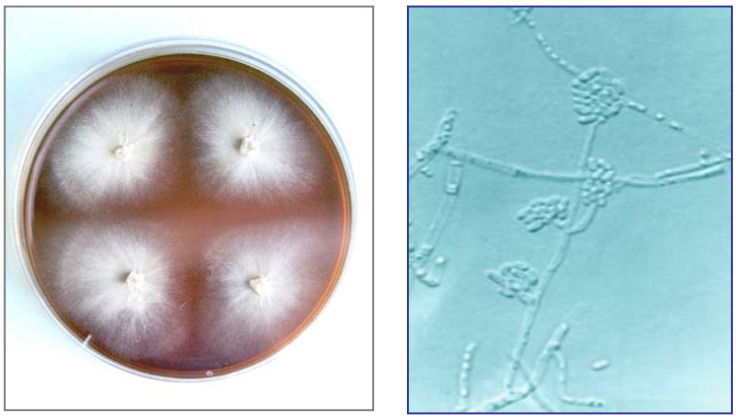

Fig. 5. *Acremonium* sp. (WK 368) isolated from tree-hole (N5), Doi Innthannon, Chiang Mai Province.

Table 5. Positive strains of alkaline-tolerant fungi isolated from different habitats/substrates.

Types of specimen	Positive Genus
Soil	*Acremonium* sp.
	Verticillium sp.
	Gliomastrix sp.
	2 strains of unknown
Sand	2 strains of *Acremonium* sp.
	Verticillium sp.
Root	4 strains of *Acremonium* sp.
	Fusarium sp.
Leaf, Leaf litter	Unknown
Wood	*Acremonium* sp.
Rock holes	2 strains of *Acremonium* sp.
	3 strains of *Stilbella* sp.
	Gliomastrix ramosa
Shell (etc)	2 strains of *Stilbella* sp.
	17 strains of *Stilbella* sp.
Tree-holes	14 strains of *Acremonium* sp.
	3 strains of *Paecilomyces* sp.
	3 strains of *Fusarium* sp.
	2 strains of *Metarhizium* sp.
	Phialophora sp.
	Scopulariopsis sp.
	Mucor sp.
	2 strains of unknown

Tree-holes are a good source of fungi (Gönczöl and Révay, 2003) and especially proved to be a good source of alkaline-tolerant fungi with positive enzyme activity, providing 45 hits from 179 strains (25%) compared with 22 from 145 strains (15%) for other samples.

It was observed that samples from all kinds of neutral habitats, especially those in tree-holes, did not supply any positive strains. However, both acid and alkaline tree-hole samples did provide positive strains, which produced all of the four alkaline enzymes assayed in high activity. Nearly 90% of all enzyme-positive strains isolated in this study belong in the order *Hypocreales*. As in Dalbøge and Lange (1998), *Fusarium* and *Trichoderma*, both members of the *Hypocreales*, are a good source of enzymes. We conclude that the *Hypocreales* represent a good source of extremozymes.

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