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CONTENTS

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Temperature Changes in Southeast Asia: 1973-2008

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ABSTRACT

This paper studied the monthly seasonally-adjusted surface temperature patterns in Southeast Asia from 1973 to 2008. The study area comprised 40 regions of 10° by 10° grid-boxes in latitudes 25°S to 25°N and longitudes 75°E to 160°E. The data were fitted with a second-order auto-regressive process to reduce auto-correlations at lags 1 and 2 months. Factor analysis was used to account for spatial correlation between grid-boxes, giving six contiguous layer regions that extended beyond the original study area to form larger regions. Exploration extended from latitudes 35°S to 25°N and longitudes 65°E to 160°E. Multivariate linear regression models were then fitted to data within these larger regions. Temperatures were found to have increased in all regions, with the increases ranging from 0.091 to 0.240°C per decade.

Keywords: Southeast Asia, Climate change, Time series analysis, Spatial correlation, Auto-correlations, Factor analysis, Multivariate linear regression model

INTRODUCTION

Climate change research is of considerable current interest. Questions on its general patterns, the mechanisms involved, reliable predictions and the effects of our current and future choices, remain at least partly unanswered.

For statistical predictions, the starting point is an analysis of the historic patterns, with reduction of their dimensionality. The available historic data of surface temperatures are of a high dimension, because both location and time coordinates play a role. Once dimensionality has been reduced, parsimonious model fitting may become possible and facilitate predictions. Natural ways to reduce the data include averaging over locations and averaging over time. Both approaches have been used, as seen from this review of prior research highlights.

Various methodologies have been used to study climate change, including mechanism-based computer simulation models or statistical techniques, as well as their combinations. For example, using annual averages, Jones et al. (1999) studied the surface air temperatures in both the southern and northern hemispheres.

They presented global fields of surface temperature change over the two 20-year periods of greatest warming during the twentieth century, 1925-44 and 1978-97. Over these periods, global temperatures rose by 0.37°C and 0.32°C, respectively. Hansen et al. (2006) also studied trends in annual mean temperatures, finding that the average (annually and globally) surface temperature has increased approximately 0.2°C per decade in the 30-year interval 1975-2005. In Australia, Collins et al. (2000) examined trends in annual counts of extreme temperature events, showing that the frequency of warm events has generally increased over the period 1957-96, while the number of cool extremes has decreased. Taniguchi et al. (2007) evaluated subsurface temperatures in four Asian cities in order to estimate the effects of surface warming due to urbanization and global warming, as well as the developmental stage of each city, over the periods 1991, 1992, 2003 and 2006. Mean surface warming in each city ranged from 1.8 to 2.8°C: Bangkok (1.8°C), Osaka (2.2°C), Seoul (2.5°C) and Tokyo (2.8°C).

Numerous studies have assessed both temperature trends and spatial correlation over land surfaces. For example, Kiraly et al. (2006) investigated correlation properties of daily temperature anomalies over land. Several thousand temperature records from the Global Daily Climatology Network were analysed by means of detrended fluctuation analysis (DFA). Short-range correlations were also evaluated by DFA and by first order autoregressive models. The strength of short- and long-range temporal correlations seemed to be coupled for large geographic areas. The spatial patterns were quite complex and had no simple dependence on, for example, elevation or distance from oceans.

Various statistical analyses have been used to model patterns of temperature change. For instance, Anisimov et al. (2007) investigated changes in air temperature in Russia. The spatial homogeneity of air temperature anomalies within each region were assessed through coefficients of correlation between the regionally averaged temperature time series and series at each station of this region, over the periods 1900-49 and 1950-2004. The minimum coefficient of multiple correlation was found to be 0.8. Hughes et al. (2006) studied the variations in the minimum/maximum temperatures of the Antarctic region using a multiple regression model with non-Gaussian correlated errors and linear autoregressive moving average (ARMA) models with innovations. The innovations had an extreme value distribution. This analysis showed an increase in the minimum monthly temperature of approximately 6.7°C over 53 years (1951-2003), without significant increase in the maximum temperatures. Griffiths et al. (2005) investigated extreme temperature changes in the Asia-Pacific region over the period 1961-2003, covering latitudes 46°N - 47°S and longitudes 80°E - 120°W. This study focused on the relationship between mean and extreme temperature in the Asia-Pacific region. The daily temperature time series were used to compute annual averages and annual standard deviations for maximum and minimum temperature. Trends and relationships were calculated using linear regression and Pearson correlation analysis.

The aim of this study is to investigate the trends and patterns in temperatures of a large specific region of Southeast Asia from 1973 to 2008. The selected region includes both land and sea, and temperature profiles of adjacent locations

are correlated. Time series analyses use a simple linear model. To observe and make use of the correlations between adjoining areas, we aggregate data from adjoining regions and fit models to data in larger regions. Thus, there is a fine location mesh at which pre-processed measurement data is available and a coarser mesh in which we generate temperature values by within subregion averaging. Factor analysis and a multivariate linear regression model are used in the spatial analysis. The linear model is also used to predict temperature changes over the next decade.

MATERIALS AND METHODS

Monthly temperatures in Southeast Asia for the 36-year period were obtained from the Climate Research Unit (CRU, 2009) and described in detail by Brohan et al. (2006). CRU provides monthly temperature averages for 5° by 5° latitude-longitude grid-boxes on the earth's surface, based on data collected from weather stations, ships and, more recently, satellites. The data incorporates 40 regions of 10° by 10° grid-boxes which were designed like ice-blocks in an igloo. These areas are located in latitude 25°S to 25°N and longitude 75°E to 160°E, and compose of all or part of 11 Southeast Asian countries, including: northern Australia, southern India, Bangladesh, Nepal, Bhutan, southern China, the Indian Ocean and the western Pacific Ocean, as shown in Figure 1.

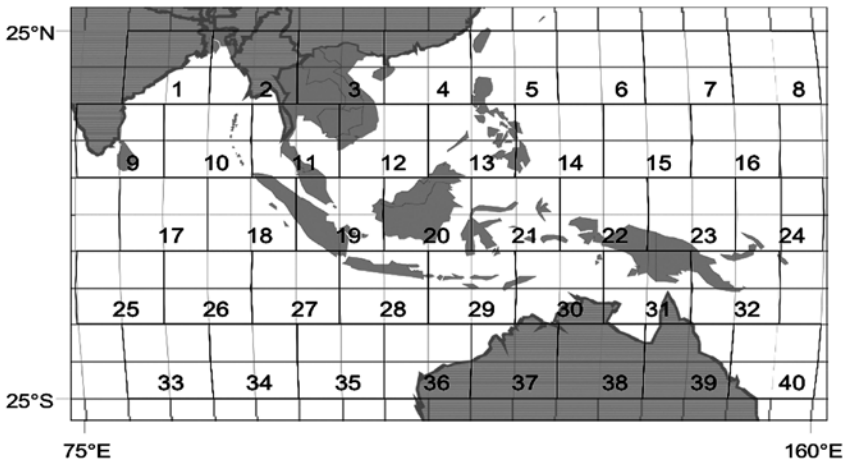

Figure 1. The study region.

Statistical methods

The data consist of 432 monthly temperatures, which were seasonally adjusted. For each grid-box, seasonal variation was removed by subtracting the monthly average and then adding back the overall mean temperature. Using a simple linear model to fit these seasonally-adjusted temperatures (Figure 3), the model takes the form:

$$y_{it} = b_{0i} + b_{1i}\, d_t \,. \,, \tag{1}$$

where y_{it} denotes the seasonally-adjusted temperature in grid-box i for month t and dt denotes the time elapsed in decades since 1973, centered at the middle of the period, that is, $d_t = (t - n/2)/120$ for a period of n months, b_{0i} is the average temperature in grid-box i over the period and b_{1i} is the estimated rate of increase in temperature per decade.

The auto-correlation coefficients measure the correlations between successive observations at different lags. The average monthly temperatures were filtered to remove auto-correlations at lags 1 and 2 months using the filter (Chatfield, 1996):

$$Z_{it} = y_{it} - a_1 y_{i,t-1} - a_2 y_{i,t-2} \,, \tag{2}$$

where coefficients a_1 and a_2 are obtained by fitting auto-regressions with two parameters to the data for each grid-box.

Factor analysis (Mardia et al., 1980) allocates variables into groups by maximizing correlations between variables within the same group and minimizing correlations between variables in different groups. This method was applied to identify correlations between the filtered monthly temperatures in the 40 grid-boxes into groups with different climate change patterns. Grid-boxes with uniqueness (the proportion of the variability that is not shared with the other variables) greater than 0.88 were omitted from the factor analysis. The factor model formulation with p factors takes the form:

$$f_{it} = \mu_j + \sum_{k=1}^{p} \lambda_j^{(k)} \phi^{(k)} \,, \tag{3}$$

where f_{it} are adjusted temperatures in month i and grid-box j, μ_j is the mean temperature of variables in grid-box j, $\lambda_j^{(k)}$ are the factor loadings at grid-box j on the k^{th} factor and $\phi^{(k)}$ are the common factors (Table 1). Data analysis and graphical displays were carried out using R (R Developement Core Team, 2009).

RESULTS

The simple linear regression model was fitted to aggregated seasonally-adjusted temperature centered to zero mean. The result in Figure 2 shows that the mean temperature increased by 0.159°C per decade over the 36-year-period, with correlation (r) 0.59 and 95% confidence interval 0.138 - 0.180°C.

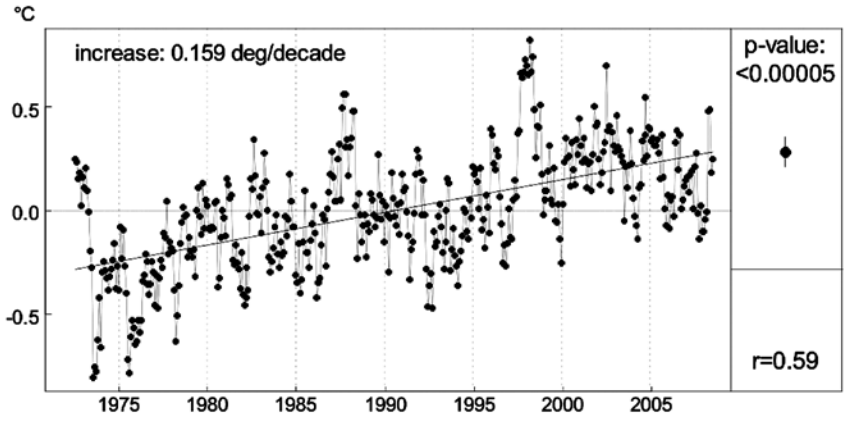

Figure 2. Mean temperature change using simple linear regression model.

Separate linear models were fitted to the seasonally adjusted temperatures for each of the 40 grid-boxes of the Southeast Asia region. Figure 3 shows that the temperatures in each grid-box have increased over the 36-year-period.

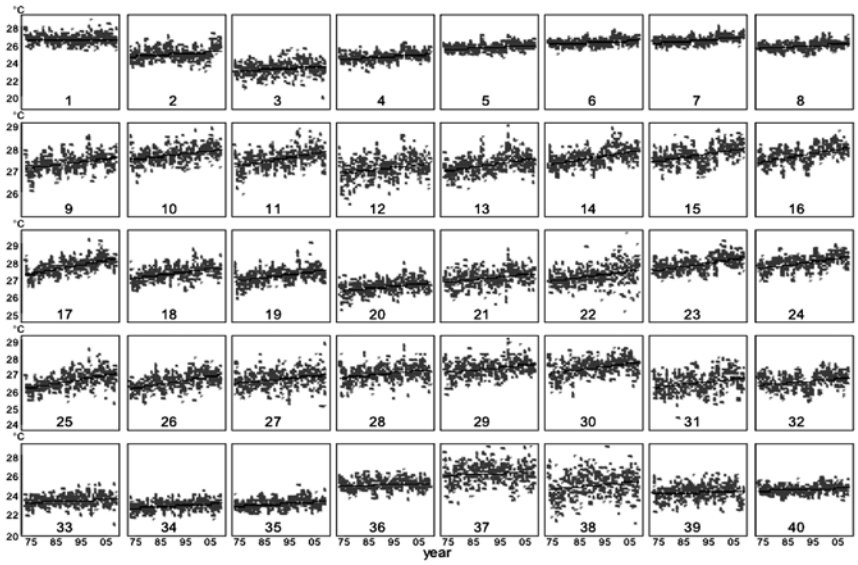

Figure 3. Temperature trends for each region, using simple linear regression models.

In the time-series analysis, the correlations in residuals from this fitted model are assumed to be stationary.

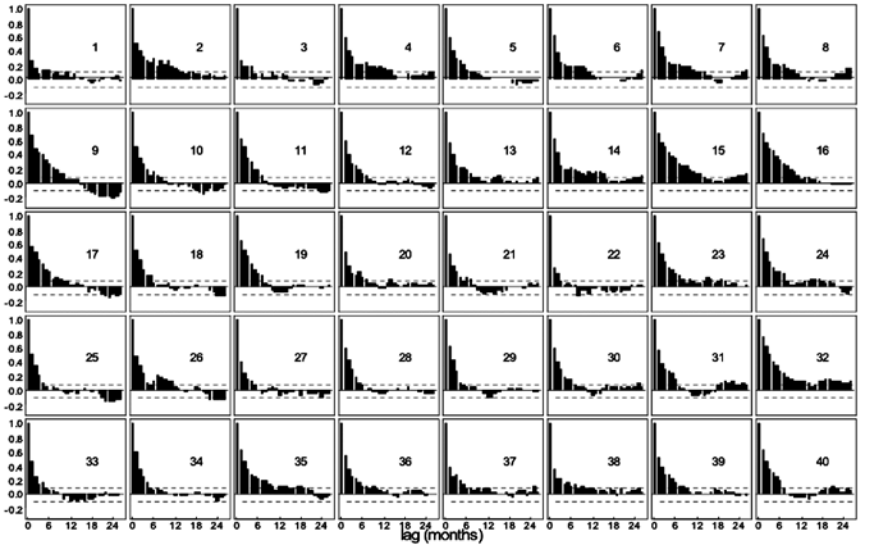

Figure 4. Auto-correlation function plots for the 40 grid-boxes.

The ACF plots in Figure 4 show that all auto-correlations are positive up to lag 26, with many being significant. To account for these significant auto-correlations, an auto-regressive process of order two was fitted to the residuals from the linear regression model.

The average values of the two parameters in the fitted 2-term auto-regressive models are $a_1 = 0.494$ and $a_2 = 0.107$; the filter has removed the auto-correlation structure as shown in Figure 5.

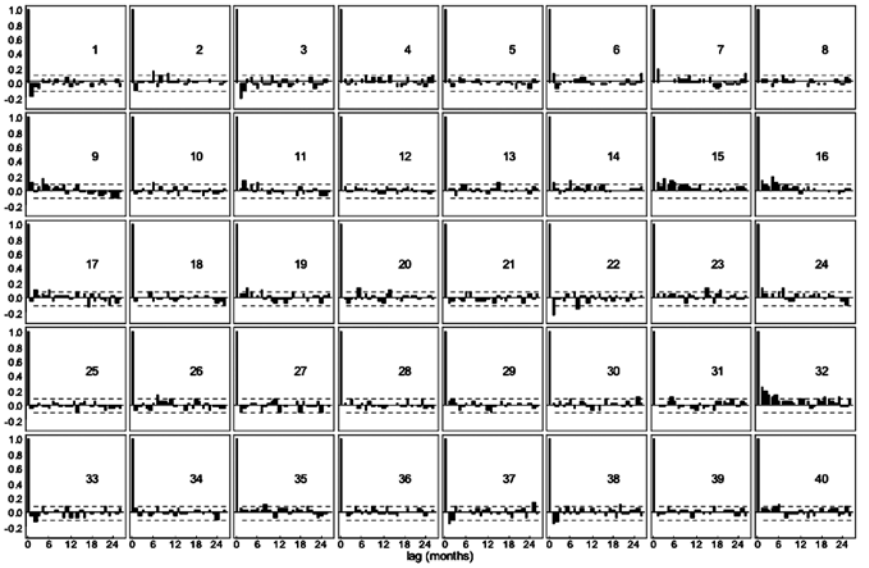

Figure 5. Auto-correlation functions for the filtered residuals.

Table 1. Results of the factor analysis.

Grid-Box	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Uniquenesses
12	0.789	0.112					0.336
4	0.694			-0.115		0.278	0.444
11	0.626			0.278		-0.173	0.434
13	0.576	0.433					0.433
3	0.567	-0.153					0.697
19	0.378	-0.175	0.264	0.275			0.562
15		0.838	-0.217				0.371
14	0.324	0.676		-0.185		0.143	0.398
23	-0.100	0.640					0.557
16		0.630	-0.234				0.652
24		0.503		0.100		-0.108	0.748
22		0.359	0.131		-0.103		0.802
30		-0.132	0.894				0.288
29	0.123	-0.172	0.757				0.410
31	-0.151		0.674				0.558
28	0.175	-0.119	0.514				0.644
32	-0.193	0.357	0.410		-0.102		0.591
21	0.136	0.294	0.368	-0.146			0.682
20	0.313		0.333			-0.102	0.698
40		0.238	0.300				0.784
17				0.710			0.516
26				0.632			0.635
25				0.510			0.743
34				0.506			0.737
18		-0.107	0.122	0.504			0.688
9	0.247	0.102	-0.236	0.460			0.681
33	-0.100			0.426			0.854
35				0.417			0.795
27			0.108	0.344		0.112	0.810
10	0.305		-0.130	0.307			0.764
38					1.009		0.005
37		-0.105			0.707		0.432
39		0.129	0.191		0.602	-0.100	0.540
6	0.118	0.104				0.761	0.318
5	0.521			-0.134		0.620	0.283
7	-0.132	0.111		0.127		0.595	0.567
8	-0.128	0.258				0.374	0.762
36		-0.105	0.110	0.141	0.163	0.148	0.884
2	0.165	0.122					0.950
1	0.119						0.973

The factor analysis gave six groups of filtered temperatures in grid-boxes comprising 6, 6, 8, 10, 3 and 4 of grid-boxes, respectively. The high loadings have been highlighted with shading. There are four grid-boxes that have a mix of factors, and three grid-boxes that have high uniqueness.

Figure 6 shows high correlations of the filtered temperatures within each factor order by six factors (left). The factor model can reduce these correlations as shown in the correlation of the residuals (right).

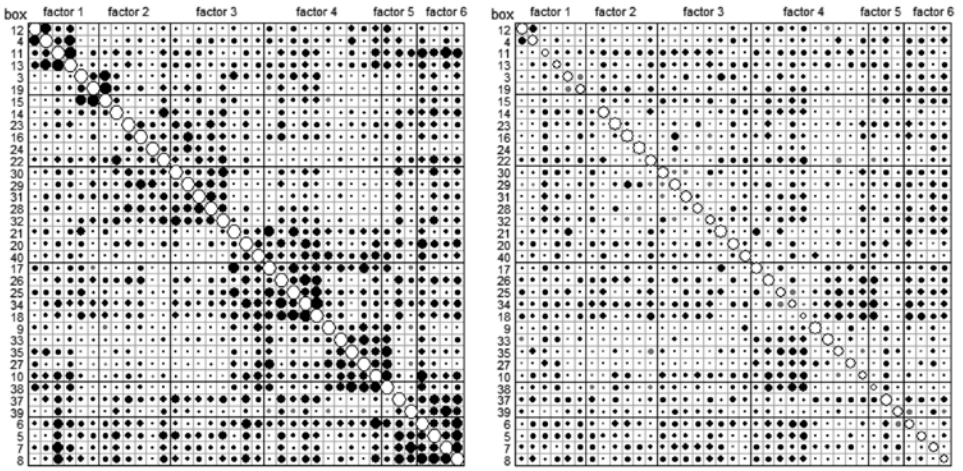

Figure 6. Bubble plots of correlations between filtered monthly temperatures in grid-boxes before (left) and after (right) fitting the factor model.

Of the 40 grid-boxes, 37 could be classified by factor analysis and combined into six regions, which are displayed in the upper part of Figure 7. Further exploration extended the area by 10° in each direction: north, south, east and west. By factor analysis, 46 from 54 of the adjoining grid-boxes were combined, as shown in the lower part of Figure 7 (after first omitting those not correlated). All the regions stay the same, except some grid-boxes.

Each factor comprised the followings regions:

- Factor 1: Southern China, Vietnam, Cambodia, Thailand, Laos, Malaysia, Singapore and Philippines.
- Factor 2: Western Pacific Ocean.
- Factor 3: Indonesia and Papua New Guinea.
- Factor 4: Southern India, Sri Lanka and the Indian Ocean.
- Factor 5: Northern Australia.
- Factor 6: Northwest Australia and the Eastern Indian Ocean

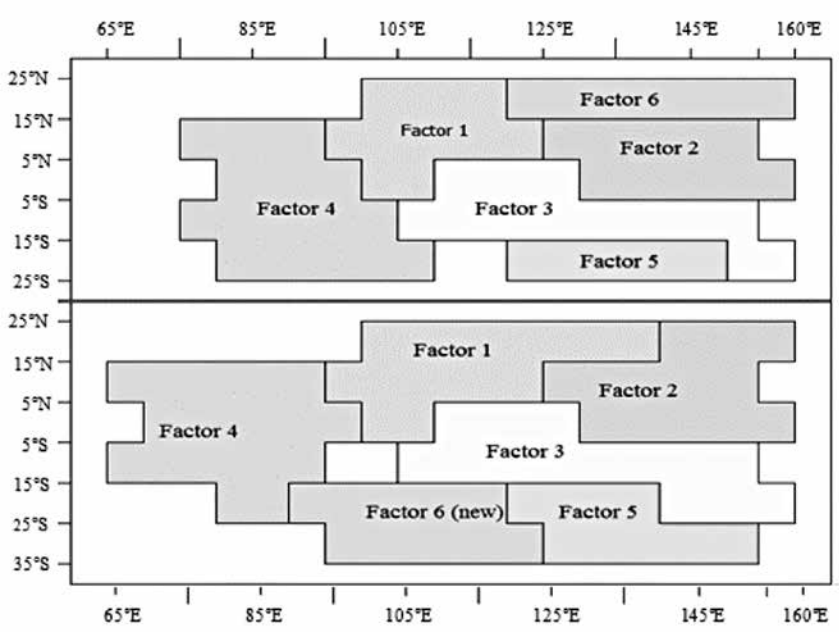

Figure 7. The adjoining grid-boxes were combined into six regions.

Multivariate linear regression models were used to fit parameters for each factor. The response variables are filtered temperatures of each grid-box in the same factor and the explanatory variable is 432 months elapsed. These models provide variance-covariance matrices of estimated temperature increases in adjoining grid-boxes of each factor. A simple linear regression model was used to analyse the average temperatures; estimated parameters are the mean estimated parameter for each relevant factor based on the average for boxes of that factor's area.

Figure 8 shows that the temperatures increased in each factor at the 95% confidence interval for the change per decade, ranging from 0.091 to 0.240°C per decade. Figure 9 shows the 95% confidence intervals for the predicted temperature change from 2009-18 for all regions in the same graph. Factor 5 had the highest range of predicted temperatures, about 0.7°C (21.8°C to 22.5°C), while Factor 2 had the lowest range, about 0.1°C (27.8°C to 27.9°C).

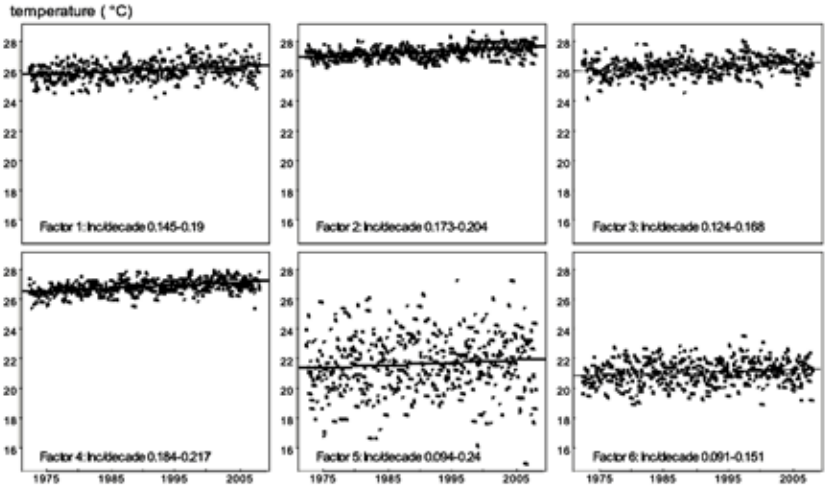

Figure 8. Temperature changes in regions defined by factors.

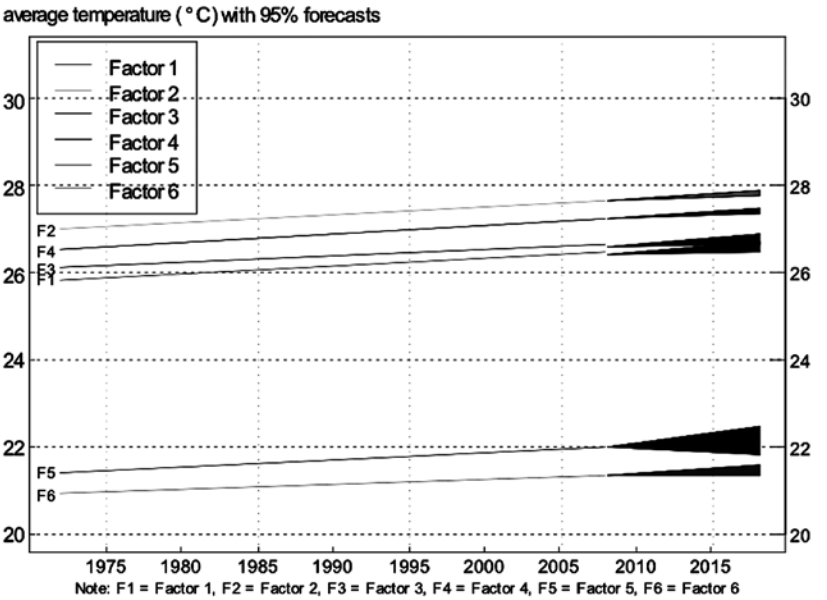

Figure 9. Temperature changes, with 95% confidence intervals, for the predicted temperature change in the next decade.

DISCUSSION

The average monthly temperatures in Southeast Asia from 1973 to 2008 were analyzed using various methods: a simple linear model, a multivariate linear regression model and factor analysis. A Linear model was fitted to the seasonally-adjusted temperatures using data collected from forty 10° by 10° grid-boxes,

covering latitudes 25°S to 25°N and longitudes 75°E to 160°E. The temperatures were filtered by removing the auto-correlation using an AR(2) process. Because of correlations between residuals in adjoining grid-boxes (spatial correlation), factor analysis was used to classify filtered monthly temperatures in grid-boxes into six regions. Multivariate linear regression model was used to fit parameters for each factor. In the extended area covering latitudes 35°S to 25°N and longitudes 65°E to 160°E factor analysis could be classified by six similar regions, except some grid-boxes. A fit of trend of each region by simple linear model, showed that temperatures have increased gradually on average during 1973-2008. Simple linear regression models were also used to predict temperature in each region in the next decade (2009-18). Region 5 had the highest range of predicted temperatures with 0.7°C.

Future studies could investigate temperature changes over longer periods using linear spline models. Moreover, the study could be extended from Southeast Asia to other areas of the world.

ACKNOWLEDGEMENTS

We are grateful to Emeritus Prof. Don McNeil for supervising our research.

REFERENCES

Anisimov, O.A., V.A. Lobanov, and S.A. Reneva. 2007. Analysis of changes in air temperature in Russia and Empirical forecast for the first quarter of the 21st century. Russian Meteorology and Hydrology. 32(10): 620-626.

Brohan, P., J. J. Kennedy, I. Harris, S. F. B. Tett, and P. D. Jones. 2006. Uncertainty estimate in regional and global observed temperature changes: A new data set from 1850. Geophysical research.111: D12106, doi:10.1029.

Chatfield, C. 1996. The Analysis of Time Series, Chapman & Hall, Melbourne.

Collins, D.A., P.M. Della-Marta, N. Plummer, and B.C. Trewin. 2000. Trends in annual frequencies of extreme temperature events in Australia. Australian Meteorology Magazine. 49: 277-292.

CRU. 2009. Climatic Research Unit, School of Environmental Sciences, University of East Anglia, Norwich, UK. Retrieved April 1, 2010, from http://www.cru.uea.ac.uk/cru/data/temperature.

Griffiths, G.M., L.E. Chambers, M.R. Haylock, M.J. Manton, N. Nicholls, H.J. Baek, Y. Choi, P.M. Della-Marta, A. Gosai, N. Iga, R. Lata, V. Laurent, L. Maitrepierre, H. Nakamigawa, N. Ouprasitwong, D. Solofa, L. Tahani, D.T. Thuy, L. Tibig, B.Trewin, K. Vediapan, and P. Zhai. 2005. Change in mean temperature as a predictor of extreme temperature change in the Asia-Pacific region, J. Climatol. 25: 1301-1330.

Hansen, J., M. Sato, R. Ruedy, K. Lo, D.W. Lea, and M. Medina-Elizade. 2006. Global temperature change. PNAS. 103. 14288-14293.

Hughes, G.L., S.S. Rao, and T.S. Rao. 2006. Statistical analysis and time-series models for minimum/maximum temperatures in the Antarctic Peninsula, The royal society, doi:10.1098/rspa.2006.1766.

Jones, P.D. M. New, D.E. Parker, S. Martin, and I.G. Rijor. 1999. Surface air temperature and its changes over the past 150 years. Reviews of Geophy-sics. 37: 173-199

Kiraly, A., I. Bartos, and I.M. Janosi. 2006. Correlation properties of daily temperature anomalies over land, J Tellus. 58A: 593-600.

Mardia, K.V., J.T. Kent, and J.M. Bibby. 1980. Multivariate Analysis, Academic Press Inc. (London) Ltd.

R Development Core Team. 2009. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, Available at URL: http://www.R-project.org. [Accessed "1 November 2009"]

Taniguchi M., T. Uemura, and K. Jago-on. 2007. Combined effects of urbanization and global warming on subsurface temperature in four Asian cities. Vadose Zone Journal. 6: 591-596.

Fertilization Management to Optimize Yield and Quality of Bana Grass

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ABSTRACT

Fertilizers containing nitrogen (N) : phosphorus (P) : potassium (K) ratios of 46:0:0, 16:20:0 and 16:12:8 were applied in combinations. Each fertilizer in both the first and the second application was provided at a rate of 75 kg ha^{-1}. The trial comprised 3 defoliation frequencies at 45, 55 and 65 d according to a randomized completed block design. Forage in four 1-m^2 areas in each plot was cut and weighed in the field. Crude protein content (P<0.05) and dry matter (DM) yield (P<0.01) of bana grass were greater with an application of fertilizer containing higher proportions of N. At only a 45 d defoliation frequency, leaf proportions (P<0.01) and the 48 h in sacco DM and neutral detergent fiber digestibility (P<0.05) were greater when the initial application of N:P:K was 16:12:8. Application of a fertilizer containing 16:12:8 10 d after cutting, followed by 46:0:0 20 d before harvesting at a 45 d defoliation frequency resulted in optimum DM yield and quality.

Keywords: Bana grass, Fertilization, Defoliation, Leaf ratio

INTRODUCTION

Growing forage with a high yield potential and good quality is important to overcome limited supply of roughage during annual drought and reduce the amounts of concentrate needed for cattle farming in the tropics. Cultivars of napier grass have been improved for yield and quality. They differ widely in terms of botanical fractions and nutritive value (Islam et al., 2003). Bana grass (*Pennisetum purpureum x Pennisetum glaucum*) is a hybrid cultivar that grows well on many soil types and is more persistent and drought resistant than napier grass (Pieterse and Rethman, 2002). It also has a high yield potential with high crude protein (CP) and digestibility (Gupta and Mhere, 1997). However, DM yield of napier grass is positively correlated with soil fertility (Bogdan, 1977) and rainfall (Pieterse and Rethman, 2002), and digestibility is negatively correlated with maturity (Chen et al., 2006).

The decline in yield with time after cutting is well documented in tropical pastures and is attributed mainly to the removal of nutrients through harvesting, and to a lesser extent also to the tie-up of the soil N (Robbins et al., 1986), P and

K (Pieterse and Rethman, 2002) in below-ground parts. In general, P and K applied alone had little influence on plant growth (Ebdon et al., 1999), while nearly all grasses respond positively to N. As pasture yield is increased, so the response to an added nutrient will continue to a higher level of application. Luxury application of N, P and K will have a harmful effect on soil (Pieterse and Rethman, 2002) and environment. Of particular concern is the high dependence of intensive dairy farming on high inputs of fertilizer N, especially for systems based on grass. Reduced efficiencies of dietary N utilization as a result of additional fertilizer N were associated with an increase in N excretion in urine (Shingfield et al., 2001). In addition, the pH of top soil noticeably decreased in plots continuously (4 years) fertilized at a high rate of N (Pieterse and Rethman, 2002). A fertility program seldom applies N, P and K individually and research is limited concerning the influence of P and K on yield and quality of bana grass. However, the effects of N, P and K may be interactive on grass quality (Christians et al., 1981), while increasing defoliation frequencies improve quality, but reduce quantity. Split applications of an appropriate N:P:K combination may improve the efficiency of fertilizer applications and, therefore, might improve yield and quality of bana grass at an appropriate defoliation frequency.

MATERIALS AND METHODS

Location of the study area

A field experiment was conducted using a second year regrowth bana grass (*Pennisetum purpureum x Pennisetum glaucum*) pasture at the Udom Dairy Farm, Banyang, Ratchaburi Province (13° 19′ N, 99° 47′ E), which is in the western part of Thailand. During the experiment, monthly maximum and minimum temperatures ranged from 34.8 to 37.0°C and 22.3 to 25.6°C, respectively, and monthly rainfall ranged from 0 to 104.9 mm. The mean monthly maximum and minimum temperatures and rainfall for the trial site over the year are shown in Table 1. The experiment was located on a Kampaengsaen soil series classified as a Non-calcic Brown Soil (54.4% sand, 32.6% silt and 13% clay) with 2-3° slope. The pH of the top soil to the depth of 30 cm was between 6.0 and 6.5. The 30 cm soil layer contained 9.5 p.p.m. available P (Bray-2) and 59 p.p.m. available K (Ammonium acetate).

Table 1. The mean monthly maximum and minimum temperatures and rainfall for the trial site, 2010.

Month	Rainfall, mm	Temperature, °C	
		Maximum	Minimum
January	6.5	32.4	19.8
February	0	35.4	22.3
March	9.6	34.7	23.4
April	0.9	37	25.6
May	39.1	36.3	25.6
June	104.9	34.8	25.4
July	140.8	32.9	24.7
August	114.8	33.1	24.6
September	145.8	32.8	24.5
October	441.5	31.4	24.2
November	87.2	31.3	23.4
December	58.7	29.1	20.9

Fertilization treatments

The experiment consisted of two consecutive defoliation cycles from 1 February to 10 June 2010, which coincides with the annual drought period. A uniform pasture of bana grass was divided into 90 plots, each 2.25 m by 7.5 m. The trial comprised 3 replications and fertilizers containing N:P:K ratios of 46:0:0, 16:20:0 and 16:12:8 were applied in combinations. The trial comprised 10 fertilization combinations that were applied as follows: 1†=46-0-0, 46-0-0; 2†=46-0-0, 16-20-0; 3†=46-0-0, 16-12-8; 4†=16-20-0, 46-0-0; 5†=16-20-0, 16-20-0; 6†=16-20-0, 16-12-8; 7†=16-12-8, 46-0-0; 8†=16-12-8, 16-20-0; 9†=16-12-8, 16-12-8; 10†= No fertilizer. Fertilization treatments were applied twice – at 10 d after harvesting and 20 d before harvesting. Each fertilizer in both the first and second application was provided at a rate of 75 kg ha^{-1}. The trial comprised 3 defoliation frequencies at 45, 55 and 65 d according to a randomized completed block design. Sprinklers irrigated the plots at approximately 30 mm per 10 d interval. At harvesting, forage in four 1-m^2 areas in each plot was cut by hand at approximately 10 cm stubble height above the soil. Fresh yield and leaf proportion were then weighed in the field. The samples were dried at 103°C for dry matter determination. The samples were also dried at 60°C and ground to 1 mm for further chemical composition and 2 mm for *in sacco* digestibility analyses.

Laboratory and statistical analyses

Ether extract (EE), CP, ash, calcium (Ca), P and DM contents of the bana grass were measured according to the AOAC (1980). Neutral detergent fiber (NDF), acid detergent fiber (ADF), neutral detergent insoluble nitrogen (NDIN) and acid detergent lignin (ADL) were determined following the method of Van Soest et

al. (1991). Total non-fiber carbohydrate (TNFC) was calculated by the equation: TNFC = 100 – CP – EE - (NDF-NDIN) - ash. Potassium content was analyzed using an Autoanalyser (SpectrAA 220 Varian, Aust.). Dry matter, NDF and CP *in sacco* digestibility were measured using the method of Orskov et al. (1980). The average yield from four 1-m^2 areas in each plot was used to represent the dry yield and leaf proportion in 1-m^2 area. For chemical composition, yield, leaf proportion and *in sacco* digestibility, the average of 2-cycle samples was used for all statistical measurements. Statistical analysis followed the linear modeling procedure of R (2009) and the difference between treatment means was analyzed by Least Squared Means.

RESULTS

Fertilizer treatment effects on yield and leaf proportions of bana grass are shown in Table 2. Dry matter yield of bana grass was greater ($P<0.01$) with an application of fertilizer containing higher proportions of N, irrespective of defoliation frequency. At a 45 d defoliation frequency, leaf proportions of bana grass were greater ($P<0.01$), both with an initial application of fertilizer containing N:P:K as 16:12:8 and without fertilization. However, at 55 d and 65 d defoliation frequencies, no significant differences were observed between fertilizer treatments for leaf proportions ($P>0.05$).

Table 2. The influence of application of various fertilizer combinations on dry matter yield and leaf proportions of bana grass.

	Fertilizer application										
Item	**1†**	**2†**	**3†**	**4†**	**5†**	**6†**	**7†**	**8†**	**9†**	**10†**	**SE**
45 d defoliation frequency											
Dry matter yield, t ha^{-1}											
Leat	7.56	7.00	6.63	6.88	3.44	3.07	7.41	3.75	3.69	2.06	0.26
Stem	7.31	6.56	6.44	6.69	3.25	2.93	5.91	3.13	2.88	1.63	0.47
Total	14.87^{a}	13.56^{b}	13.07^{b}	13.57^{b}	6.69^{c}	6.00^{c}	13.32^{b}	6.88^{c}	6.57^{c}	3.69^{d}	0.91
Leaf,%	50.84^{b}	51.62^{b}	50.73^{b}	50.70^{b}	51.42^{b}	51.17^{b}	55.63^{a}	54.51^{a}	56.16^{a}	55.83^{a}	1.99
55 d defoliation frequency											
Dry matter yield, t ha^{-1}											
Leaf	8.31	7.75	7.38	6.69	4.81	4.43	7.74	4.56	4.44	2.31	0.56
Stem	8.81	7.50	7.38	7.06	4.56	4.20	7.11	4.25	4.08	2.00	0.60
Total	17.12^{a}	15.25^{b}	14.76^{b}	13.75^{b}	9.37^{c}	8.63^{c}	14.85^{b}	8.81^{c}	8.52^{c}	4.31^{d}	1.51
Leaf,%	48.54	50.82	50.00	48.65	51.33	51.30	52.14	51.76	52.11	53.60	1.66
65 d defoliation frequency											
Dry matter yield, t ha^{-1}											
Leaf	10.00	9.06	9.13	8.75	6.38	6.56	8.88	6.69	7.31	2.75	0.66
Stem	11.19	9.81	10.18	9.63	7.88	8.63	10.06	8.00	7.69	2.75	0.74
Total	21.19^{a}	18.87^{b}	19.31^{b}	18.38^{b}	14.26^{c}	15.19^{c}	18.94^{b}	14.69^{c}	15.00^{c}	5.50^{d}	1.70
Leaf,%	47.19	48.01	47.28	47.61	44.70	43.19	46.88	45.54	48.73	50.00	1.71

Note: $^{a, b, c \text{ and } d}$ Means within a row without a common superscript letter differ ($P<0.01$). Split applications of fertilizers (10 d after and 20 d before harvesting) are as follows: 1†=46-0-0, 46-0-0; 2†=46-0-0, 16-20-0; 3†=46-0-0, 16-12-8; 4†=16-20-0, 46-0-0; 5†=16-20-0, 16-20-0; 6†=16-20-0, 16-12-8; 7†=16-12-8, 46-0-0; 8†=16-12-8, 16-20-0; 9†=16-12-8, 16-12-8; 10†= No fertilizer.

Crude protein contents of bana grass ranged from 46 to 102 g kgDM^{-1}. They were greater when fertilizers containing higher proportions of N were applied (Table 3). No significant differences were detected (P>0.05) between fertilizers containing P and K for contents of P (between 11.9 and 33.6 g kgDM^{-1}) and K (from 11.9 to 13.2 g kgDM^{-1}) of bana grass. Fertilizer applications did not have an effect (P>0.05) on contents of EE (12.0-28.0 g kgDM^{-1}), NDF (640-735 g kgDM^{-1}), ADF (380-479 g kgDM^{-1}), ADL (36-79 g kgDM^{-1}), ash (98-164 g kgDM^{-1}), TNFC (97-166 g kgDM^{-1}) and Ca (3.7-6.0 g kgDM^{-1}).

Table 3. The influence of application of various fertilizer combinations on crude protein contents of bana grass.

	Fertilizer application										
Item	**1†**	**2†**	**3†**	**4†**	**5†**	**6†**	**7†**	**8†**	**9†**	**10†**	**SE**
Crude protein contents, g kgDM^{-1}											
At 45 d cut	102^{a}	90^{b}	80^{b}	86^{b}	83^{b}	77^{b}	84^{b}	83^{b}	77^{b}	52^{c}	11
At 55 d cut	96^{a}	82^{b}	79^{b}	81^{b}	79^{b}	73^{b}	80^{b}	76^{b}	72^{b}	50^{c}	13
At 65 d cut	88^{a}	70^{b}	74^{b}	78^{b}	75^{b}	70^{b}	77^{b}	72^{b}	70^{b}	46^{c}	13

Note: $^{a, b, c \text{ and } d}$ Means within a row without a common superscript letter differ (P<0.01). Split applications of fertilizers (10 d after and 20 d before harvesting) are as follows: 1†=46-0-0, 46-0-0; 2†=46-0-0, 16-20-0; 3†=46-0-0, 16-12-8; 4†=16-20-0, 46-0-0; 5†=16-20-0, 16-20-0; 6†=16-20-0, 16-12-8; 7†=16-12-8, 46-0-0; 8†=16-12-8, 16-20-0; 9†=16-12-8, 16-12-8; 10†= No fertilizer.

Data for 48 h *in sacco* digestibility of bana grass are presented in Table 4. At 55 d and 65 d defoliation frequencies, no significant differences were found (P>0.05) between fertilizer treatments for the 48 h *in sacco* DM, CP and NDF digestibility. At a 45 d defoliation frequency, the 48 h *in sacco* DM and NDF digestibility were greater (P<0.05) when the initial application of N:P:K fertilizer was 16:12:8. However, the 48 h *in sacco* DM, NDF and CP digestibility were lowest for the unfertilized bana grass (P<0.05), irrespective of defoliation frequency.

Table 4. The influence of application of various fertilizer combinations on *in sacco* DM, CP and NDF digestibility of bana grass.

	Fertilizer application										
Item	**1†**	**2†**	**3†**	**4†**	**5†**	**6†**	**7†**	**8†**	**9†**	**10†**	**SE**
The 48 h *in sacco* digestibility, g kgDM^{-1} at a 45 d defoliation frequency											
Dry matter	589^{b}	590^{b}	583^{b}	579^{b}	590^{b}	581^{b}	630^{a}	628^{a}	624^{a}	531^{c}	22
Neutral detergent fiber	525^{b}	526^{b}	518^{b}	520^{b}	518^{b}	523^{b}	559^{a}	549^{a}	550^{a}	467^{c}	23
Crude protein	595^{a}	600^{a}	588^{a}	583^{a}	599^{a}	590^{a}	603^{a}	602^{a}	609^{a}	531^{b}	28
The 48 h *in sacco* digestibility, g kgDM^{-1} at a 55 d defoliation frequency											
Dry matter	550^{a}	543^{a}	546^{a}	539^{a}	542^{a}	549^{a}	564^{a}	552^{a}	559^{a}	499^{b}	29
Neutral detergent fiber	490^{a}	497^{a}	488^{a}	480^{a}	495^{a}	490^{a}	499^{a}	497^{a}	492^{a}	421^{b}	25
Crude protein	557^{a}	544^{a}	550^{a}	546^{a}	555^{a}	556^{a}	572^{a}	561^{a}	561^{a}	505^{b}	35
The 48 h *in sacco* digestibility, g kgDM^{-1} at a 65 d defoliation frequency											
Dry matter	501^{a}	497^{a}	504^{a}	491^{a}	503^{a}	507^{a}	520^{a}	511^{a}	508^{a}	444^{b}	30
Neutral detergent fiber	432^{a}	435^{a}	432^{a}	440^{a}	437^{a}	429^{a}	444^{a}	437^{a}	430^{a}	389^{b}	26
Crude protein	519^{a}	523^{a}	515^{a}	508^{a}	520^{a}	520^{a}	534a	516^{a}	518^{a}	449^{b}	40

Note: $^{a, b,c \text{ and } d}$ Means within a row without a common superscript letter differ (P<0.05). Split applications of fertilizers (10 d after and 20 d before harvesting) are as follows: 1†=46-0-0, 46-0-0; 2†=46-0-0, 16-20-0; 3†=46-0-0, 16-12-8; 4†=16-20-0, 46-0-0; 5†=16-20-0, 16-20-0; 6†=16-20-0, 16-12-8; 7†=16-12-8, 46-0-0; 8†=16-12-8, 16-20-0; 9†=16-12-8, 16-12-8; 10†= No fertilizer.

DISCUSSION

In this study, multiple cuts of regrowth bana grass in an irrigated area were conducted during the drought period, presumably representing the response to fertilization on DM yield and leaf/stem ratio during rainy months. Normally, N is regularly applied while P and K are applied only once a year as basal fertilizers. However, luxury application of N, P and K fertilizer results in a decrease in pH of the topsoil (Pieterse and Rethman, 2002) and contributes to negative environmental impacts, such as excess P encouraging nuisance aquatic weed growth. For this reason, an appropriate N:P:K combination was applied in this study, as it was likely to improve the efficiency of fertilizer application in an irrigated area and, therefore, might improve yield and quality of bana grass at an appropriate defoliation frequency.

Effects of N, P and K individually on forage growth and quality as reported in the literature are primarily dependent on soil fertility, rainfall, maturity and forage variety. Powell and Fussell (1993) showed the importance of N and P in increasing forage yield and quality of pearl millet. Conversely, P and K had little influence on Kentucky bluegrass growth (Ebdon et al., 1999). Growth, quality and total nonstructural carbohydrate accumulation of Bermuda grass differed due to rates of N and K fertilization (Trenholm et al., 1998). Pieterse and Rethman (2002) demonstrated the influence of N in increasing DM yield of bana grass. In this study, DM yield of bana grass increased with increasing applications of fertilizer containing higher proportions of N, whereas increasing application of a fertilizer containing higher proportions of P and K had no effect.

Leaf proportion is important in determining forage nutritive values. In general, there is a progressive decline in leaf proportion as plants develop from a leafy vegetative stage toward maturity (Blaser, 1964). However, leaf and stem growth can vary due to environmental factors, particularly soil fertility (Batten et al., 1984). An optimum supply of N, P and K in combination is necessary for shoot growth (Ebdon et al., 1999), although the number of shoots increased with increasing N rate up to 200 kg ha^{-1} and decreased thereafter (Rusland et al., 1993). Maintenance fertilization with P and K to Brachiaria pasture is essential to sustain pasture productivity (Boddy et al., 2004). Furthermore, K fertilization has an important influence on root growth (Trenholm et al., 1998), having been shown to influence the recovery rate of Kentucky bluegrass turf from summer drought (Schmidt and Brueninger, 1981). At a 45 d defoliation frequency, leaf proportion of bana grass with an initial application (10 d after cutting) of 16:12:8 was significantly higher than those with an initial application of 46:0:0 and 16:20:0. Consequently, an initial application of fertilizer containing some K (i.e., 16:12:8) may be the optimum combination fertilizer for root and shoot growth of bana grass.

Nitrogen (Longnecker et al., 1993) and P (Rodriquez et al., 1998) are important in expansion of leaf area and P has an important effect on the rate of leaf primordial initiation in the stem apex and the size of individual leaves (Rodriquez et al., 1998). At a 45 d defoliation frequency, increasing yields of bana grass were associated with higher levels of N fertilization, probably associated with faster growth rate of stem length (Rusland et al., 1993). An initial application (10 d after cutting) of fertilizer containing 16:12:8 followed (20 d before harvesting) by 46:0:0 may produce the optimum growth rate of stem and leaf. Although the trial design makes it difficult to interpret interaction between nutrients, synergistic effects of N, P and K cannot be ignored. The increased leaf proportion was not found in bana grass at a 55 and 65 d defoliation frequency, because the effect of N on stem growth was probably greater than the effects of P on leaf growth.

The nutritive value of tropical grass is primarily determined by the CP content (Leng et al., 1993) and NDF digestibility (Flores et al., 1993), which are the major factors influencing voluntary intake rate by ruminants in the tropics. An increase in the rate of N fertilization will increase the content of CP of napier grass, although the same effect does not occur with P and K (Pieterse and Rethman, 2002). Adding P to brome grass (McCartney et al., 1998) and K to Coastal Bermuda grass (Cripps et al., 1989) increased P and K uptake in the herbage. A similar result of the CP content for fertilizers containing a higher proportion of N applied to bana grass was also observed in this study. In addition, under 45 d defoliation frequency, an initial application of fertilizer containing N:P:K as 16:12:8 resulted in an increase in DM and NDF digestibility. This increment possibly resulted from an increase in leaf proportion, as mentioned above. However, an increase in leaf proportion found in the unfertilized bana grass resulted in a decrease in DM, CP and NDF digestibility. This reduction is possibly due to higher contents of structural materials, such as NDF (684 vs 636 g $kgDM^{-1}$) and lignin (47 vs 40 g $kgDM^{-1}$), and a lower content of TNFC (138 vs

166 g $kgDM^{-1}$), as compared to those treatments with fertilization.

CONCLUSION

Increasing yields of bana grass were consistent with higher levels of N fertilization in all defoliation frequencies, while leaf proportion and DM and NDF digestibility at a 45 d defoliation frequency increased with an initial application of 16:12:8 fertilizer. At a 45 d defoliation frequency, an initial application (10 d after harvesting) of 16:12:8 fertilizer with the second application (20 d before harvesting) of 46:0:0 fertilizer resulted in an optimum DM yield, and an increase in leaf proportion and DM and NDF digestibility. More information is needed to improve protein nutrition, while reducing fertilizer application, and this could be done by growing bana grass in association with a legume such as cowpea.

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REFERENCES

Association of Official Analytical Chemists. 1980. Official Methods of Analysis. 13th ed. AOAC, Washington, DC.

Batten, G.D., M.A. Khan, and B.R. Cullis. 1984. Yield responses by modern wheat genotypes to phosphate fertilizer and their implications for breeding. Euphytica 33: 81-89. 10.1007/BF00022753

Blazer, R.E. 1964. Effects of fertility levels and stage of maturity on forage nutritive value. J. Anim. Sci. 23: 246-253.

Boddy, R.M., R. Macedo, R.M. Tarré, E. Ferreira, O.C. de Oliveira, C.P. de Rezende, R.B. Cantarutti, J. M. Pereire, B.J.R. Alves, and S. Urqiaga. 2004. Nitrogen cycling in Brachiaria pastures: the key to understanding the process of pasture decline. Agric. Ecosys. Environ. 103: 389-403. 10.1016/j.agee.2003.12.010

Bogdan, A.V. 1977. Tropical pasture and fodder plants. Longman Inc: New York, USA.

Chen, C.S., S.M. Wang, and Hsu, J.T. 2006. Factors affecting *in vitro* digestibility of napier grass. Asian-Aust. J. Anim. Sci. 19: 507-513.

Christians, N.E., D.P. Martin, and K.J. Karnok, 1981. The interactions among nitrogen, phosphorus and potassium on the establishment, quality, and growth of Kentucky bluegrass (poa pratensis L. Merion). Guelph. Ontario Agric. Coll., University of Guelph, Guelph, ON. p. 341-348. In R.W. Sheard (ed). Proceeding of the 4th International. Turfgrass Research Conferrence.

Cripps, R.W., J.L. Young, and A.T. Leonard. 1989. Effects of potassium and lime applied for coastal Bermuda grass production on sandy soil. Amer. Soil Sci. Soc. J. 53: 127-132.

Ebdon, J.S., A.M. Petrovic, and R.A. White. 1999. Interaction of nitrogen, phosphorus and potassium on evapotranspiration rate and growth of Kentucky bluegrass. Crop Sci. 39: 209-218.

Flores, J.A., J.E. Moore, and L.E. Sollenberger. 1993. Determinants of forage quality in Pensacola bahiagrass and mott elephantgrass. J. Anim. Sci. 71: 1606-1614.

Gupta, S.C., and O. Mhere. 1997. Identification of superior pearl millet by napier hybrids and napiers in Zimbabwe. Afric. Crop Sci. J. 5: 229-237.

Islam, M.R., C.K. Saha, N.R. Sarker, M.A. Jalil, and M. Hasanuzzaman. 2003. Effect of variety on proportion of botanical fractions and nutritive value of different napier grass (*Pennisetum purpureum*) and relationship between botanical fractions and nutritive value. Asian-Aust. J. Anim. Sci. 16: 837-842.

Leng, R.A., N. Jessop, and J. Kanjanapruthipong. 1993. Control of feed intake and the efficiency of utilization of feed by ruminants. University of New England, Armidale, Australia. p. 70-88. In: D.J. Farrell (ed.). Recent Advances in Animal Nutrition in Australia.

Longnecker, N., E.J.M. Kirby, and A. Robson. 1993. Leaf emergence, tiller growth and apical development of nitrogen deficient spring wheat. Crop Sci. 33: 154-160. 10.1006/anbo.1994.1087

McCartney, D.H., S. Bittman, P.R. Horton, J. Waddington, and W.F. Nuttall. 1998. Uptake of N, P and S in fertilized pasture herbage and herbage yield response to fertilizer as affected by soil nutrients. Can. J. Soil Sci. 78: 241-247.

Orskov, E.R., F.D. Hovel, and F. Mould. 1980. The use of nylon bag technique for the evaluation of feedstuffs. Trop. Anim. Prod. 5: 195-213.

Pieterse, P.A., and N.F.G. Rethman. 2002. The influence of nitrogen fertilization and soil pH on the dry matter yield and forage quality of *Pennisetum purpureum* and *P. purpureum x P. glaucum* hybrids. Trop. Grassl. 36: 83-89.

Powell, J.M., and L.K. Fussell. 1993. Nutrient and structural carbohydrate partitioning in pearl millet. Agron. J. 85: 862-866.

Radin, J.W., and E.M.P. Idenbock, 1984. Hydraulic conductance as a factor limiting leaf expansion of phosphorus deficient cotton plants. Plant Physiol. 75: 372-377.

Robbins, G.B., K.B. Rickert, and L.R. Humphreys. 1986. Productivity decline in sown tropical grass pastures with age: the problem and possible solutions. Proc. Aust. Anim. Prod. 16: 319-322.

Rodri′quez, D., W.G. Keltjens, and J. Goudriaan. 1998. Plant leaf area expansion and assimilate production in wheat (*Triticum aestivum L.*) growing under low phosphorus conditions. Plant Soil 200: 227-240. 10.1023/A:1004310217694

Rusland, G.A., L.E. Sollenberger, and C.S. Jones. Jr. 1993. Nitrogen fertilization effects on planting stock characteristics and establishment performance of dwarf elephantgrass. Agron. J. 85: 857-861.

R Development Core Team. 2009. A Language and Environment for Statistical computing, R Foundation for Statistical Computing. Vienna, Austria.

Schmidt, R.E., and J.M. Brueninger. 1981. The effect of fertilization on recovery of Kentucky bluegrass turf from summer drought. Guelph, Canada. In: R.W. Sheard, (ed.). Proceeding of the 4th International Turfgrass Research Conferrence

Shingfield, K.J., S. Jaakkolaand, and P. Huhtanen. 2001. Effects of level of nitrogen fertilizer application and various nitrogenous supplements on milk production and nitrogen utilization of dairy cows given grass silage-based diets. Anim. Sci. 73,: 541-554.

Trenholm, L.E., A.E.,Dudeck, J.B. Sartain, and J.L. Cisar. 1998. Burmudagrass growth, total nonstructural carbohydrate concentration, and quality as influenced by nitrogen and potassium. Crop Sci. 38: 168-174.

Acid-adapted Arbuscular Mycorrhizal Fungi Promote Growth of Legumes in Phosphorus-Deficient Acid Soil

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ABSTRACT

Soil acidity is a major limiting factor for upland crops. Arbuscular mycorrhizal fungi (AMF) help improve soil fertility through a fallow enriching tree, Macaranga denticulata, and directly enhance growth of many crops, but its benefit to legumes in acid soil are not known. Three experiments evaluated the benefits from AMF on legumes growing on acidic, low phosphorus soil (pH 5, 11 mg P kg^{-1} by Bray II). In Experiment 1, root zone soil and root fragments of M. denticulata significantly increased cowpea (Vigna unguiculata) growth and P uptake. In experiment 2, CMU22 – a strain of Acaulospora morrowiae propagated from a single spore in the rhizosphere of mimosa (Mimosa invisa) – growing in soil with pH 5 and 11 mg P kg^{-1} was as effective as soil from the root zone of M. denticulata on cowpea and mimosa growth. In experiment 3, cowpea growing in soil with pH 5 and 11 mg P kg^{-1} was inoculated with varying rates of mimosa root zone soil containing CMU22 and CMU22 spores. Both types of inoculum promoted cowpea growth, but at a low rate of 100 spores $plant^{-1}$. Root zone soil that contained infected root fragments and hyphae, as well as spores, was more effective. Arbuscular mycorrhizal fungi adapted to acidic, low P soils have been shown to be effective in alleviating acid soil stress in legumes, with CMU22, an Acaulospora morrowiae, especially well adapted to acid soil.

Keywords: Acid soil, Arbuscular mycorrhizal fungi, Legumes, Phosphorus

INTRODUCTION

Soil acidity is a major limiting factor of legume growth (Munns and Fox, 1977). In acid soil, factors such as toxicity of aluminium (Al) or manganese (Mn) or deficiencies of phosphorus (P), molybdenum (Mo), calcium (Ca) and magnesium (Mg) can limit growth and nitrogen fixation by legumes (Marschner, 1995), with P deficiency the most common (Maddox and Soileau, 1991). Lime and chemical fertilizer, especially phosphatic fertilizer, can ameliorate acid soil

problems (Maddox and Soileau, 1991), but often the cost or logistics of applying these chemicals are prohibitive or surface application cannot solve subsoil acidity (Parkpian et al., 1991).

This raises a question whether the soil acidity problem in legume crops, especially in upland cropping systems where legumes provide sources of cheap protein as well as enhance soil fertility through symbiotic nitrogen fixation, can be solved in other ways. Interestingly, in an upland, shifting-cultivation system on acidic, low P soil (pH 4-6, 3-15 mg P kg^{-1} by Bray II) (Yimyam, 2006a) in Huai Teecha village, Mae Hong Son Province, Thailand, farmers grow legumes – such as cowpea (*Vigna unguiculata*), yardlong bean (*V. unguicultata sesquipedalis*), winged bean (*Psophocarpus tetragonolobus*) and other legumes – without visible symptoms of stress, while also obtaining good yields from their upland rice and other crops (Yimyam, 2006a). High densities of *Macaranga denticulata,* a fallow enriching tree, have been shown to be associated with accumulation of nutrients and higher yield of upland rice (Yimyam et al., 2003). *M. denticulata,* on the other hand, has been found to be highly dependent on arbuscular mycorrhizal fungi (AMF) for growth and nutrient accumulation on acidic, low P soil (Youpensuk et al., 2004). The local population of AMF associated with *M. denticulata* at Huai Teecha is especially diverse and abundant (Youpensuk et al., 2004). Moreover, they are effective in improving growth of many crop species, including rubber (Kanyasone, 2009), coffee (Yimyam, 2006b) and tangerine (Youpensuk et al., 2008). Food crops of shifting cultivation at Huai Teecha, including upland rice, Job's tears and sorghum, have also been shown to benefit from association with AMF (Wongmo, 2008).

In spite of this, no information is available on the association between AMF and local legumes and how the symbiosis might benefit adaptation by legumes to the acidic, upland soils. Whether these AMF can directly benefit legumes in acid soil is the focus of this study. Three pot experiments were conducted to evaluate the effectiveness of acid-adapted AMF from Huai Teecha village to alleviate acid soil stress in legumes.

MATERIALS AND METHODS

Three pot experiments were conducted in a greenhouse at Chiang Mai University, Thailand in 2008 and 2009.

Growth medium preparation

The plant growth medium was prepared from a mixture of sand and soil. Sansai soil (0-30 cm depth) was collected from Mae Hia Agricultural Research Station and Training Center of Chiang Mai University. The soil had the following properties: 3 mg Bray II P kg^{-1}, pH (1:1 H_2O) 5.9, organic matter 1.98 % (w/w), total nitrogen 0.07 % (w/w), extractable K 40.6 mg kg^{-1}, CEC 7.5 cmol kg^{-1}. The soil was air dried, broken up and then sieved to pass a 5 mm screen. The sieved soil was mixed with washed river quartz sand in a 2:1 ratio (w/w) and placed in plastic bags at 3.6 kg bag^{-1}. The pH of the mixture was adjusted to 5 by adding

0.909 g $Al_2(SO_4)_3$ $18H_2O$ kg^{-1}. Available soil P was adjusted to 11 mg Bray II P kg^{-1} by adding 55.5 mg KH_2PO_4 kg^{-1}. Basal nutrients were as follows (mg kg^{-1}): K_2SO_4=71, $CaCl_2.H_2O$=94, $MnSO_4.H_2O$=10, $ZnSO_4.7H_2O$=5, $CuSO_4.5H_2O$=2.1, H_3BO_3=0.8, $CoSO_4.7H_2O$=0.36 and $Na_2MoO_4.2H_2O$=0.18.The above chemicals were mixed thoroughly through the soil. The growth medium was autoclaved at 121°C for one hour.

Inoculum preparation

1. Soil from the root zone of *M. denticulata* was collected from a shifting cultivation field at Teecha, Sob Moei District, Mae Hong Son Province in the rainy season of 2008. The soil, pH 5.5 and 3.8.mg P kg^{-1} (Bray II), contained 68 AMF spores g^{-1} of mixed species and genera7 (*Glomus* 73.5%, *Acaulospora* 24.7%, *Gigaspora* 1.1% and *Scutellospora* 0.7%), as well as root fragments and hyphae. This was used as the root zone soil inoculum, designated Ma. Inoculum rates were varied by adding autoclaved soil inoculum.

2. Fresh root fragments of *M. denticulate* in Ma were washed free of soil and used as an AMF inoculum, designated RF.

3. Single spores of the many different AMF types in Ma were multiplied in association with spineless mimosa (*Mimosa invisa* Mart) growing in autoclaved soil (121°C for 1 hour), pH 5 and 4.2 mg P kg^{-1}. Although the Ma contained spores of *Glomus, Acaulospora, Scutellospora* and *Gigaspora* (in the ratio 69:28:4:2), after 3 months only one strain of Acaulospora morrowiae, CMU22, was able to multiply in abundance. The mimosa root zone soil, air dried and ground to pass a 5 mm mesh, contained 103 spores g^{-1}, and was used as mimosa root zone soil inoculum, designated Mi. Inoculum rates were varied by adding autoclaved soil inoculum.

4. Spores of CMU22 were extracted from Mi. They were surface sterilized for 5 minutes in 70% ethanol and washed in sterilized water 3 times before being used as spore inoculum, designated Ac.

Experiment 1

Experiment 1 was designed in a randomized complete block design with 3 replications. Five treatments of AMF inoculation were conducted, with one pot as an experimental unit. The plant used was cowpea (*Vigna unguiculata* L. Walp.cv. Ubon Rajathanee). Seeds were surface sterilized by soaking in 70% ethanol for 5 minutes, then washed by sterilized water. Six surface sterilized seeds were sown in each 5 L drained plastic pot containing 3.6 kg of the steam-sterilized growth medium. The surface soil in each pot contained 3 holes, with 2 seeds sown in each hole. Two types of inoculum treatments were applied to the seeds in each hole: 1) 10 g of infected *Macaranga denticulata* root fragment (RF) and 2) 10 g of rhizosphere soil from *M. denticulata* (Ma) containing 100, 250 or 500 spores (Ma100, Ma250 and Ma500 respectively). Un-inoculated cowpeas were used as a control. Each hole was inoculated with 2 ml of appropriate rhizobium suspension (CP002) at 10^9 cells ml^{-1} (the suspension was provided by the Department of Soil Science, Faculty of Agriculture, Chiang Mai University). Three sets of pots

with complete treatments and replications were set up for 3 harvests at 15, 35 and 46 days after emergence (V4, flowering and pod filling stage, respectively). Seedlings emerged at 3 days after sowing. Plants were thinned to 3 plants per pot (1 plant hole^{-1}) at 1 week after emergence. At harvest, shoots were cut at ground level then oven dried at 75°C for 48 hours before weighing. Roots were washed free of soil. Root nodules were collected and oven dried before weighing. Fresh roots were weighed before cutting into pieces of approximately 1 cm in length. A root sub-sample of one-tenth total root fresh weight was randomly taken from every pot for assessment of AMF colonization. The remaining roots were oven dried. Fresh root sub-samples were cleared in 10% KOH before staining with 0.05% trypan blue in lactoglycerol. Root colonization percentage was assessed using the intercept method (Brundrett et al., 1996) under a compound microscope. Thirty-two 1-cm pieces of roots were examined for each sub-sample. Shoot and root P concentrations were measured by the molybdovanadate-phosphoric acid method (Murphy and Riley, 1962).

Experiment 2

Experiment 2 applied three types of inoculum to mimosa and cowpea (cv. Ubon Rajathanee) in 3 replications, arranged in a randomized complete block design, with one 5 L plastic pot containing 3.6 kg growth medium as an experimental unit. Seeds of the 2 legumes were surface sterilized with 70% ethanol for 5 minutes and washed 3 times with sterilized water before sowing. There was one hole in each pot. Twenty seeds of mimosa or 5 seeds of cowpea were sown in each pot. Three AMF inoculum types were applied to the seeds in each pot: 1) 22 g of soil from the root zone of *Macaranga* containing 1,500 spores of mixed AMF species (Ma); 2) 1500 spores pot^{-1} of *Acaulospora morrowiae* CMU22 (Ac) plus 22 g autoclaved soil inoculum and 3) 22 g pot^{-1} of autoclaved soil inoculum as a control treatment. Appropriate rhizobium inoculation (10^9 cells ml^{-1} suspension) was applied at the rate of 5 ml pot^{-1}. Cowpea seedlings were thinned to 3 per pot and mimosa seedlings to 10 per pot at 11 days after sowing. At 59 days after sowing, the plants were harvested, weighed and AMF colonization and shoot and root P determined, as described in Experiment 1.

Experiment 3

Experiment 3 involved 7 AMF inoculation treatments, arranged in a randomized complete block design with 3 replications. Cowpea (cv. Ubon Rajathanee) seeds were surface sterilized and sown, six seeds in each 5 L drained plastic pot containing 3.6 kg of growth medium. The surface soil in each pot contained 3 holes, and 2 seeds were sown in each hole. Three types of inoculum treatments were applied to the seeds in each hole: 1) 10 g of mimosa root zone soil containing 100, 250 and 500 spores of *A. morrowiae* CMU22 (Mi100, Mi250 and Mi500, respectively); 2) 100, 250 and 500 extracted spores of *A. morrowiae* CMU22 (Ac100, Ac 250 and Ac500, respectively) and 3) nil-inoculation. Each hole was inoculated with 2 ml of appropriate rhizobium suspension at 10^9 cells ml^{-1}. Plants were thinned to 3 plants per pot at 1 week after emergence. Plants were harvested

at 46 days after emergence (pod filling stage). Biomass yield, P concentration in shoot and root, and root colonization were measured, as described in Experiment 1.

Data were analyzed using analysis of variance and difference among treatments was compared using least significant different (LSD) value ($p \leq 0.05$). The data in percentage were transformed by arcsine before analysis. Data of total dry weight in Experiment 2 were log transformed before analysis. Correlation coefficients were calculated to determine relationship between parameters.

RESULTS

Experiment 1

Root colonization by AMF was evident at 15 days after emergence (Table 1). Increasing the spore rate from Ma100 to Ma500 increased root colonization, from 4.6 to 15.1% at Day 15. By Day 35, over 60% of the root system had been colonized by AMF, with no difference between the spore rates used. The RF inoculum was also very effective and the roots of plants inoculated with RF had the highest level of root colonization at Day 35. However, by Day 46 all inoculated treatments had similar levels of root colonization (Table 1). Infection of roots in the control treatment was nil or very low.

Table 1. Root colonization of cowpea inoculated with *Macaranga denticulata* root fragments (RF) or rhizosphere soil (Ma with three spore loads: 100, 250 and 500 spores) containing AMF inoculum at 15, 35 and 46 days after emergence (Experiment 1).

Inoculation treatment	Days after emergence		
	15	35	46
	Root colonization (%)		
Control	1.1 (1.1)	0 (0.0)	0.2 (0.2)
RF	7.1 (3.9)	86.1 (2.9)	82.6 (9.2)
Ma100	4.6 (4.1)	61.8 (6.6)	70.0 (10.3)
Ma250	10.6 (4.9)	67.8 (4.5)	73.5 (4.7)
Ma500	15.1 (4.1)	73.8 (1.6)	78.5 (4.6)

Note: Numbers in parenthesis are standard errors.

Shoot dry weight did not respond to AMF at Day 15, but the effect of AMF on shoot weight became apparent at Day 35 (Table 2). Plants inoculated with RF had the same shoot weight as those inoculated with Ma100, which was double that of the control. Shoot weight continued to increase with increasing inoculum rate to Ma500. The same response was found at Day 46 (Table 2).

In contrast to the shoot, root dry weight did not respond to AMF until Day 46 (Table 2). At this stage, all inoculated treatments had higher root weight than the control. Root weight increased with inoculum rate, from Ma100 to Ma500.

Plants inoculated with RF had the same root weight as those inoculated with Ma250 (Table 2).

Table 2. Shoot and root dry weights of cowpea inoculated with *Macaranga denticulata* root fragments (RF) or rhizosphere soil (Ma with three spore loads: 100, 250 and 500 spores) containing AMF inoculum at 15, 35 and 46 days after emergence (Experiment 1).

Inoculation treatment	Days after emergence		
	15	35	46
	Shoot dry weight (g plant^{-1})		
Control	0.37	0.74 c	0.9 c
RF	0.32	1.55 b	2.1 b
Ma100	0.26	1.54 b	1.9 b
Ma250	0.29	2.11 ab	2.8 ab
Ma500	0.37	2.48 a	3.2 a
F-test	NS	**	**
	Root dry weight (g plant^{-1})		
Control	0.24	0.43	0.56 c
RF	0.22	0.81	1.40 ab
Ma100	0.24	0.76	1.06 b
Ma250	0.24	0.85	1.28 ab
Ma500	0.22	0.95	1.58 a
F-test	NS	NS	**

Note: NS = not significant. ** = significant at $P < 0.01$. Means in the same column followed by different letters indicate significant difference ($P < 0.05$).

At Day 15, cowpea had tiny visible nodules, but they could not be quantified. At Day 35, all inoculated plants had the same nodule dry weight (Table 3), but this changed by Day 46. At Day 46, increasing the inoculum rate from Ma100 to Ma500 increased nodule weight by 66%. Plants inoculated with RF had the same nodule weight as Ma100 plants (Table 3).

Similar to the growth response, inoculation with Ma enhanced the total plant P content from Day 35 and by Day 46 plants inoculated with the higher spore rate had accumulated the highest amount of P. Plants inoculated with RF acquired P similar to Ma100 plants (Table 3).

Table 3. Nodule dry weight and total P content of cowpea inoculated with *Macaranga denticulata* root fragments (RF) or rhizosphere soil (Ma with three spore loads: 100, 250 and 500 spores) containing AMF inoculum at 15, 35 and 46 days after emergence (Experiment 1).

Inoculation treatment	Days after emergence		
	15	35	46
	Nodule dry weight (mg plant^{-1})		
Control	-	7.9 b	7.7 c
RF	-	56.0 a	80.9 b
Ma100	-	50.3 a	72.8 b
Ma250	-	64.0 a	95.9 ab
Ma500	-	76.4 a	121.2 a
F-test	-	**	**
	Total P content (mg plant^{-1})		
Control	0.96	1.11 b	1.26 d
RF	0.73	3.30 a	4.17 bc
Ma100	0.81	2.93 a	3.76 c
Ma250	1.03	3.84 a	4.97 ab
Ma500	1.08	4.28 a	5.68 a
F-test	NS	*	**

Note: NS = non-significant difference. * = significant at $P < 0.05$. ** = significant at $P < 0.01$. Means in the same column followed by different letters indicate significant difference ($P < 0.05$).

Experiment 2

Spores of *Acaulospora morrowiae* and spores of mixed AMF species were equally effective in colonizing and promoting growth of cowpea and mimosa. In cowpea, 0.4% root colonization was found in the un-inoculated control and much higher root colonization occurred in Ac and Ma (36.5 and 53.6 %, respectively, Table 4). In mimosa, root colonization was 32.8% in Ac and 46.1% in Ma plants. Inoculation with Ac or Ma increased total dry weight equally, even though in mimosa or cowpea (Table 4). Nodule dry weight of cowpea was not affected by AMF, but both Ac and Ma increased nodule dry weight of mimosa (Table 4). The total P content of both legumes was increased by inoculation (Table 4).

Table 4. Root colonization total dry weight nodule dry weight and total P content of cowpea and mimosa inoculated with spores of *Acaulospora morrowiae* CMU22 (Ac) or mixed species (Ma) at 59 days after sowing (Experiment 2).

Treatment	Root colonization (%)	Total dry weight (g plant^{-1})	Nodule dry weight (mg plant^{-1})	Total P content (mg plant^{-1})
		Cowpea		
control	0.4 b	1.97 b	27.2	1.82 b
Ac	36.5 a	3.09 a	55.4	3.91 a
Ma	53.6 a	2.94 a	80.8	3.41 a
F-test	**	*	NS	**
		Mimosa		
control	0.0 b	0.15 b	0.03 b	0.17 b
Ac	32.8 a	0.47 a	18.70 a	0.86 a
Ma	46.2 a	0.71 a	14.80 a	0.94 a
F-test	**	*	*	*

Note: * = significant at $P < 0.05$. ** = significant at $P < 0.01$. Means in the same column of each plant followed by different letters indicate significant difference ($P < 0.05$).

Experiment 3

Root colonization of inoculated treatments ranged between 46 and 53%, with no significant difference between inoculum types or spore rates (Figure 1A). The un-inoculated control cowpea had very low root colonization (0.2%). No effect of spore rate on total dry weight was found in Mi. By contrast, the lowest spore rate in Ac (Ac100) depressed total dry weight by 37% compared to Ac250 (Figure 1B). The nodule dry weight in all inoculated treatments was higher than in the un-inoculated plants (Figure 2A). In Mi, nodule dry weight was not affected by spore rate, whereas in Ac, nodule weight was depressed by 30% at Ac100 compared to Ac500 (Figure 2A). All types of inoculum increase the total P content in cowpea similar to the growth response. Spore rate had no effect on the P content of Mi plants, but in Ac increasing the spore rate from Ac100 to Ac250 increased the total P content by 25% (Figure 2B).

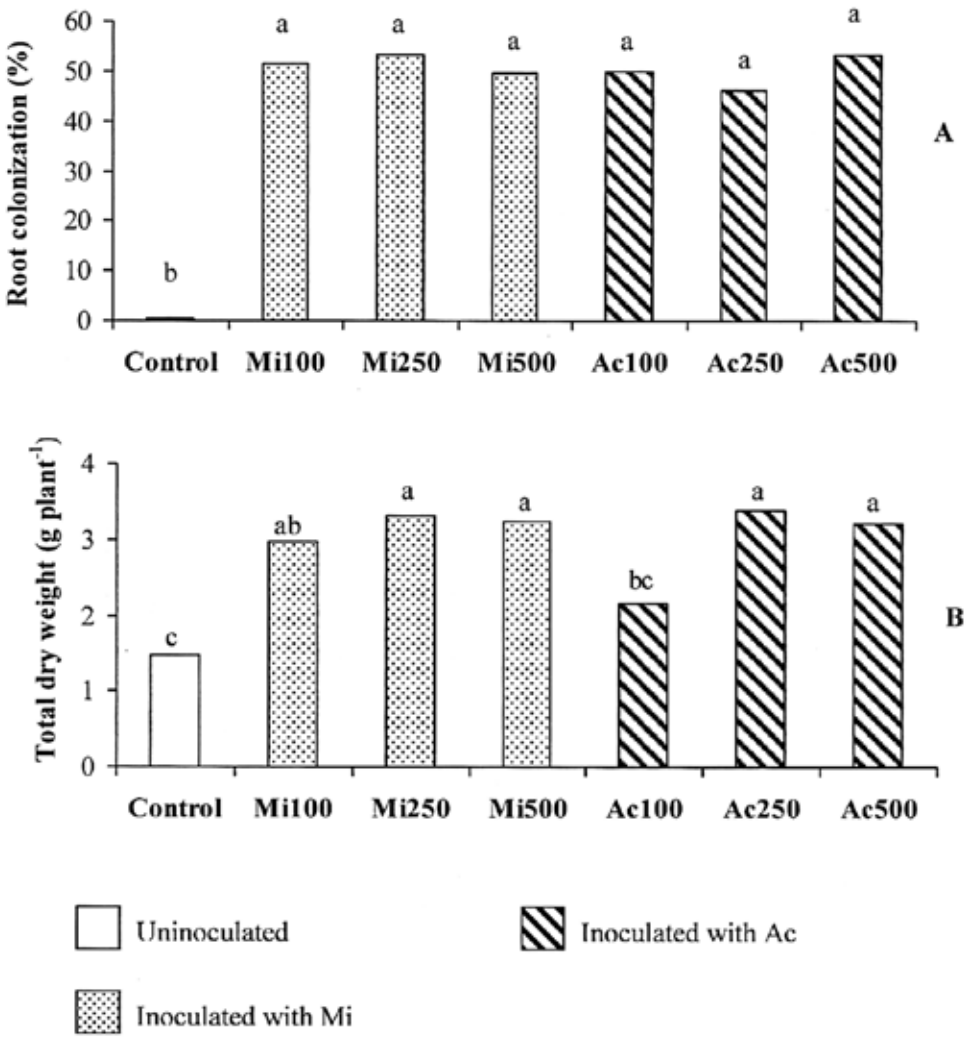

Figure 1. Root colonization (A), Total dry weight (B) of cowpea inoculated with *Acaulospora morrowiae* CMU22 spores (Ac) or *A. morrowiae* soil inoculum (Mi) at three spore rates. Each bar is the mean of 3 replications. The different lowercase letters indicate significant difference by $LSD_{0.05}$ (Experiment 3).

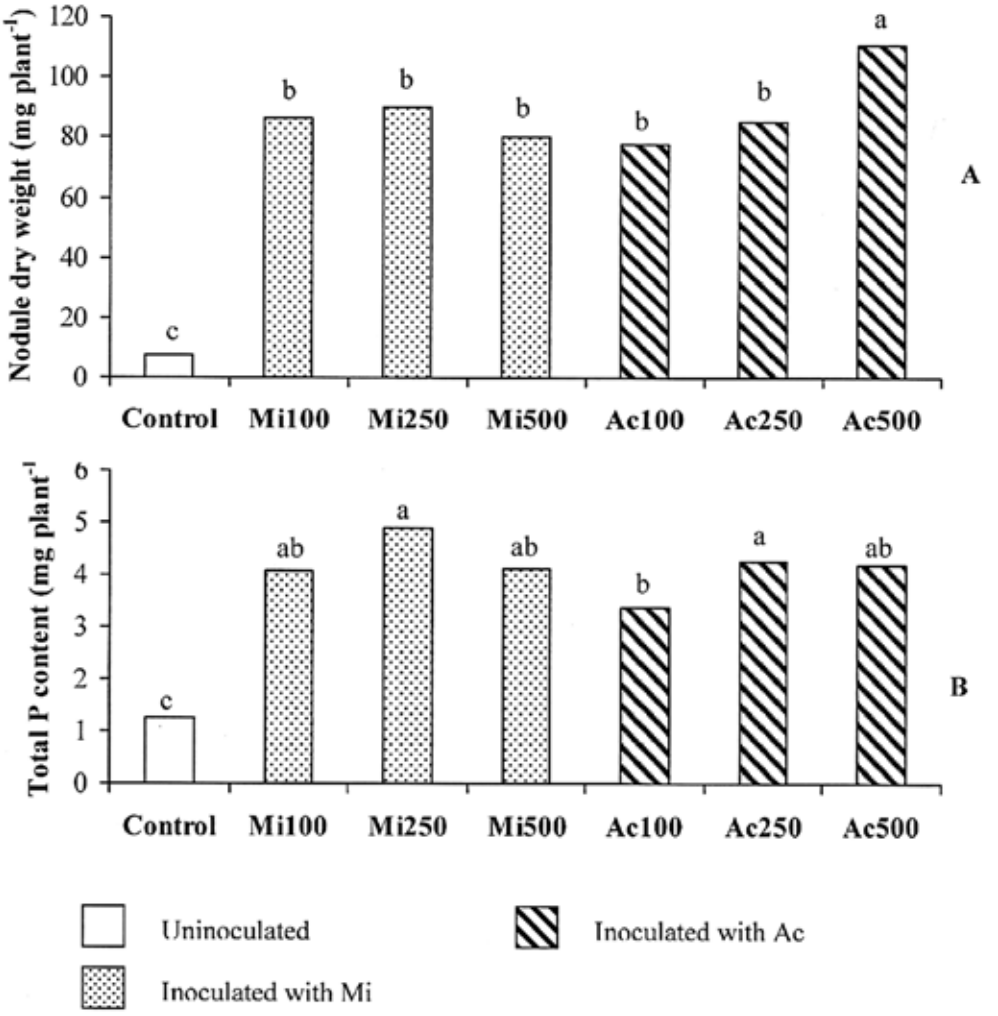

Figure 2. Total dry weight (A) and Total P content (B) of cowpea inoculated with *Acaulospora morrowiae* CMU22 spores (Ac) or *A. morrowiae* soil inoculum (Mi) at three spore rates. Each bar is the mean of 3 replications. The different lowercase letters indicate significant difference by $LSD_{0.05}$ (Experiment 3).

DISCUSSION

Growth stimulation of cowpea by Ma or RF in Experiment 1 indicated that both types of inoculum had potential to colonize and alleviate acid soil stress in cowpea (Tables 1 and 2). The effect appeared in shoot growth before root growth and the response was greater in the shoot (Table 2). This phenomenon is common in plants associated with AMF because competition for photosynthate between the root and fungi could cause root growth to have less response to AMF than shoot growth (Marschner, 1995). When shoot weight was used as a growth indicator, soil inoculum (Ma) was slightly more effective than root fragment inoculum (RF) (Table 2). This could be caused by the soil inoculum containing more infection units. Soil inoculum contains not only AMF spores but also root fragments and AMF hyphae (Brundrett et al.,1996). The higher colonizing ability of Ma showed in root colonization at Day 15 (V4 stage) (Table 1). The root colonization at V4 determined the growth of the plant at flowering and pod filling (35 and 46 days respectively) (Figure 1), although root colonization at pod filling was not different between treatments (Table 1). These results indicated that root colonization in the early stage of growth was very important and influenced growth of the host plant. This was evidenced by a strong positive correlation between root colonization at V4 and shoot dry weight at pod filling (Figure 3). The inoculated plants had more growth because they were able to uptake more P. Figure 4 shows a strong positive correlation between total P content and total dry weight.

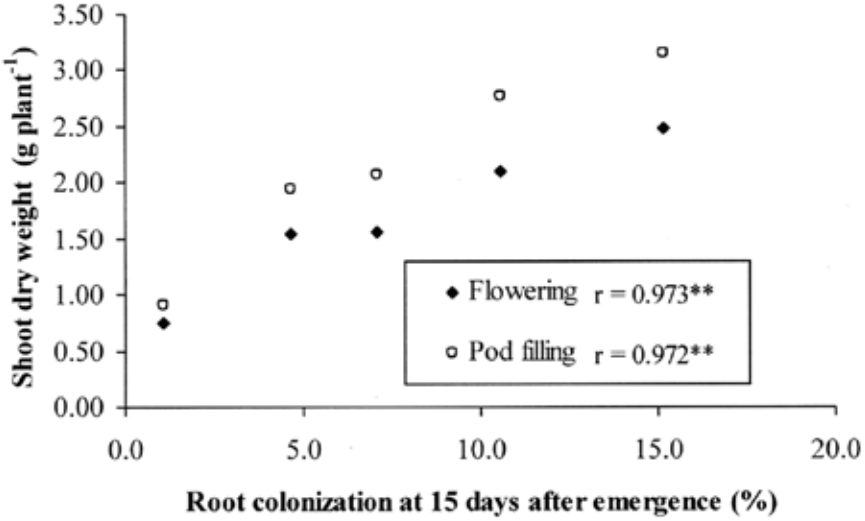

Figure 3. Correlation between root colonization at 15 days after sowing (V4) and shoot dry weight at 35 and 46 days after sowing (flowering and pod filling) r = correlation coefficient, **=significant at $P<0.01$ (Experiment 1).

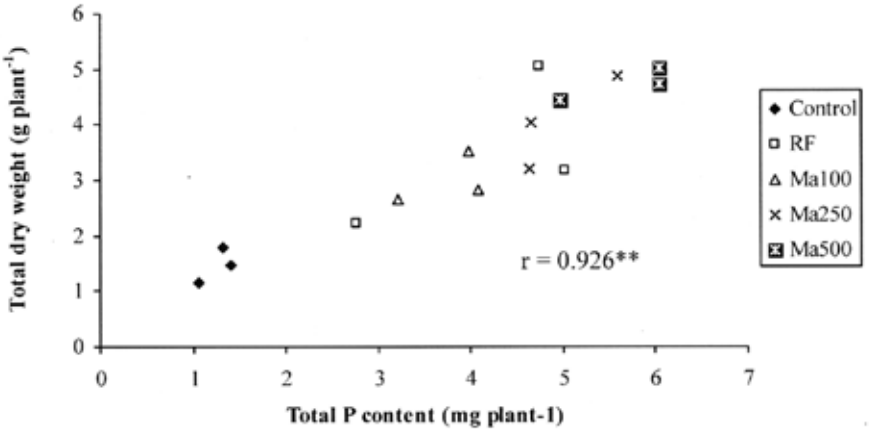

Figure 4. Correlation between total P content and total dry weight of cowpea at 46 days after emergence r = correlation coefficient, **=significant at $P<0.01$ (Experiment 1).

Arbuscular mycorrhizal fungi are well known for improving P uptake of the host plant (Plassard and Dell, 2010). The fungi not only increase the surface area for nutrient uptake through extensive penetration of hyphae into soil (Marschner, 1995) but also some fungi secrete siderophores (Haselwandter, 2008) that enhance P availability by reducing precipitation of phosphate in acid soil (Hu and Boyer, 1996). In acid soil, Al^{3+} is released from unreactive $Al(OH)_3$ into the soil solution and binds with phosphate ions to make P unavailable to plants (Harter, 2002).

In Experiment 2, the Ma and Ac inocula had similar effectiveness in alleviating acid soil stress in cowpea and mimosa (Table 4). This indicated that the CMU22 strain of *Acaulospora morrowiae* was an effective AMF strain that warranted further testing. A spore of this strain was multiplied with mimosa growing in an acidic, low P soil and this inoculum was used to establish that *A. morrowiae* was effective not only in mimosa but also in cowpea. This result shows that CMU22 had low specificity for a plant host. This finding substantiates previous reports that AMF from the same source in Tee Cha village could stimulate the growth of many crop species, including rubber (Kanyasone, 2009), coffee (Yimyam, 2006b), tangerine (Youpensuk et al.,2008), upland rice, Job's tears and sorghum (Wongmo, 2008). In Experiment 3, a lower inoculum dose of Mi achieved the same result as a higher inoculum dose of Ac. To stimulate legume growth, Mi needed 100 spores $plant^{-1}$, while Ac needed at least 250 spores $plant^{-1}$ (Figure 3).

In conclusion, arbuscular mycorrhizal fungi from acidic, low P soils of Tee Cha village have been shown to be effective in alleviating acid soil stress in cowpea, with *Acaulospora morrowiae* CMU22 especially well adapted to acid soil.

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REFERENCES

Brundrett M. N. Bougher. B. Dell, T. Grove, and N. Malajczuk. 1996. Working with Mycorrhizas in Forest and Agriculture. The Australian Centre for International Agricultural Research (ACAIR) Monograph No. 32, Canberra, Australia.

Harter R.D. 2002. Acid Soils of the tropics [Internet]. c2002-2011. Nort Fort Myers (FL) 33917 USA: ECHO Technical Note; [cited 2011 Feb 13]. Available from: http://people.umass.edu/psoil370/Syllabus-files/Acid_Soils_of_the_Tropics.pdf

Haselwandter K. 2008. Structure and function of siderophores produced by mycorrhizal fungi. Mineral Mag 72, 61-64. 10.1180/minmag.2008.072.1.61

Hu X., and G.L. Boyer. 1996. Siderophore-Mediated Aluminum Uptake by Bacillus megaterium ATCC 19213. Appl Environ Microb 62, 4044-4048.

Kanyasone V. 2009. Arbuscular Mycorrhizal Fungi (AMF) for Rubber Seedlings and Agroforestry in Northern Lao P.D.R. MS Thesis, Chiang Mai University.

Maddox J.J., and J.M. Soileau. 1991. Effects of phosphate fertilization, lime amendments and inoculation withVA-mycorrhizal fungi on soybeans in an acid soil. Plant Soil 134, 83-93.

Marschner H. 1995. Mineral Nutrition of Higher Plant. 2nd ed, Academic Press, London, 899 p.

Munns D.N., and R.L. Fox. 1977. Comparative lime requirements of tropical and temperate legumes. Plant Soil 46, 533-548. 10.1007/BF0015912

Murphy V., and J.P. Riley. 1962. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27, 31–36.

Parkpian P., P. Pongsakul, and P. Sangtong. 1991. Characteristics of acid soils in Thailand: A review. In Plant-Soil Interaction at Low pH. Eds. R J Wright, V C Baligar and R P Murrmann. pp 397-405. Proceeding of the Second International Symposium on Plant-Soil Interactions at Low pH. Beckley, West Virginia.

Plassard C., and B. Dell. 2010. Phosphorus nutrition of mycorrhizal trees. Tree Physiol 30, 1129-1139. 10.1093/treephys/tpq063

Wongmo J. 2008. Influences of Arbuscular Mycorrhizal Fungi on Different Food Crops. PhD Thesis, Chiang Mai University.

Yimyam N. 2006a. Fallow regeneration and upland rice yield variation in a system of shifting cultivation with Pada (Macaranga denticulatal (BL.) Muell. Arg) as the fallow enriching species in northern Thailand. PhD thesis, Chiang Mai University.

Yimyam N. 2006b. Arbuscular micorrhizal fungi on growth of arabica coffee seedling. Highland Research and Training Center, Chiang Mai University. 20 p.

Yimyam N., K. Rerkasem, and B. Rerkasem. 2003. Fallow enrichment with pada (Macaranga denticulata (Bl.) Muell. Arg.) trees in rotational shifting cultivation in Northern Thailand. Agroforest Syst. 57: 79-86. 10.1023/A:1023949628251

Youpensuk S., B. Dell, S. Lumyong, and B. Rerkasem. 2004. Arbuscular mycorrhizal fungi from the rhizosphere of a fallow enriching tree, Macaranga denticulata Muell. Arg. and their effect on the host plant. Agroforest Syst 60: 239-246. 10.1023/B:AGFO.0000024417.88703.65

Youpensuk S., S. Lordkaew, and B. Rerkasem. 2008. Arbuscular mycorrhizal fungi association with tangerine (Citrus reticulata) in northern Thailand and their effect on the host plant. Sci Asia 34, 259-264. 10.2306/scienceasia1513-1874.2008.34.259

Quality Control of Mango Fruit during Postharvest by Near Infrared Spectroscopy

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ABSTRACT

Near infrared spectroscopy (NIRS) is a prominent technique for non-destructive fruit quality assessment. This research applied NIRS to control mango quality during postharvest management, harvesting, cold storage and shelf storage. Mango fruits cv. Nam Dok Mai Si Thong were harvested in three stages of fruit maturity; 100, 110 and 120 days after fruit set (DAFS). Mangoes were then divided into three groups. The first group was used to measure the quality at harvest. Mango fruits were measured using short wavelength spectra (700-1100 nm) by NIRsystem 6500 with a fiber optic probe. Physical (color and firmness) and chemical properties (total soluble solids (TSS), tiratable acidity (TA) and dry matter (DM)) were analyzed by conventional methods. The partial least square regression (PLSR) was used to develop the calibration model using The Unscrambler® version 9.8 (CAMO, Oslo, Norway). The means of the data were compared using the least significant difference (LSD). The second group was used to measure quality after cold storage, and was comprised of mango fruits at two harvesting stages (100 and 110 DAFS). Fruits from each stage were stored in cold conditions (13°C, 80-90%RH) for 21 days before the spectra were measured using the NIRsystem 6500. The physical and chemical properties were analyzed. PLSR model development and study of variance followed the same procedure as with the first group. For the third group, mango fruits at two harvesting stages, 100 and 110 DAFS, were stored in cold conditions (13°C, 80-90%RH) for 21 days, after which all samples were kept in the same chamber at 22°C for an additional 4 and 7 days. Spectral data measuring, physical and chemical properties, the PLSR model development and analysis of

variance followed the same method as in the first and second groups to evaluate quality after cold storage and shelf storage. The color, firmness, TSS, TA, DM and TSS/TA of mango fruit changed, depending on postharvest management. The PLSR models for TSS, TA and DM obtained preferable results in terms of coefficient of determination (R^2), standard error of calibration (SEC), standard error of prediction (SEP) and the average of difference between actual value and the NIRS predicted value (bias). The best results were for the PLSR model of DM, which had ratio of standard deviation of reference data in validation set to SEP (RPD) of 3.24. Therefore, NIRS can be applied to control the quality of mango fruit during postharvest management.

Keywords: Mango, Postharvest, Near infrared spectroscopy

INTRODUCTION

Mango is a popular fruit in Asia, Europe and America. Several varieties of mango are available in world markets, including the Kensington, Keitt, Kent, Irwin and Nam Dok Mai. All markets require fruit of high quality, with a long shelf life. Mango fruit quality depends up on many factors, particularly postharvest management (Haidar and Demisse, 1999). These can be divided into three main steps: harvesting, cold storage and shelf storage at the point of sale. Quality changes during postharvest management are the major problems for mango exportation to other continents. Moreover, quality during one stage affects the subsequent stages. Therefore, postharvest management should reduce physical and chemical changes in color, texture, carbohydrates and acidity (Mukherjee, 1997; Nakasone and Paull, 1998; Saranwong et al., 2004 and Tefera et al., 2007). Moreover, the quality has to be evaluated throughout the entire process.

Usually, mango fruit quality is assessed by traditional destructive techniques, which are time-consuming and costly. Therefore, this research focuses on the potential for utilizing new nondestructive techniques. The quality of each fruit should be determined in accordance with consumer needs. Then near infrared spectroscopy (NIRS) was used to assess the postharvest quality of mango fruit. NIRS has been widely used in quality evaluation of many agricultural products such as peaches, apples, melons, citrus and kiwi fruit (Iwamoto et al., 1995). It has the advantages of providing a nondestructive, low-cost, rapid, repeatable and chemical-free analysis. It is very simple to use and can be performed by unskilled personnel (Osborne, 1986 and Osborne et al., 1993).

MATERIALS AND METHODS

Mango fruits cv. Nam Dok Mai Si Thong from orchards in Phrao, Chiang Mai, Thailand were used in this research. This study included three steps of postharvest management: harvesting, cold storage and shelf storage at the point of sale. Mango fruit were harvested in three stages of fruit maturity: 100, 110 and 120 days after fruit set (DAFS). The harvested fruits were divided into three

treatment groups.

The first group was used to measure mango quality at the harvesting stage. One hundred mango fruits at each stage were completely protected from wetting with clear plastic sheets before dipping in a low temperature bath (EYELA, Japan) for 20 minutes at 25°C (Figure 1). Then mangoes were measured using the NIRsystem 6500 (Foss NIRsystem, Silver Spring, USA) at a wavelength range of 700-1100 nm using a fiber optic probe (Figure 2). Physical (color and firmness) and chemical properties (total soluble solids (TSS), tiratable acidity (TA) and dry matter (DM)) were then analyzed. Peel and flesh color were measured by colorimeter (ColorQuest XE, Hunterlab, USA). Firmness of mango flesh was measured by a texture analyzer (TAXtplus, Stable Micro systems, UK) with 2 mm diameter. The maximum value was recorded by the probe while passing through the flesh in force unit, Newton (N). TSS was determined by digital refractometer (PAL-1, ANTAGO, Japan) and TA was titrated with standard sodium hydroxide (0.1 N NaOH) to the end point at pH 8.2 using an autotitrator (Titroline easy, Schott, Germany). Then DM was determined by putting the sample into a vacuum oven (VD53, Binder, USA) and calculated by comparing the weight difference before and after drying. Also, the partial least square regression (PLSR) was used to develop the calibration model in conjunction with The Unscrambler® version 9.8 (CAMO, Oslo, Norway) to evaluate mango fruit quality at harvest, the samples were divided into two sets for calibration and validation. The means of data were compared using the least significant difference test (LSD).

The second group included mango fruits from two harvesting stages (100 and 110 DAFS) and was used to study the effects of cold storage on quality. A total of 200 fruits (100 fruit per harvesting stage) were used. Each mango fruit was stored in a cold room (13°C, 80-90%RH) for 21 days. Samples were dipped into the low temperature bath to equalize the temperature of whole fruit at 25°C and then the spectra were measured using the NIRsystem 6500. The physical and chemical properties were analyzed using the same method as the first group. To study the quality change during cold storage, The Unscrambler® version 9.8 was used to develop a PLSR model for evaluating mango fruit quality during cold storage. The means of data were compared using LSD.

The third group was used to assess the effects of the shelf-life period on mango fruit quality. A total of 400 mango fruits at two harvesting stages, 100 and 110 DAFS (200 fruit per stage), were stored in the cold (13°C, 80-90%RH) for 21 days. Then all fruit samples were kept in the same chamber at 22°C for an additional 4 and 7 days to simulate the shelf-life period, followed by dipping in a low-temperature bath. Mango fruit spectral data were measured using the NIRsystem 6500 (wavelength 700-1100 nm) and physical and chemical properties were analyzed. PLSR was also used to develop the calibration models to evaluate quality after cold storage and shelf-life period following the methodology of the first two groups. Data means were also compared by using LSD.

Figure 1. Mango fruit with the temperature controlled at 25°C by dipping in a low-temperature bath.

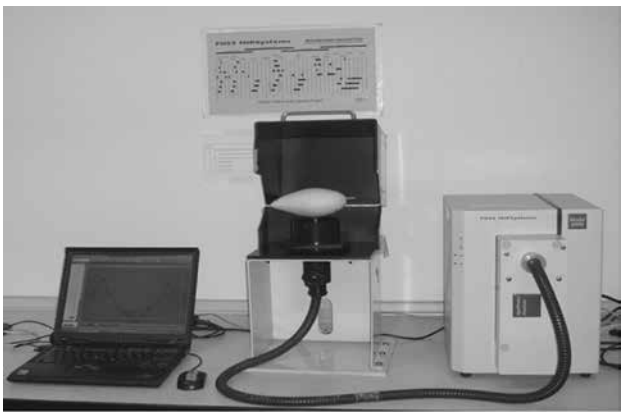

Figure 2. NIRSystem 6500 with a fiber optic probe was used to measure the spectrum of mango fruit.

RESULTS

Mango fruit spectrum

The original spectra of mango fruit showed a clear peak at 980 nm. The absorbance of water molecules in mango fruit decreased when the harvest time increased. For harvest times of DAFS absorbance was 1.21, 1.18 and 1.15, respectively (Figure 3). After the fruit were stored at 13°C for 21 days and kept at 22°C for 4 and 7 days, the absorbance value decreased. They were 1.02, 0.94, 1.06 and 1.08, respectively. To reduce the effect of overlapping peak and baseline shift, the second derivative technique was used to transform the spectral data, lowering the peak of the original spectra at 980 nm to 962 nm (Figure 4).

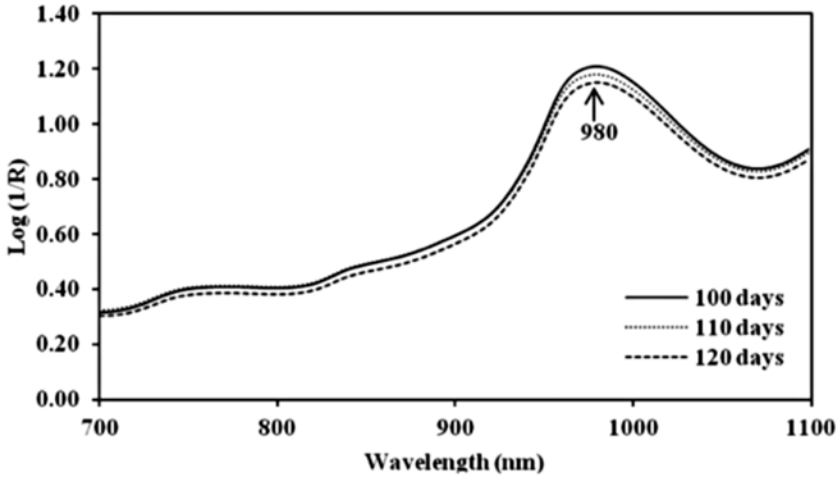

Figure 3. Means original spectra of mango fruit harvested at 100, 110 and 120 DAFS.

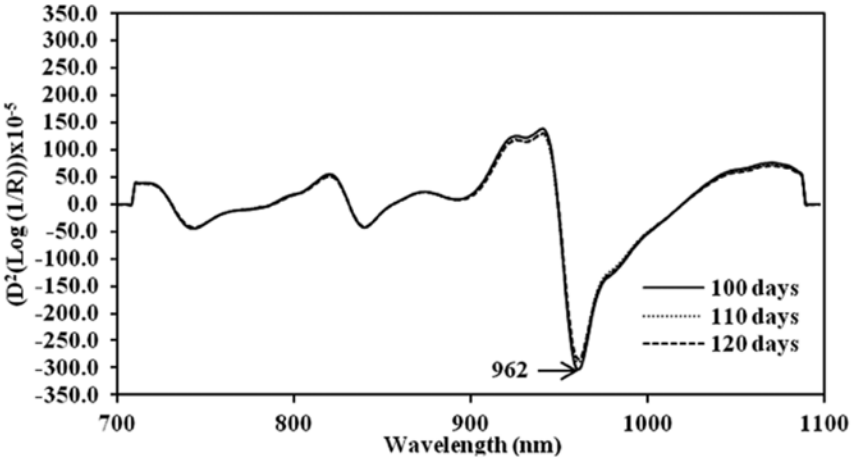

Figure 4. Means second derivative spectra of mango fruit harvested at 100, 110 and 120 DAFS.

Color and firmness of mango fruit

Peel and flesh color of mango fruit at three harvesting times (100, 110 and 120 DAFS) were characterized in terms of L*, C* and h° and were measured with a color meter based on the CIELAB color system, with L* varying from 100 (perfect white) to zero (black). C*(croma) and h°(hue angle) values are calculated based on a* and b* value according to the following equations: $C^* = [(a^*)^2+(b^*)^2]^{0.5}$ and $h^\circ = \tan^{-1}[a^*/b^*]$. C* describes the length of the color vector and h° determines the position of the vector (Jesus Ornelas-Paz et al., 2008). The changes of peel and flesh color of mango fruit during postharvest management

(Figure 5 and 6) were significantly different at P<0.05 (Table not shown). L* and h° slightly decreased, while C* slightly increased.

Firmness of mango flesh measured at three harvesting times was 67.41, 66.26 and 48.05 N, respectively. The result showed that firmness rapidly decreased when stored at 13 °C for 21 days and kept at 22°C for an additional 4 and 7 days. There were significant differences at P<0.05 (Figure 7).

Chemical properties of mango fruits were measured at 100, 110 and 120 DAFS. Total soluble solids (TSS) were 14.05%, 13.00% and 18.56%; titratable acidity (TA) were 2.06%, 1.80% and 1.23%; dry matter (DM) were 21.03%, 22.63% and 24.28%; and the ratios of TSS and TA (TSS/TA) were 7.01, 7.63 and 16.49, respectively. They also changed after cold storage and shelf storage. All values were significantly different at P<0.05 (Table not shown). TSS greatly increased during cold storage (80-90%RH) and lowered to 17-18% after shelf storage. Meanwhile, TA decreased after cold and shelf storage. DM slightly changed after cold and shelf storage (Figure 8).

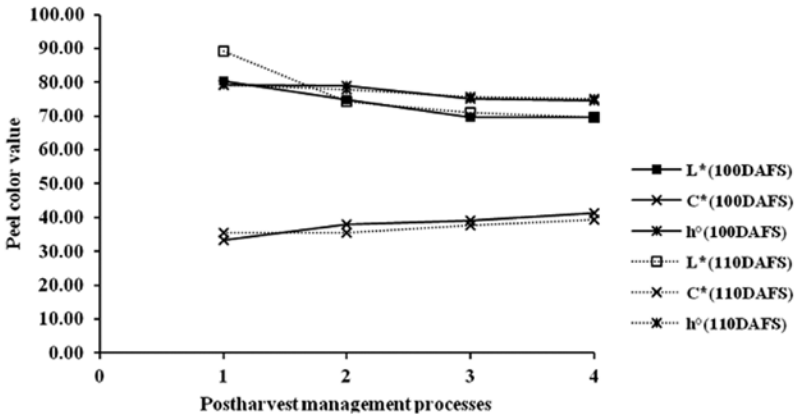

Figure 5. Changes of peel color values (L*, C*, h°) of mango fruit harvested at 100 and 110 DAFS (1), stored at 13°C for 21 days (2) and kept at 22°C for 4 (3) and 7 days (4).

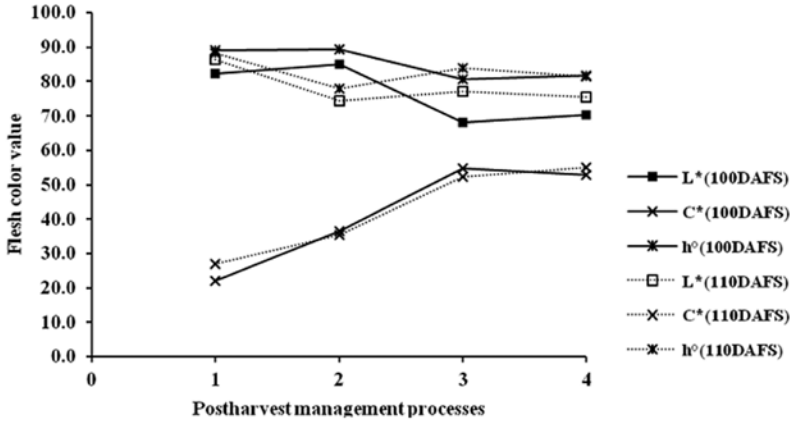

Figure 6. Changes of flesh color values (L*,C*, h°) of mango fruit harvested at 100 and 110 DAFS (1), stored at 13°C for 21 days (2) and kept at 22°C for 4 (3) and 7 days (4).

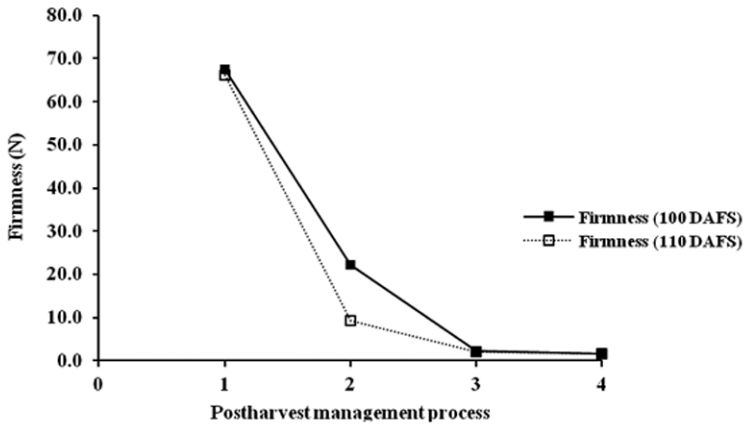

Figure 7. Changes of firmness of mango fruit harvested at 100 and 110 DAFS (1), stored at 13°C for 21 days (2) and kept at 22°C for 4 (3) and 7 days (4).

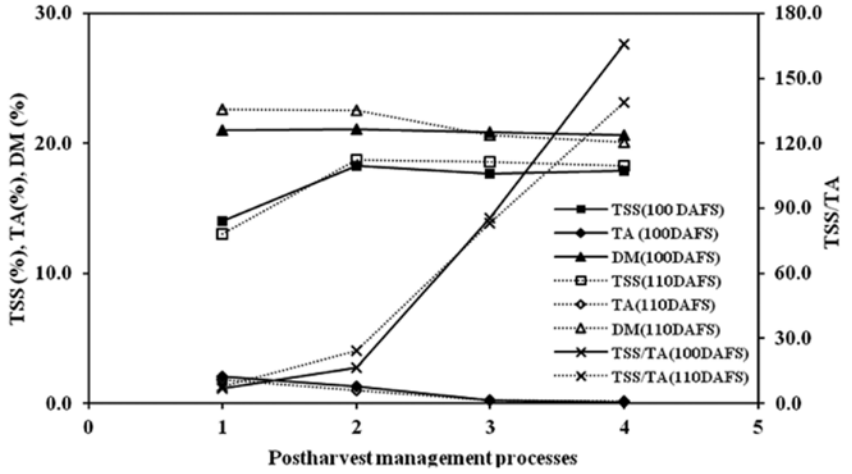

Figure 8. Changes of TSS, TA, DM and TSS/TA of mango fruit harvested at 100 and 110 DAFS (1), stored at 13°C for 21 days (2) and kept at 22°C for 4 (3) and 7 days (4).

Calibration models

The spectral data of mango fruit were treated by various mathematical methods such as Savitzky-Golay smoothing, standard normal variate (SNV), multiplicative scatter correction (MSC) and Savitzky-Golay second derivative before they were used to develop the PLSR calibration models for TSS, TA and DM at each process of postharvest management. The wavelength range was selected using the same wavelength range of PLSR models of puree mixtures of six substances: glucose and sucrose (900-1000 nm), citric acid and malic acid (800-1000 nm), starch (900-1000 nm) and cellulose (800-1000 nm) from previous studies. PLSR model of TSS at harvest was developed using the treated spectral data with Savitzky-Golay second derivative (10 nm average for left and right sides) in the 800-1000 nm wavelength range. The coefficient of determination (R^2), standard error of calibration (SEC), standard error of prediction (SEP), the average of the difference between actual value and NIR predicted value (bias) and ratio of standard deviation of reference data in validation set to SEP (RPD) were 0.87, 1.04%, 1.06%, 0.07% and 2.60, respectively. Before the PLSR model for TSS at cold storage was developed, the spectral data were treated by the MSC and Savitzky-Golay second derivative (10 nm average for left and right sides), obtaining an R^2 of 0.84, SEC of 0.73%, SEP of 0.79%, bias of 0.12% and an RPD of 2.24. To develop the PLSR model for TSS after shelf storage, the spectra were treated by Savitzky-Golay smoothing and second derivative (20 nm average for left and right sides) of the entire process. R^2, SEC, SEP, bias and RPD were 0.84, 0.51%, 0.56%, -0.02% and 2.36, respectively. The best model was developed using the treated spectral data with only the Savitzky-Golay second derivative (10 nm average for left and right sides) of the entire process, obtaining an R^2 of 0.87, SEC of 0.92%, SEP of 0.94%, bias of 0.02% and RPD of 2.69 (Table 1).

PLSR models of TA at harvest, cold storage, shelf storage and the entire process were developed following the same step as the model for TSS. The results at harvest were an R^2 of 0.77, SEC of 0.22%, SEP of 0.23%, bias of 0.04% and RPD of 1.96. The results at cold storage were 0.74, 0.22%, 0.19%, 0.02% and 1.98, respectively. The results at shelf storage were 0.58, 0.04%, 0.04%, 0% and 1.66, respectively, and for the entire process 0.84, 0.29%, 0.28%, 0% and 2.66, respectively (Table1).

To develop the PLSR model for DM, the spectra of mango fruit at each postharvest process were treated using the same mathematical methods. The result of R^2, SEC, SEP, bias and RPD at harvest were 0.83, 0.83%w/w, 0.86%w/w, 0.19%w/w and 2.29. The results at cold storage were 0.85, 0.60%w/w, 0.73%w/w, 0.22%w/w and 3.88, respectively. The results at shelf storage were 0.94, 0.59%w/w, 0.66%w/w, 0.07%w/w and 3.98, respectively, and for the whole process were 0.89, 0.83%w/w, 0.86%w/w, 0.11%w/w and 3.24, respectively (Table 1).

The scatter plot of chemical analysis data of mango fruit, TSS and DM, in both calibration and validation sets, for the entire process showed a high correlation between actual values (conventional analysis method) and predicted values, with the exception of TA (Figures 9, 10 and 11). Meanwhile, the regression coefficient plot exhibited the highest value at various wavelengths, which affected the PLSR model. The results also showed that TSS and DM gave high values at the same wavelengths: 816, 904, 944 and 976 nm, whereas TA had peaks at 778, 888, 956 and 986nm (Figure 12).

Table 1. PLSR calibration results of TSS, TA and DM of mango fruit at harvest, cold storage (13°C, 21 days), shelf storage (22°C, 4 and 7 days) and the entire process.

Constituent	Pre-treatment	Wavelength (nm)	F	R^2	SEC	SEP	Bias	RPD
TSS-Harvesting	2nd Derivative	800-1000	5	0.87	1.04	1.06	0.07	2.60
TSS-Storage	MSC+2nd Derivative	750-1000	6	0.84	0.73	0.79	0.12	2.24
TSS-Shelf	SM+ 2nd Derivative	800-1000	6	0.84	0.51	0.56	-0.02	2.36
TSS-Whole processes	**2nd Derivative**	**800-1000**	**6**	**0.87**	**0.92**	**0.94**	**0.02**	**2.69**
TA-Harvesting	2nd Derivative	800-1000	7	0.77	0.22	0.23	0.04	1.96
TA-Storage	SM+ 2nd Derivative	750-1000	7	0.74	0.22	0.19	0.02	1.98
TA-Shelf	2nd Derivative	800-1000	7	0.58	0.04	0.04	0	1.66
TA- Whole processes	**2nd Derivative**	**750-1000**	**7**	**0.84**	**0.29**	**0.28**	**0**	**2.66**
DM-Harvesting	SM+2nd Derivative	750-1000	6	0.83	0.83	0.86	0.19	2.29
DM -Storage	SM+ 2nd Derivative	750-1000	7	0.85	0.60	0.73	0.22	3.88
DM -Shelf	SM+ 2nd Derivative	800-1000	6	0.94	0.59	0.66	0.07	3.98
DM - Whole processes	**SM+ 2nd Derivative**	**800-1000**	**6**	**0.89**	**0.83**	**0.86**	**0.11**	**3.24**

Note: TSS: total soluble solids (%); TA: titratable acidity (%); DM: dry matter (%w/w); SM: smoothing; 2nd Derivative: second derivative; F: number of factors used in the calibration equation; R^2: coefficient of determination; SEC: standard error of calibration; SEP: standard error of prediction; Bias: average of the difference between actual value and NIR predicted value; RPD: ratio of standard deviation of reference data in validation set to SEP.

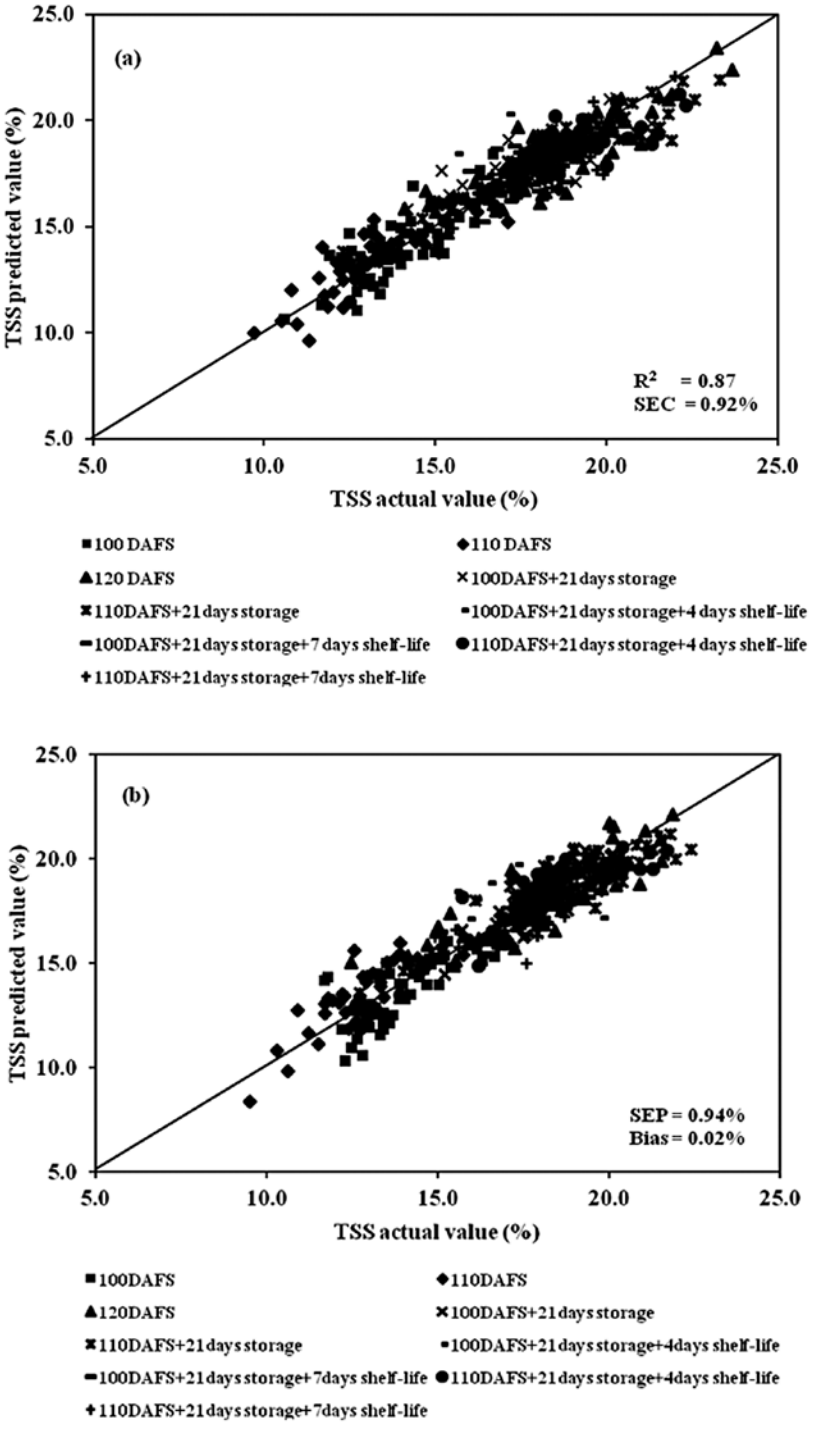

Figure 9. Scatter plot of PLSR calibration results for mango fruit TSS in the whole process of postharvest management (harvesting, cold storage and shelf storage): (a) calibration set and (b) validation set.

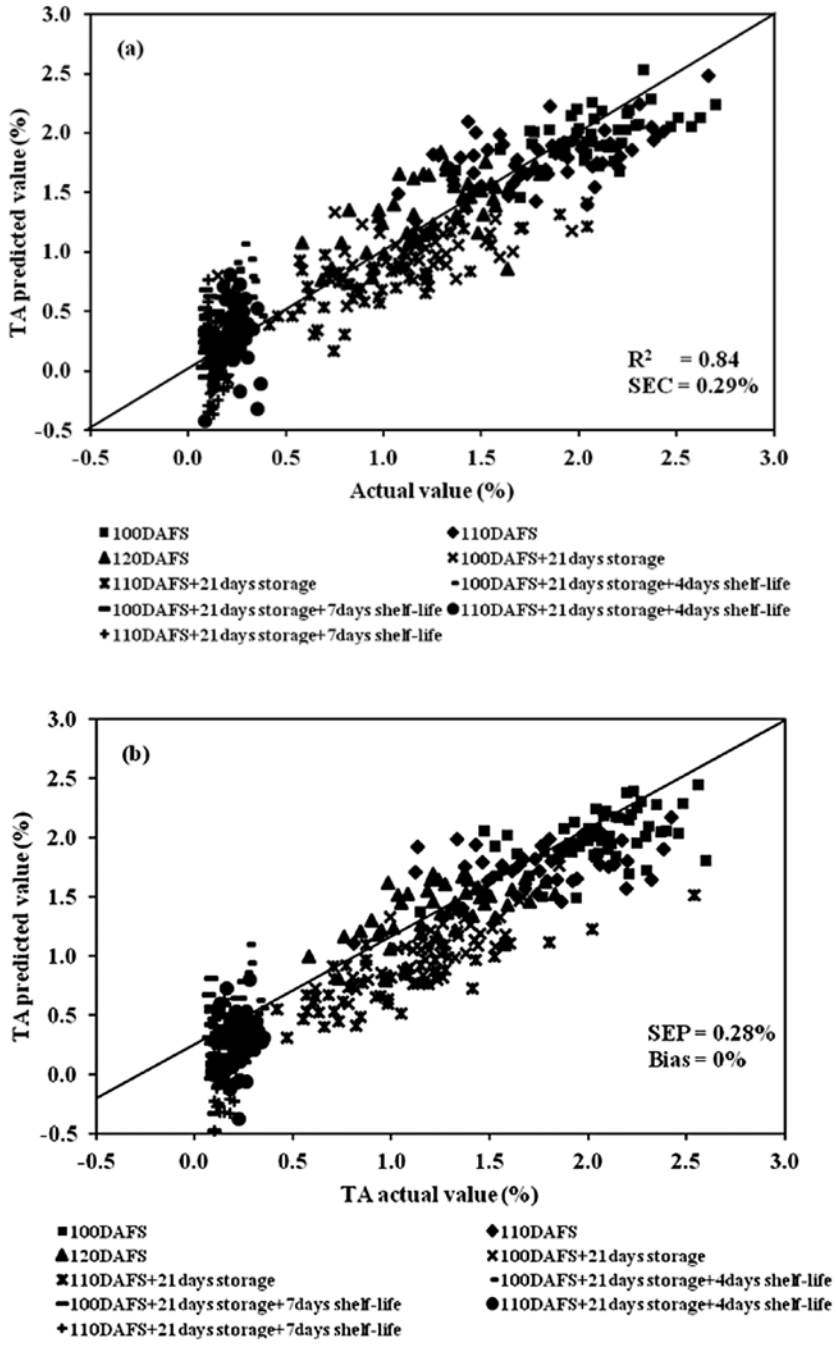

Figure 10. Scatter plot of PLSR calibration results of mango fruit TA for the entire postharvest process (harvest, cold storage and shelf storage): (a) calibration set and (b) validation set.

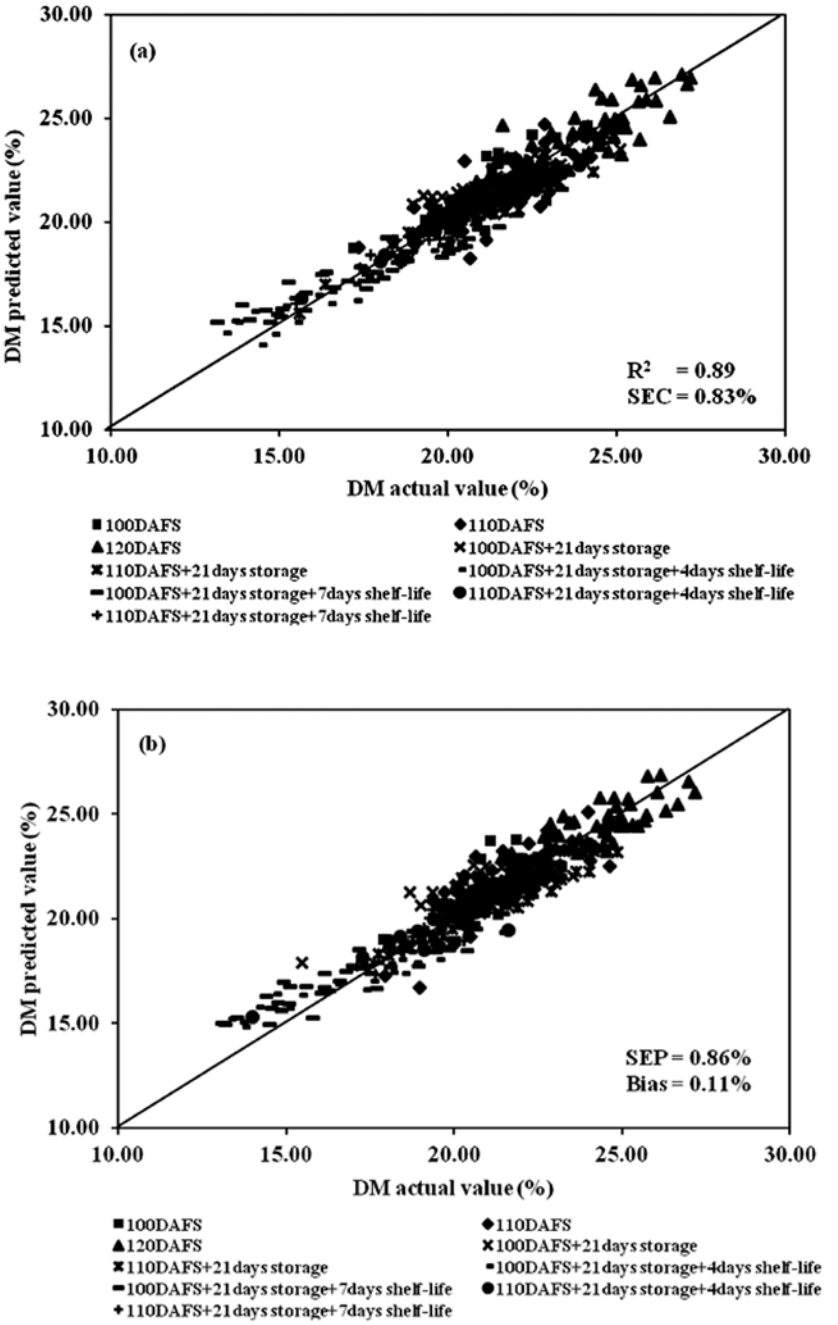

Figure 11. Scatter plot of PLSR calibration results of mango fruit DM for the whole process of postharvest management (harvesting, cold storage and shelf storage): (a) calibration set and (b) validation set.

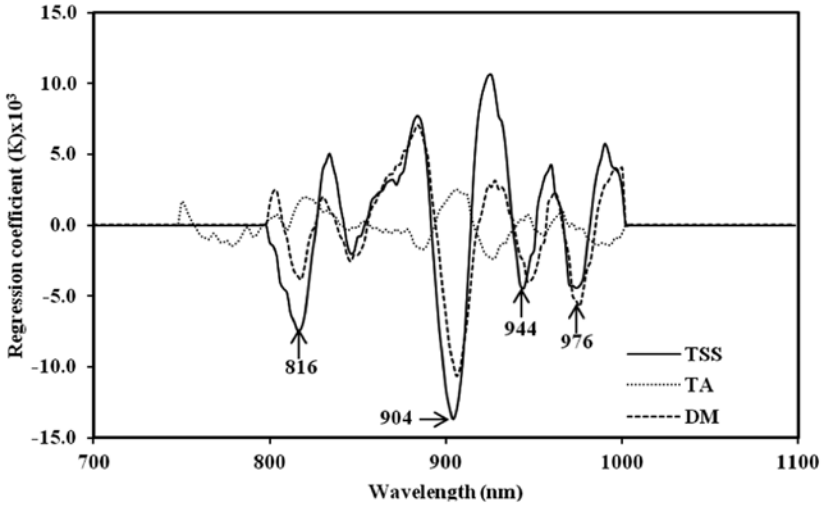

Figure 12. Regression coefficient plot of PLSR calibration models of TSS, TA and DM of mango fruit in the whole process of postharvest management (harvesting, cold storage and shelf storage).

DISCUSSION

Only a clear peak at 980 nm was found on the original spectra of mango fruit from the three harvest times. Mango fruit during cold and shelf storage showed a water absorption band at 978 nm (Saranwong et al., 2003; Theanjumpol et al., 2010). Since it is the main component of mango fruit and the molecule can absorb many wavelength regions in NIRS, such as 770 and 970 nm, this can interfere with the absorption band of other chemical components (Mosenin, 1984). Meanwhile, the absorbance at the water band decreased during the whole process, since weight loss of fruit occured during cold storage. Similar to Tefera et al. (2007), the physiological weight loss of mango fruit occured during storage at a temperature range of 14-19°C and at a relative humidity of 70-82%. After spectral data were adjusted to reduce the effects of baseline shift, overlapping peaks and light scattering (Osborne, 1993 and Williams and Norris, 2001), the peaks shifted slightly, an effect of the pretreatment method. Derivative methods were used to remove or suppress constant background signals and enhance the visual resolution. Each derivative reduces the polynomial order by one, so constant offset is removed. This transformed the linear term into a constant one, thus removing linear tilting of the graph. The second derivative transferred peak maxima into minima and vice versa (Heise and Winzen, 2002).

Color is an important food quality parameter affecting consumer acceptance (Crisosto et al., 2003). When color values were considered, L* and h° values slightly decreased while C* slightly increased to coincide with the increasing of the intensity of the yellow-orange epidermis color and mesocarp (Jesus Ornelas-Paz et al., 2008). This is likely a result of the ripening process. The loss of

green color is due to degradation of the chlorophyll structure, which associates with the synthesis of carotenoids, with pigment ranging from yellow to red. It remains a compound and intact in the tissue, even when extensive senescence has occurred. Carotenoids may be synthesized during the development stage of plants, but remain masked by the presence of chlorophyll. Moreover, it can be synthesized during fruit ripening. Following the degradation of chlorophyll, the carotenoid pigments become visible (Wills et al., 1998 and Vazquez-Caicedo et al., 2005). Texture softening occurs during storage and ripening, which directly affects fruit shelf life and quality (Yashoda et al., 2007). Since cell wall hydrolases increase activity during ripening, this results in disassembly, depolymerization and dissolution of pectin and other hemicellulosic polysaccharides (Fry, 1995). Moreover the cell wall degrading enzymes, glycanase and glycosidase, were indentified in ripe mango (Yashoda et al., 2007). In addition, in climacteric fruit the rapid synthesis of polygalacturonase (PG) activity coincides with considerable textural alteration (loss of firmness) and conversion of pectic polysaccharides into water-soluble galacturonides during ripening (Lasan et al., 1986). TSS of mango fruit increased substantially after harvest until storage, and remained constant during the shelf-storage period. This is because mango is a climacteric fruit, which tends to have increased soluble solid concentration until a maximum is reached at the fully-ripe stage, followed by a decreasing trend when the fruit reaches full senescence (Jha et al., 2006 and Tefera et al., 2007). In contrast, TA slightly decreased from harvest to storage for 4 days and then remained almost constant during shelf storage for 7 days. TA decreased dramatically during ripening. This is associated with rapid ripening and senescence of mangoes when stored at higher temperatures. Changes in TA and pH are based on changes in citric, malic and ascorbic acid (Tefera et al., 2007). TSS/TA increased substantially during cold storage and shelf storage for 4 and 7 days because of its relation to TSS and TA. DM of mango fruit were composed of carbohydrates – starch and sugar – that changed slightly during cold and shelf storage. During the ripening process, starch is hydrolyzed to simple sugars (Kumar et al., 1994). Morover, DM relates to shelf life; mango fruit has a high percentage of dry matter and consequently a short shelf life (Hofman et al., 1997). So mango fruit quality components in terms of color, texture, TSS, TA, TSS/TA and DM changed during postharvest management. These occurred after storage at 13°C. This is similar to the findings of Lederman et al. (1997) who found that mango fruit normally ripen when stored at 14°C.

The PLSR provided the results of TSS, TA and DM in terms of R^2, SEC, SEP and bias of mango fruit. The results of the entire process were better than each process since it was obtained from a variety of samples (Osborne et al., 1993; William and Norris, 2001 and Siesler et al., 2002). Particularly, RPD values were 2.69, 2.66 and 3.24, respectively. RPD values of TSS and TA PLSR models were in the range of 2.4-3.0, which can be used for rough screening. Meanwhile, the RPD of DM PLSR model was in the range of 3.1-4.9, which can be applied in screening (William and Norris, 2001). This is similar to considerable prior research reporting that NIRS can be used to assess the quality of fruit, such as

soluble solids content (SSC) and acidity (pH) of Satsuma mandarin oranges (Gomez et al., 2006), SSC and TA of Valencia Late orange (Cayuela, 2008) and SSC of apple (Fan et al., 2009).

This research reveals that NIRS can be applied to screen the quality of mango fruit during postharvest management stages including harvesting period, cold storage and shelf storage. By using the calibration models, the quality of mango fruit in term of TSS, TA and DM can be predicted. However, the exact quality should be confirmed by consumer preferences. NIRS could be installed in the sorting line system for automatica screening of mango fruit quality.

ACKNOWLEDGEMENTS

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REFERENCES

Cayuela, J.A. 2008. Vis/NIR soluble solids prediction in intact oranges (Citrus sinensis L.) cv. Valencia Late by reflectance. Postharvest Biology and Technology 47: 75-80. 10.1016/j.postharvbio.2007.05.005

Crisosto, C.H., G.M. Crisosto, and P. Metheney. 2003. Consumer acceptance of 'Brook' and 'Bing' cherries is mainly dependent on fruit SSC and visual skin color. Postharvest Biology and Technology 28: 159-167. 10.1016/S0925-5214(02)00173-4

Fan, G., J. Zha, R. Du, and L. Gao. 2009. Determination of soluble solids and firmness of apples be Vis/NIR transmittance. Journal of Food Engineering 93: 416-420. 10.1016/j.jfoodeng.2009.02.006

Fry, S.C. 1995. Polysaccharide-modifying enzymes in the plant cell wall. Annual Reviews of Plant Physiology and Plant Molecular Biology 46: 497-520.

Haidar, J., and T. Demisse. 1999. Malnutrition and xerophthagma in rural communities of Ethiopia. East Africa Medical Journal 10: 590-593.

Heise, H.M., and R. Winzen. 2002. Chemometrics in Near Infrared Spectroscopy. p.125-162. In H.W. Siesler, Y. Ozaki, S. Kawata, and H.M. Heise. (eds.), Near-Infrared Spectroscopy: Principle, Instrument, Application. WILEY-VCH Verlag GmbH. Germany.

Hofman, P.J., L.G. Smith, D.C. Joyce, G.I. Johnson, and G.F. Meiburg. 1997. Bagging of mango (Mangifera indica cv. Keitt) fruit influences fruit quality and mineral composition. Postharvest Biology and Technology 12: 83-91. 10.1016/S0925-5214(97)00039-2

Iwamoto, M., S. Kawano, and Y. Ozaki. 1995. An overview of research and development of near infrared spectroscopy in Japan. Journal of Near Infrared Spectroscopy 3: 179-189.

Jha, S.N., A.R.P. Kingsly, and S. Chopra. 2006. Physical and mechanical properties of mango during growth and storage for determination of maturity. Journal of Food Engineering 72: 73-76. 10.1016/j.jfoodeng.2004.11.020

Jesus Ornelas-Paz, J.D., E.M. Yahia, and A.A. Gardea. 2008. Changes in external and internal color during postharvest ripening of 'Manila' and 'Ataulfo' mango fruit and relationship with carotenoid content determined by liquid chromatography –Apcl+-time-of-flight mass spectrometry. Postharvest Biology and Technology 50: 145-152. 10.1016/j.postharvbio.2008.05.001

Kumar, S., D.K Das, A.K. Singh, and U.S. Prasad. 1994. Sucrose metabolism during maturation and ripening of mango cultivars. Plant Physiology and Biochemistry 21: 27-32.

Lazan, H., Z. M. Ali, L.K. Wah, J. Voon, and G.R. Chaplin. 1986. The potential role of polygalacturonase in pectin degradation and softening of mango fruit. ASEAN Food Journal 2: 93-98.

Lederman, I.E., G. Zauberman, A. Weksler, I. Rot, and Y. Fuchs. 1997. Ethylene-forming capacity during cold storage and chilling injury development in 'Keitt' mango fruit. Postharvest Biology and Technology 10: 107-112.

Mohsenin, N.N. 1984. Electromagnatic Radiation Properties of Foods and Agricultural Products. Gordon and Breach, Science Publishers, Inc. USA. 673 pp.

Mukherjee, S. K. 1997. Introduction: Botany and Importance. p. 1-19. In R. E. Litz, (ed) The Mango:Botany, Production and Uses. CABI Publishing. UK.

Nakasone, H. Y., and R. E. Paull. 1998. Tropical Fruit. CABI International. UK. 445 pp.

Osborne, B. G. 1986. Near Infrared spectroscopy in Food Analysis. p. 1-14. In R. A. Meyers (ed), Encyclopedia of Analytical Chemistry. John Wiley&Sons Ltd, Chichester.

Osborne, B. G., T. Fearn, and P. H. Hindle. 1993. Practical NIR Spectroscopy with Applications in Food and Beverage Analysis. Longman Group UK Limited1986, UK. p. 227.

Saranwong, S., J. Sornsrivichai, and S. Kawano. 2003. Performance of a portable NIR instrument for brix value determination of intact mango fruit. Postharvest Biology and Technology 11:175-181.

Saranwong, S., J. Sornsrivichai, and S. Kawano. 2004. Prediction of ripe-stage eating quality of mango fruit from its harvest quality measured nondestructively by near infrared spectroscopy. Postharvest Biology and Technology 31: 137-145. 10.1016/j.postharvbio.2003.08.007

Siesler, H.W., Y. Ozaki, S. Kawata, and H.M. Heise. 2002. Near-Infrared Spectroscopy: Principle, Instrument, Application. WILEY-VCH Verlag GmbH, Germany(2002).

Tefera, A., T. Seyoum, and K. Woldetsadik. 2007. Effect of disinfection, packaging and storage environment on the shelf life of mango. Biosystems Engineering 96(2): 201-212. 10.1016/j.biosystemseng.2006.10.006

Theanjumpol, P., V. Sardsud, and G. Self. 2010. Effect of mango fruit sampling position on quality assessment by near infrared spectroscopy. Journal of Agricultural Science 41(1) (Suppl.): 409-412.

Vazquez-Caicedo, A.L. P. Sruanmsiri, R. Carle, and S. Neidhart. 2005. Accumulation of all- tran-β-carotene and its 9-cis and 13 – cis stereoisomer during postharvest ripening of nine Thai mango cultivar. Journal of Agricultural and Food Chemistry 53: 4827-4835. 10.1021/jf048168h

Wills, R., B. McGlasson, D. Graham, and D. Joyce. 1998. Postharvest: An Introduction to the Physiology & Handling of Fruit, Vegetable & Ornamantals. 4 th (ed). Hyde Park Press. Australia. 262p.

Williams, P., and K. Norris. 2001. Near-Infrared Technology in the Agricultural and Food Industries. 2nd (ed). American Association of Cereal Chemists, Inc, USA.

Yoshida, H.M., T.N. Prabha, and R.N. Tharanathan. 2007. Mango ripening-Role of carbohydrases in tissue softening. Food Chemistry 102: 691-698.

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Biotechnological Valorization of Cashew Apple: a Review

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ABSTRACT

Cashew apple, the peduncle of cashew fruit, is an agricultural waste byproduct from harvesting cashew nuts. Cashew apple juice contains about 10% reducing sugar. Its bagasse contains about 20% of cellulose. The byproducts can be used as a substrate for several microbial fermentation processes. Wine and bioethanol were produced by Saccharomyces cerevisiae. Probiotic beverage and lactic acid were produced by Lactobacillus casei. Biosurfactants-rhamnolipids, emulsan and surfactin were synthesized by Pseudomonas aeruginosa, Acinetobacter calcoaceticus and Bacillus subtilis, respectively. Tannase and pectinase were produced during solid-state fermentation of Aspergillus spp. Prebiotic oligosaccharides were synthesized by the activity of dextransucrase produced by Leuconostoc spp. Cashew apple is a potential substrate for producing a variety of products, depending on the type of microorganisms used.

Keywords: Cashew apple, Ethanol, Biosurfactant, Beverage, Enzyme, Oligosaccharide

CASHEW APPLE

Cashew (*Anacardium occidentale*) is a tropical evergreen tree cultivated in a range of countries, including India, Vietnam, Brazil and Thailand (Clay, 2004). It is grown for the cashew nut industry. The peduncle, or cashew apple (Figure 1), is a waste byproduct of the cashew nut harvest. The cashew apple contains about 10 g of total sugar and 200 mg of ascorbic acid per 100 ml juice, as shown in Table 1 (Figueiredo et al., 2002; Attri, 2009). Most cashew apple is left in the field as agricultural waste (Figure 2). The weight of the leftover cashew apple is about 10 times of the harvested nuts (Attri, 2009). Global production of cashew nuts was 1.6 million tons in 2000, implying almost 16 million tons of cashew apples were underutilized.

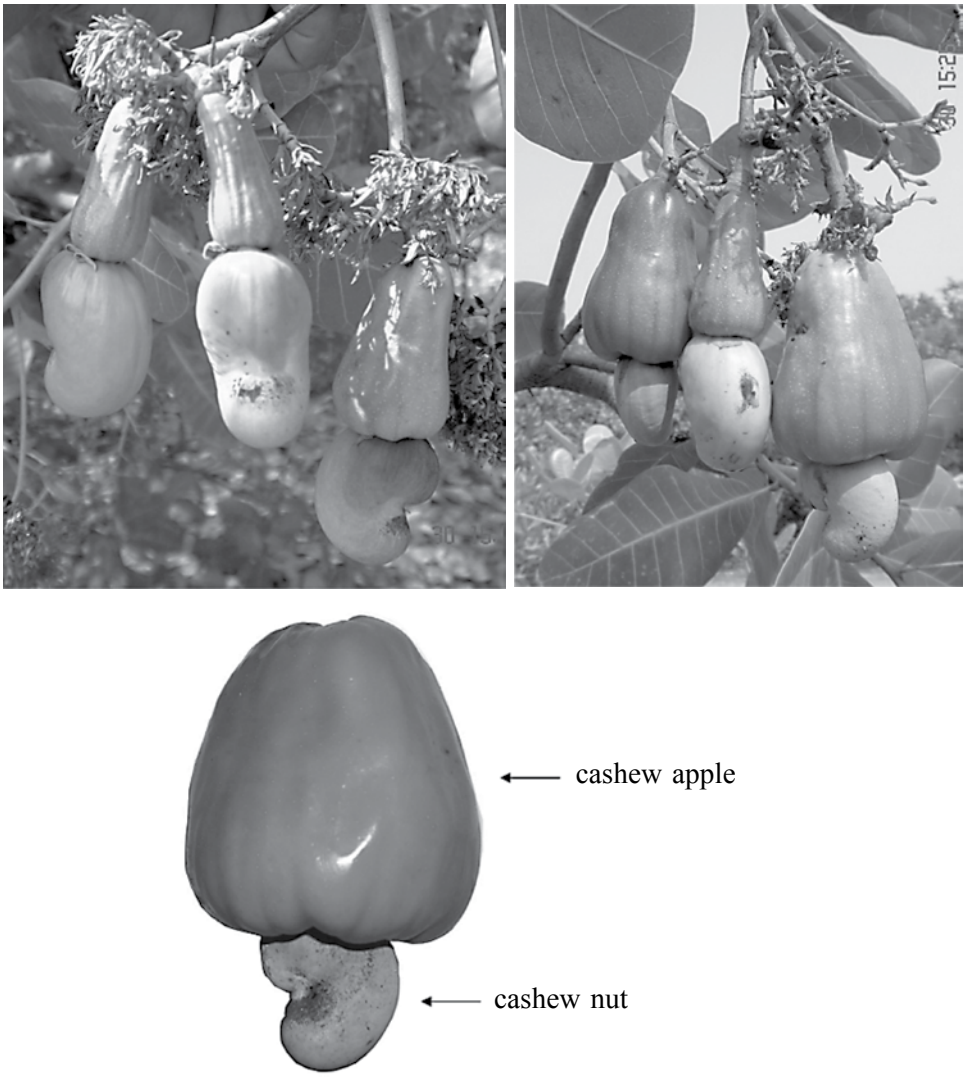


Figure 1. Cashew fruit, cashew apple and cashew nut.

Table 1. Chemical composition of cashew apple juice and bagasse.

Composition	Value	References
Cashew apple juice		
Total soluble solid (% w/v)	7.4-14.5	Oduwole et al. (2001); Zepka et al. (2009)
Reducing sugar (% w/v)	9.04-10.4	Oduwole et al. (2001); Honorato et al. (2007)
Glucose (% w/v)	3.85-4.63	Azevedo and Rodrigues (2000); Honorato and Rodrigues (2010)
Fructose (% w/v)	3.90-4.52	Azevedo and Rodrigues (2000); Honorato and Rodrigues (2010)
Sucrose (% w/v)	0.042-0.051	Azevedo and Rodrigues (2000)
Total acidity (% as malic acid)	0.29-1.1	Inyang and Abah (1997)
Malic acid (% w/v)	0.4	Rocha et al. (2007)
Citric acid (% w/v)	0.42-0.64	Azevedo and Rodrigues (2000)
Ascorbic acid (mg/100 ml)	104-293.5	Oduwole et al. (2001); Assunção and Mercadante (2003)
pH	3.5-4.6	Michodjehoun-Mestres et al. (2009); Zepka et al. (2009)
Total tannins (mg/100 g)	0.6	Rocha et al. (2007)
Condensed tannins (mg/100 g)	0.2	Rocha et al. (2007)
Carotene (mg/100 g)	0.03-0.74	Rocha et al. (2007)
Cashew apple bagasse		
Cellulose (%)	19.21-24.3	Rocha et al. (2009a); Rodrigues et al. (2011)
Hemicellulose (%)	12.05-12.5	Rocha et al. (2009a); Rodrigues et al. (2011)
Lignin (%)	22.5-38.11	Rocha et al. (2009a); Rodrigues et al. (2011)
Protein (%)	14.2	Rocha et al. (2009a)
Non-fiber carbohydrate (%)	11.3	Rocha et al. (2009a)

Figure 2. Cashew apple waste produced during harvesting of the cashew nut.

Cashew apples have the potential to be processed into juice, syrup, jam, ice cream, candy, chutney, pickle, and other products (Rabelo et al., 2009). Cashew apples can also be utilized through biotechnology, which depending on the substrates and microorganisms can yield a variety of products.

This review aims to summarize the current research regarding the potential of cashew apples to be fermented into different products, including: wine, bioethanol, enzymes, biosurfactants, probiotic beverages, lactic acid and oligosaccharides (Figure 3)

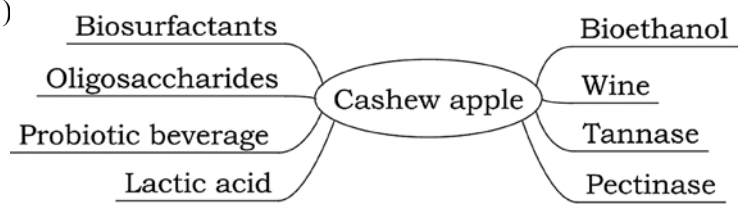


Figure 3. Potential products from fermentation of cashew apple.

PRE-FERMENTATION TREATMENT

The cashew apple can initially be decontaminated by washing in 100 ppm chlorine water before juice extraction (Muir-Beckford and Badrie, 2000).

Tannins are a group of phenolic compounds that can form strong complexes with proteins and other macromolecules. The cashew apple contains about 0.6 mg tannins/100 g juice (Rocha et al., 2007). The tannins can form complexes with salivary protein and glycoprotein, resulting in astringency (Fontoin et al., 2008). Ingested tannin could inhibit digestive enzymes and affect the utilization of nutrients (Chung et al., 1998a). However, tannins also have beneficial health effects, including: acceleration of blood clotting, reduction of blood pressure, treatment of burn wounds, modulation of immune response as well as antimicrobial and anticarcinogenic properties (Chung et al., 1998b; Chokotho and Hasselt, 2005). Removal of tannins from cashew apples can be accomplished by adding proteins (e.g., gelatin) or starch (e.g., cassava starch, rice gruel, sago), followed by filtration or siphoning (Jayalekshmy and John, 2004; Cormier, 2008). Among these tannin-precipitating agents, gelatin was the most commonly used. However, different levels of gelatin (ranging from 0.3 to 1.0% w/v) have been reported. The cost-effective amount of gelatin for precipitating tannins in cashew apples should be evaluated.

Pectinase can be added to increase the extraction yield and clarification of fruit juice (Gummadi et al., 2007). Pectinase is a group of enzymes, composed of pectin lyase, pectinesterase and polygalacturonase. However, pectin degradation caused by pectinesterase during fermentation releases methanol into the products. For example, application of pectinase (Rapidase ADEX-D at 100 g/ton) in apple juice increased methanol content in apple spirit from 51.9 mg/100 ml (no pectinase treatment) to 398.7 mg/100 ml, higher than the United States FDA limit for fruit spirits at 280 mg/100 ml (Zhang et al., 2011). Increasing of

methanol content could be prevented by the use of pectin lyase instead of mixed pectinase containing pentinesterase (Wu et al., 2007).

Due to its high mineral content, adding minerals to cashew apple juice may not be necessary for production of dextransucrase, which is used for the synthesis of dextran from sucrose (Rabelo et al., 2009; Honorato and Rodrigues, 2010). Must has been nourished before wine fermentation by adding Becoplex (consisting of 10 mg vitamin B_1, 3 mg B_2, 1 mg B_6 and 50 mg vitamin C), which served as coenzymes for the microorganism. The B-complex vitamins were essential for lactic acid bacteria, because the microorganism cannot synthesize them. Diammonium phosphate, a widely used assimilable nitrogen for wine yeast, can be added at 2.2% (Muir-Beckford and Badrie, 2000; Ribéreau-Gayon et al., 2006).

Depending on the microorganisms used in fermentation, the pH of the medium may be adjusted to the optimum pH of the microorganisms. In cashew wine production, the pH of must was adjusted down from pH 4.7-5.1 to pH 3.5 with citric acid (Muir-Beckford and Badrie, 2000). The pH of fermentation media were adjusted to 7.0 for production of biosurfactants from cashew apple juice by *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* (Rocha et al., 2006; Rocha et al., 2007).

Elimination of wild microorganisms before wine fermentation can be accomplished by adding 50 ppm sodium metabisulfite (Muir-Beckford and Badrie, 2000). However, sodium bisulfate may cause off-flavor in wine and was also banned in the United States due to health concerns about the sodium content (Rivard, 2009). Potassium metabisulfite, with lower sulfur dioxide content, can be used instead (Sanchez, 2008). Filtration of the juice through 0.45 μm filter or exposing to ultraviolet radiation for 1 h can also be used (Rocha et al., 2006; Rocha et al., 2007). However, turbidity and juice color may interfere with exposure to ultraviolet light. Therefore, any ultraviolet process should be followed by filtration through 0.2 μm membrane for sterilization purposes (Udeh, 2004).

WINE AND BIOETHANOL

Cashew apple juice contained about 10% (w/v) of total sugar. Production of bioethanol from this level of sugar resulted in about 4.4% (w/v) of final ethanol concentration (Pinheiro et al., 2008). However, for production of cashew apple wine, initial sugar content was usually adjusted to above 20% (w/v) by adding sucrose to obtain a higher final ethanol concentration. The size of yeast inocula ranged between 0.1 to 12% (v/v) (Sudheer Kumar et al., 2009; Ogunjobi and Ogunwolu, 2010). However, an inoculum size of 10^5 cells/ml was desirable for wine making, because it provided high concentration of esters, lactones and free monoterpenes, while higher alcohols and medium chain fatty acids were less than other inoculum sizes (Carrau et al., 2010). Fermentation usually took place under ambient temperature for at least two weeks under static conditions. However, fermentation time depended on the fermentation temperature. For example, wine fermentation at 15°C required 500 h to reach dryness (less than 2 g sugar/l), while fermentation at 28°C required only 184 h (Molina et al., 2007). Final ethanol

concentration ranged between 5 to 12% (Muir-Beckford and Badrie, 2000; Silva et al., 2007; Attri, 2009; Ogunjobi and Ogunwolu, 2010). Figure 4 shows the process for producing cashew apple wine. Fermentation conditions, initial sugar concentration and final ethanol concentration of cashew apple wine and ethanol are shown in Table 2.

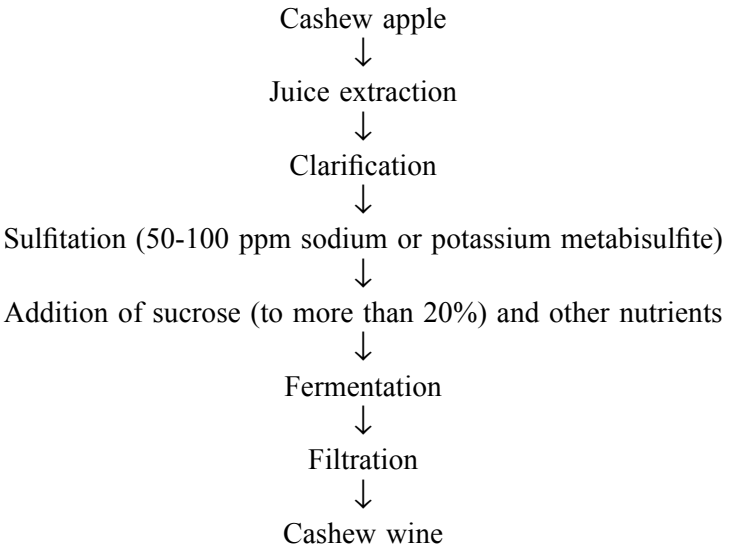


Figure 4. Processing diagram of cashew apple wine.

Osme GC-olfactory analysis revealed that the sweet, fruity and cashew-like aroma of cashew apple wine was contributed by ester compounds, mainly methyl 3-methyl butyrate, ethyl 3-methyl butyrate, methyl butyrate, ethyl butyrate, *trans*-ethyl crotonate and methyl 3-methyl pentanoate. The sweaty odor of 2-methyl butanoic acid was a primary reason for the unpleasant characteristic of the wine (Garruti et al., 2006b). Fermenting the wine at 18°C produced higher concentrations of fruity and sweet flavor compounds and lower concentrations of undesirable compounds when compared with fermentation at 30°C (Garruti et al., 2006a).

Cashew apple wine could also be produced from dried cashew apple. Because cashew apple is highly perishable and not available throughout the year, preservation of cashew apple can be accomplished by drying and grinding into cashew apple powder. This powder can be mixed with water at 75 g/L to prepare must for wine fermentation, which had initial total soluble solids of 20.0%. Alcohol content of wine from cashew apple powder was 7.0% v/v, lower than wine from fresh cashew apple juice (9.2% v/v). Although wine from cashew apple powder was light brown in color, its sensory scores were comparable to wine from fresh cashew apple juice and higher than commercial kola wine, cocoa wine and tea wine (Ogunjobi and Ogunwolu, 2010).

Cashew juice extraction leaves bagasse of about 20% of the total fruit weight.

Table 2. Processing conditions and final ethanol concentration of cashew apple wine and bioethanol.

Products	Microorganism	Yeast added (% v/v)	Initial total soluble solid (% w/v)	Fermentation time	Fermentation temperature (°C)	Final total soluble solid (% w/v)	Final ethanol concentration (% w/v)	References
Dry and sweet wine	*Saccharomyces cerevisiae* var. ellipsoideus	0.3	21 (dry) 23 (sweet)	3 weeks	23	4.1-4.3 (dry) 9.3-9.5	11.59-11.69 (dry) 11.86-11.90 (sweet)	Muir-Beckford and Badrie (2000)
Wine (2 step fermentations)	Fleishmann™ *Saccharomyces cerevisiae*	2	Step 1: 150 Step 2: 170	Step 1: 15 h Step 2: 33 h		10, 88	10.29	Silva et al. (2007)
Wine from cashew apple powder and fresh juice	*Saccharomyces cerevisiae* (Baker's yeast)	0.1	20.0 % TSS	14 days	28	7.0 (powder), 9.2 (fresh)	5.2 (powder), 6.0 (fresh)	Ogunjobi and Ogunwolu (2010)
Wine	Active S. *cerevisiae* var. ellipsoideus	5	20, 22, 24	15 days	28-30	12.4, 12.8, 13.2	7.81, 8.25, 8.90	Attri (2009)
Bioethanol	*Saccharomyces cerevisiae* var. ellipsoideus	5	15	Aeration 24 h, static 2 weeks	28	3%	7.70	Joseph (2010)
Bioethanol	*Saccharomyces cerevisiae*	0.2	26.5	32 h	32		6.5	Neelakandan et al. (2010)
Bioethanol	*Zymomonas mobilis* MTCC 090	10	28.5	37.15 h	32		12.64	Karuppaiya et al. (2009)
Bioethanol	*Saccharomyces cerevisiae* (baker yeast)	1	8.77, 10.31	4 h, 6h	30		4.28, 4.44	Pinheiro et al. (2008)

This bagasse contains 19-24% cellulose, 12% hemicelluloses and 22-38% lignin on a dry-weight basis (Rocha et al., 2009a; Rodrigues et al., 2011). Cellulose is a polymer of glucose units linked by β-glycosidic bond that can be hydrolyzed by β-glycosidase. Glucose obtained from enzymatic hydrolysis can be used for ethanol production. However, the cellulose molecules are naturally packed in a crystalline structure and associated with hemicelluloses and lignin. As a result, the cellulose molecules are inaccessible for enzymatic hydrolysis. Thus, pretreatment is required for removal of lignin to improve enzymatic saccharification (Laxman and Lachke, 2009).

Many pretreatment methods were introduced to improve enzymatic hydrolysis of cellulose. Steam explosion is widely used in the industry. Among chemical treatments, alkaline treatment is the most successful. Pretreatment of cashew apple bagasse in alkaline solution was shown to be effective for increase the availability of cellulose for enzymatic hydrolysis. Pretreatment of cashew apple bagasse by autoclaving (121°C, 15 min) in 0.8 M sulfuric acid followed by autoclaving in 4% sodium hydroxide solution for 30 min was more effective than using the acid solution alone. The autoclave was vented within 10 min of the cycle end. Cellulase released 52.4 g/L of glucose from a mixture containing 16% w/v of alkaline treated bagasse. After fermentation for 6 h, 20 g/L of ethanol was obtained (Rocha et al., 2009a).

Cashew apple bagasse contains about 12% hemicelluloses. Xylose is the most abundant monomer unit of the hemicelluloses. However, native strains of *Saccharomyces cerevisiae* cannot utilize xylose. But some native strains of *Pichia, Candida* and *Kluyveromyces*, as well as genetically modified *S. cerevisiae* strain, can convert xylose to ethanol (Rocha et al., 2011).

ENZYMES

Tannase

Tannase, or tannin acyl hydrolase (EC 3.1.1.20), is an enzyme that catalyzes the hydrolysis reaction of hydrolysable tannin and gallic acid esters. The products of the reaction are gallic acid and glucose, which can be utilized by microorganisms for energy metabolism (Rodrigues et al., 2008). Tannase is widely produced by the fungi in the genus of *Aspergillus* and *Penicillium*. Some yeast and bacteria also have tannase producing capability. Tannase has been used for production of gallic acid – a substrate for the manufacturing of propyl gallate and trimethoprim. Tannase has also been used for clarification of wine and fruit juices to prevent haze formation and sedimentation (Belur and Mugeraya, 2011).

Tannase production from cashew apple bagasse can be acheived by solid-state fermentation of *Aspergillus oryzae*. The optimal moisture content for producing tannase was about 40%. Higher or lower moisture content decreased the enzyme production rate. Microbial production of tannase required an inducer-tannin. Due to the presence of tannin in cashew apple (0.64 mg/100 g cashew apple pulp), tannase activity was detectable after inoculation of the fungi (Campos et al., 2002). One unit of tannase activity was the amount of enzyme that catalyzed the production

of 1 μmol of gallic acid/min under assay condition. However, addition of tannic acid at 2.5% w/w increased tannase activity more than fourfold. Supplementation with higher concentrations of tannic acid caused growth inhibition, resulting in less enzyme synthesis. Organic nitrogen sources such as peptone and yeast extract had no effect on enzyme synthesis due to complex formation between tannin and protein. In contrast, an inorganic counterpart, e.g. ammonium sulphate, increased enzyme production. Supplementation with ammonium sulphate at 2.5% was suitable for better productivity of tannase. Tannase activity and productivity reached its maximum (3.42 U/g_{ds} and 0.128 U/g_{ds}.h, respectively) at fermentation times between 24 to 48 h, before decreasing thereafter (Rodrigues et al., 2007).

Inoculum size also played an important role in tannase production, like other products produced by solid-state fermentation. Increasing size of inocula helped improve enzyme production. A temperature range between 30-35°C was suitable for tannase production by *A. oryzae*. Moreover, tannase activity was also increased by supplementation with sucrose and starch, but not glucose (Rodrigues et al., 2008). However, tannase produced from cashew apple bagasse was lower than tamarind seed, wheat bran or jamun leaves (Table 3).

Pectinase

Pectin or pectic substances are complex polysaccharides containing galacturonic acid as a basic monomer. The carboxyl groups of some galacturonic acids are methylesterified, with the degree of methoxylation used to determine the quality of pectin. Pectinases are a group of enzymes that catalyze the reaction for degrading pectic substances. Pectinase are divided into three groups: (1) protopectinases that degrade insoluble protopectin to polymerized soluble pectin; (2) esterases that act on the ester linkage and depolymerase that acts on the main polymer chain and (3) depolymerases that hydrolyse glycosidic bonds between galacturonic acid moieties and play a major role in pectin breakdown during fruit ripening (Jayani et al., 2005). Pectinases have many uses in the food industry, including clarification of fruit juice, extraction of juice and oil and treatment of wastewater (Gummadi et al., 2007).

Pectin esterase can be prepared by solid-state fermentation of fruit waste containing pectin, e.g. cashew apple, banana, pineapple and grape, by *Aspergillus* sp. The cashew apple bagasse was dried to a moisture content of 8 to 10% (w/w) and inoculated with *A. foetidus* at $2x10^7$ spore/g for 6 days. A combination of urea and ammonium sulphate (1.5% and 5% of waste mass, respectively) was a suitable nitrogen source for growth of the fungi in cashew apple. The highest activity of pectin esterase in cashew apple waste (0.29 U/mg) was obtained by a fermentation temperature of 40°C for 8 days. However, the enzyme activity was lower than that prepared from grape waste (0.35 U/mg), but higher than a mixture of orange bagasse and wheat bran (0.071 U/mg) (Silva et al., 2005; Venkatesh et al., 2009).

Many factors influenced polygalacturonase production by *Aspergillus niger* CCT0916 in cashew apple bagasse. Moisture content positively effected polygalacturonase and pectinolytic activities (study range was between 30 to 50% wb).

Table 3. Tannase produced from cashew apple bagasse compared with other substrates.

Raw material	Microorganisms	Initial moisture (%)	Nutrient supplementation	Fermentation condition	Tannase activity	References
Cashew apple bagasse	*Aspergillus oryzae* (10^7 spores/g)	40.4	2.5% tannic acid, 1% ammonium sulphate	30°C, 48 h	3.42 U/gds*	Rodrigues et al. (2007)
Cashew apple bagasse	*Aspergillus oryzae* (10^7 spores/g)	40.4	2.5% tannic acid, 2.5% ammonium sulphate, 1% sucrose	30°C, 48 h	4.63 U/gds	Rodrigues et al. (2008)
Tamarind seed powder	*Aspergillus niger* ATCC 16620 (33×10^9 spores/5 g)	65.75	1% glycerol, 1% potassium nitrate	30°C, 120 h	6.44 U/gds	Sabu et al. (2005)
Palm kernel cake	*Aspergillus niger* ATCC 16620 (11×10^9 spores/5 g)	53.5	5% tannic acid	30°C, 96 h	13.03 U/gds	Sabu et al. (2005)
Coffee husk	*Lactobacillus* sp. ASR S1 (8×10^8 cells/5 g)	50	0.6% tannic acid	33°C, 72 h	0.85 U/gds	Sabu et al. (2006)
Jamun leaves	*Aspergillus ruber*	1 g substrate: 2 ml tap water (pH 5.5)	Carbon and nitrogen source had no positive effect	30°C, 96 h	69 U/gds	Kumar et al. (2007)
Wheat bran	*Aspergillus aculeatus* DBF9	80	5% tannic acid	30°C, 72 h	8.16 U/g	Banerjee et al. (2007)

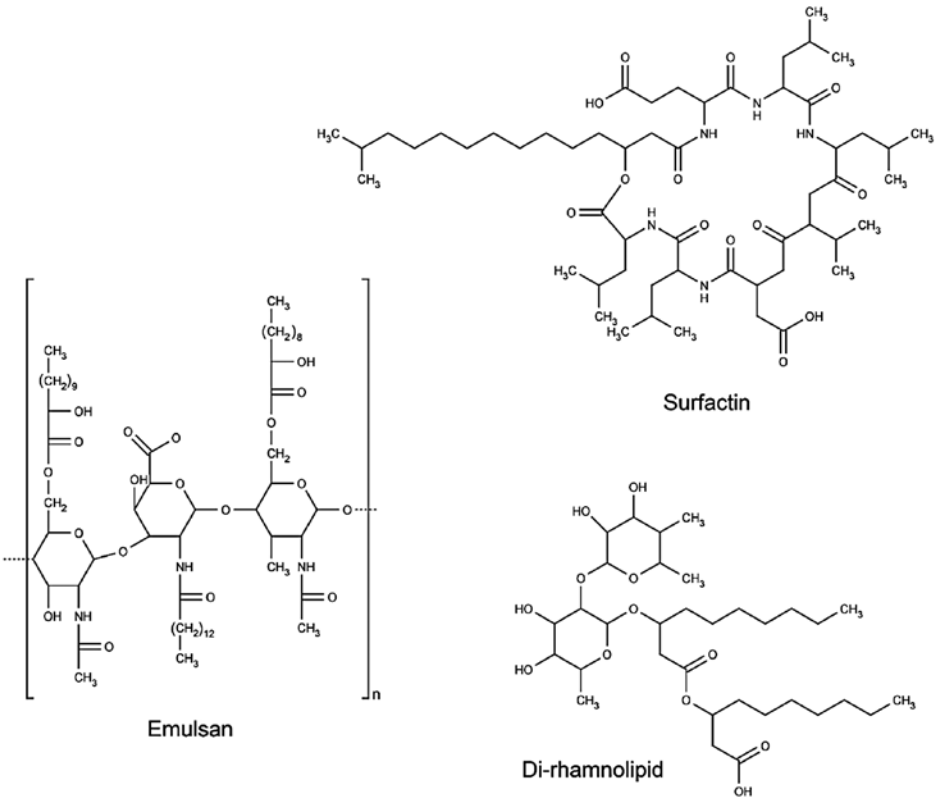
Note: *gds = gram per dry substrate.

Ammonium sulphate (range from 0.5 to 1.5%) negatively effected enzyme production (Alcântara et al., 2010). In another study, treatment using an ammonium sulphate concentration of 1.5% resulted in the highest polygalacturonase activity, although this treatment also included other factors, including spore concentration of 10^6 spores/g medium, temperature of 35°C and fermentation period of 29 h (Alcântara and da Silva, 2011).

Various solvents can extract the enzyme from the fermentation medium. Distilled water was better than calcium chloride solution for extracting pectin esterase (Venkatesh et al., 2009). For polygalacturonase, 200 mM acetate buffer pH 4.5 was used (Alcântara et al., 2010). Water and acetate buffer were not compared for cashew apple. However, for extracting polygalacturonase fermented from wheat bran, water was better than acetate buffer for extracting the enzyme produced by *Aspergiilus carbonarius* (Singh et al., 1999). In another study, acetate buffer was better than water for extracting the enzyme produced by *Aspergillus niger*. These contradictory results may be due to extraction time and temperature, which significantly affected enzyme activity (Castilho et al., 2000). Nevertheless, adding sodium sulphate to either water or acetate buffer increased enzyme recovery (Singh et al., 1999).

BIOSURFACTANTS

Surfactants are surface-active compounds that can decrease superfacial and interfacial tension between solids, liquids and gases (Rocha et al., 2009b). Currently, most surfactants are chemically synthesized, resulting in toxic and non-biodegradable compounds. Biosurfactants produced by various microorganisms offer more environmentally friendly alternatives. Examples of biosurfactants are shown in Figure 5. Biosurfactants can be used in food, pharmaceutical and environmental applications as emulsifying, foaming, detergency, wetting, dispersing and solubilizing agents (Rocha et al., 2006). However, barriers to their use include high cost and low yield. Lower cost substrates and simpler substrates that reduce purification steps could help counter this. The yield problem could be overcome by process optimization (Makkar et al., 2011). Cashew apple, an agro-industrial



waste, is a potential substrate for producing biosurfactants.

Figure 5. Examples of biosurfactants (adapted from: Banat et al., 2010).

The biosurfactant rhamnolipid from *Pseudomonas* species has been studied extensively (Banat et al., 2010). Rhamnolipid is a glycolipid containing rhamnose and 3-hydroxy fatty acid. Biosurfactant can be produced from cashew apple juice by *P. aeruginosa* ATCC 10145. The highest reduction of surface tension (50.0 to 29.5 dyne/cm, or 41.0%) was obtained when cashew apple juice was supplemented with 5 g/L peptone. Suitable biosurfactants should reduce the surface tension of the medium to less than 30 dyne/cm. The highest surfactant production was 3.86 g/L, after fermentation at 30°C for 48 h. Emulsification activity was determined by mixing cell-free supernatant and hydrocarbon; then the emulsion height after 24 h was measured and calculated as a percentage of the total solution height. The emulsion activity of the biosurfactant was the highest with soy oil (71.79%) and the lowest with kerosene (16.50%). Although cashew apple juice contains glucose and fructose in equal amounts, only glucose was used by *P. aeruginosa* ATCC 10145 while the fructose concentration remained constant. Rhamnolipid

could be purified by solvent extraction using chloroform/methanol in a 2:1 ratio (Rocha et al., 2007).

Emulsan is a lipopolysaccharide biosurfactant comprised of a sugar backbone linked with fatty acids (Castro et al., 2008). Many microorganisms are capable of producing bioemulsan, but *Acinetobacter calcoaceticus* has been widely studied. Bioemulsan is used in the food, agriculture, bioremeditation, detergent and cosmetic industries (Rosenberg and Ron, 1997). Cashew apple juice could be used as a substrate for production of emulsan by *A. calcoaceticus* RAG-1. The medium showed emulsifying activity with kerosene of 58.8% after 34 h of fermentation, while the surface tension was decreased about 17% (Rocha et al., 2006). Thus, bioemulsan has higher emulsifying activity, but lower surface activity than that of rhamnolipid. Generally, high-molecular-weight polymers, such as emulsan, are effective in emulsion stabilization and ineffective in surface tension reduction (Banat et al., 2010).

Surfactin is a cyclic lipopeptide biosurfactant produced by *Bacillus subtilis.* Surface activity of surfactin is higher than that of sodium lauryl sulfate. Surfactin has potential applications in the healthcare and environmental sectors. Surfactin can be used as a blood-clotting inhibitor (Sen, 2010). Surfactin exhibits antibiotic properties. It has a non-specific antibacterial property, which can disrupt the cell membranes of both Gram-positive and Gram-negative bacteria. A study of 20 multidrug-resistant bacteria showed that all strains, especially *Enterococcus faecalis*, were sensitive to surfactin (Fernandes et al., 2007). Antiviral properties of surfactin include inactivation of herpes and retroviruses. Surfactin possess antitumor and antiproliferative activities against cancer cell lines (Seydlová and Svobodová, 2008).

Surfactin production from cashew apple juice by various strains of *B. subtilis* has been studied. *B. subtilis* LAMI008 was inoculated in clarified cashew apple juice supplemented with mineral medium and produce surfactin at a concentration of 3.5 g/L after 24 h of fermentation. Surface tension of the medium was reduced by 21%. The emulsification index with kerosene was 65% (Rocha et al., 2009b). *B. subtilis* LAMI005 produced surfactin in the same medium at 123 mg/L after 48 h of fermentation. Surface tension of the medium was decreased by 25%. The emulsification index was 67% and 51% with kerosene and soybean oil, respectively. Moreover, critical micelle concentration was 2.5-fold lower than a medium using glucose and fructose as carbon sources (Giro et al., 2009). Yeast extract was important for producing surfactin; no reduction in surface tension was observed without yeast extract in the medium (Rocha et al., 2008). A summary of

Table 4. Surface tension and emulsification activity of fermented cashew apple juice.

Substrate	Biosurfactant produced	Fermentation conditions	Microorganism	Surface tension			Emulsification activity (%)		References
				Initial (dyne/cm)	Fermented (dyne/cm)	Reduction (%)	Kerosene	Soy oil	
Cashew apple juice	rhamnolipid	Shaking at 150 rpm, 30°C for 72 h	*P. aeruginosa* ATCC 10145	66.0	44.4	32.8			Rocha et al. (2007)
Cashew apple juice supplemented with peptone	rhamnolipid	Shaking at 150 rpm, 30°C for 24 to 48 h	*P. aeruginosa* ATCC 10145	50.0	29.5	41.0	16.5	71.79	Rocha et al. (2007)
Cashew apple juice	emulsan	Shaking at 150 rpm, 30°C for 34 to 44 h	*Acinetobacter calcoaceticus* RAG-1	76.0	63.0	17.1	58.8		Rocha et al. (2006)
Cashew apple juice supplemented with yeast extract	surfactin	Shaking at 180 rpm, 30°C for 24 h to 72 h	*Bacillus subtilis* LAMI008	50.3	39.6	21.4	65		Rocha et al. (2009b)
Cashew apple juice	surfactin	48 h	*Bacillus subtilis* LAMI005	38.5	29.0	24.7	66.7	51.15	Giro et al. (2009)

biosurfactants produced from cashew apple juice is shown in Table 4.

PROBIOTIC BEVERAGE AND LACTIC ACID

Probiotics are microorganisms that survive ingestion in certain numbers and provide health benefits to the host beyond general nutrition (Prado et al., 2008). Probiotics have many health benefits, including stimulating the immune system, preventing pathogens, reducing gastrointestinal tract disease, preventing cancer and reducing food allergies (Swennen et al., 2006).

Traditionally, probiotics are presented in dairy products. However, probiotics are increasingly being offered in non-dairy products, which have many advantages over the dairy products, e.g. casein allergy, lactose intolerance and cholesterol content (Prado et al., 2008). Many fruits and vegetables have proven to be good media for probiotics, including pineapple, orange, mango, beet, cabbage and cashew apple juice (Yoon et al., 2005; Yoon et al., 2006; Pereira et al., 2011).

Probiotic foods should have minimal counts of 7 log CFU/mL. Probiotic beverages produced from cashew apple juice, using *Lactobacillus casei* NRRL B-442, had viable cell counts of more than 8 log CFU/mL throughout 42 days of storage. *L. casei* overcame spoilage microorganisms, although heat treatment of the medium was not used in this study. The optimum fermentation condition was 30°C, initial pH 6.4, inoculation at 7.48 log CFU/mL and fermentation for 16 h, based on viable cells count and a final pH level below 4.6, which inhibited pathogenic microorganisms. The first 28 days of storage showed increasing viability, making this period most suitable for consumption with maximum benefits. Even with viability loss after 28 days due to the pH falling below 4.0, cell viability was still higher than 8 CFU/ml for at least 42 days (Pereira et al., 2011).

Lactobacillus spp. can also be used for lactic acid production. Lactic acid can be produced by chemical and fermentation processes. The chemical process produces a racemic mixture of lactic acid. The *D*-lactic acid was not metabolized by humans. Absorption of large amount of *D*-isomer can cause encephalopathy and acidosis (Uribarri et al., 1998). Lactic acid obtained by fermentation contained about 90% *L*-lactic acid, an isomer used in the food industry (Guilherme et al., 2011).

A study of cashew apple juice (25 to 37.5 g/L reducing sugar obtained by dilution) fermentation with *L. casei* NRRL B-442 found that the reducing sugar concentration had a significant effect on lactic acid production through carbohydrate metabolism. High concentration of reducing sugar increased lactic acid productivity until the concentration reached 60 g/L. After this point, lactic acid production decreased due to substrate inhibition. Ammonium sulfate affected the biomass because nitrogen is needed for creation of cells wall. The optimal condition for lactic acid production was 6 g/L ammonium sulfate (12% w/w nitrogen/carbon ratio), pH 6.5 and 37°C – at which lactic acid yield and productivity were about 95% and 2.3 g/L·h, respectively. However, the condition yielding the highest productivity may not be the most economical, given it was obtained from a low initial concentration of reducing sugar and, therefore, the lactic acid produced in each batch was not high (Silveira et al., 2012; Guilherme et al., 2011).

PREBIOTIC OLIGOSACCHARIDES

Prebiotics are food ingredients that are not digested and absorbed in the upper part of the gastrointestinal tract, but rather enter the large intestine to become substrates for probiotic bacteria, e.g. lactobacilli and bifidobacteria. Among prebiotics, non-digestible oligosaccharides have received the most attention (Swennen et al., 2006).

Dextransucrase (EC 2.4.1.5) is a glycosyltranferase enzyme that synthesizes dextran from sucrose. If a carbohydrate other than sucrose was in the medium, the enzyme pathway was shifted from dextran synthesis to oligosaccharide synthesis. Glycosyl moiety is transferred from a donor molecule (sucrose) to an acceptor by α-1,6-glycosidic bond (Figure 6). Acceptor molecules can be mono-, di-, oligosaccharides and also the products of this enzyme. In the latter case, the acceptors become longer, producing oligosaccharides or polysaccharides. During transfer of the glycosyl unit, fructose is left as a by-product that can be used to monitor the process. Dextransucrase is produced by certain lactic acid bacteria, e.g. *Leuconostoc mesenteroides* (Demuth et al., 2000; Chagas et al., 2007; Rabelo et al., 2009).

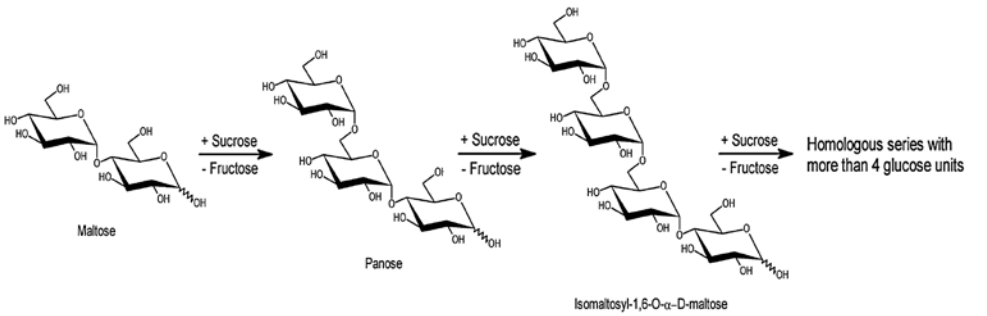


Figure 6. Dextransucrase acceptor reaction with maltose (adapted from: Rodrigues et al., 2005).

Oligosaccharides are produced by enzymatic method in two main steps: (i) production of dextransucrase and (ii) synthesis of oligosaccharides by the crude or purified dextransucrase obtained from the first step.

Cashew apple is a good substrate for dextransucrase production. *L. mesenteroides* NRRL B-512F was able to produce dextransucrase with high activity in a medium containing cashew apple juice (diluted to 5 g/L reducing sugar) and 5 g/L sucrose, without addition of other nutrients. Due to the fact that the primary sugars in cashew apple juice are glucose and fructose, adding sucrose is required to induce the enzyme. Dextransucrase activity in cashew apple was at least 3.5 times higher than synthetic medium. Juice supplementation with phosphate and yeast extract increased cell biomass (Chagas et al., 2007).

The stability of dextransucrase depended on the specific strain of microorganism. Dextransucrase from *L. citreum* B-742 had optimum activity at pH 6.5. This is also the optimum pH for *Leuconostoc* spp. The falling pH level throughout

the fermentation process should be stopped when the pH of the medium reaches 5.5 because dextransucrase is denatured at a pH lower than 5.0 (Rabelo et al., 2009). The enzyme from *L. mesenteroides* B-512F had optimum stability at pH 5.2 (Rodrigues et al., 2005). Controlling the pH during fermentation at 6.5 results in decreased enzyme activity as dextransucrase activity from this microbe was not stable at this pH level (Chagas et al., 2007). The effect of controlling the pH level on the stability of dextransucrase from *L. citreum* B-742 has not yet been investigated.

Stability of dextransucrase in cashew apple juice (27.35 g/L of fructose, 22.47 g/L of glucose, 50 g/L of added sucrose, 20 g/L of yeast extract and 20 g/L of K_2HPO_4) was higher than that in synthetic medium (50 g/L of sucrose, 20 g/L of yeast extract, 20 g/L of K_2HPO_4 and minerals). Synthetic medium was used to investigate whether stability of the enzyme was caused by fermentation metabolites or by the cashew apple juice itself. Activity of dextransucrase from *L. citreum* B-742 and *L. mesenteroides* B-512F in synthetic crude fermented broth was completely lost after 20 h and 6 h, respectively. Thus, enzyme precipitation and stabilization should be performed immediately after fermentation. However, in cashew apple juice medium, the enzyme from *L. citreum* B-742 was stable for 48 h at 25°C and 20 h at 30°C. Maximum enzyme activity was obtained at 25°C after 20 h and 30°C at 3 h (Rabelo et al., 2011). The enzyme from *L. mesenteroides* B-512F was stable at least 30 h at pH 4.5 to 5.5. In addition, at pH 5.5, relative activity of the enzyme increased fivefold at the 30 h reaction time. Cashew apple juice from both fermented and non-fermented conditions maintained activity of dextransucrase. The partially purified enzyme was stable for 96 h at pH 5.5, 30°C in non-fermented cashew apple juice. However, the juice compositions responsible for stabilizing dextransucrase have not been studied (Honorato and Rodrigues, 2010).

The second step is an oligosaccharide synthesis. A study of oligosaccharide synthesis by crude enzyme from *L. citreum* B-742 used substrate media containing sucrose (25 to 75 g/L) and reducing sugar (62.5 to 125 g/L). Sucrose was an added disaccharide, while glucose and fructose were reducing sugars from concentrated cashew apple juice. It was found that oligosaccharide yield depended on the sugar composition of the medium. Both sucrose and reducing sugar had positive effects on oligosaccharide concentration. However, in terms of oligosaccharide yield, only reducing sugar had a positive effect and sucrose had no significant effect. The increment of acceptor concentration shifted the acceptor mechanism toward oligosaccharide synthesis instead of highly-polymerized dextran production. High concentration of sucrose and low concentration of reducing sugar enhanced dextran formation. The optimal medium condition for high oligosaccharide yield contained sucrose below 60 g/L and reducing sugar above 100 g/L. The reducing sugar substrate was almost totally consumed within 72 h (Rabelo et al., 2009).

Oligosaccharides could also be produced by direct inoculation of *L. mesenteroides* into cashew apple juice. Sucrose was added to the medium for dextransucrase induction. Fermentation was conducted while shaking at 30°C for 24 h., producing oligosaccharides with up to six degrees of polymerization,

similar to the synthetic medium. Prebiotic effect of fermented cashew apple juice was tested using the probiotic *Lactobacillus johnsonii* NRRL B-2178. *In vitro* growth of *L. johnsonii* in fermented cashew apple juice was about three times higher than non-fermented juice. Although reducing sugar in fermented cashew apple juice was about five times lower than MRS broth containing fructose as the carbon source, the growth of *L. johnsonii* in both media was not significant (Vergara et al., 2010).

Levan, a fructose polymer synthesized by levansucrase (EC 2.4.1.10), is another polymer similar to dextran. This enzyme releases fructose from sucrose and adds it to the acceptor (Tanaka et al., 1979 and Yoo et al., 2004). *Zymomonas mobilis* has been widely studied for levan production (Bekers et al., 2001; de Paula et al., 2008; Ernandes and Garcia-Cruz, 2011). Levan production from cashew apple juice has not been studied.

Because cashew apple juice contains sucrose concentrations of less than 1 g/L (Azevedo and Rodrigues, 2000), and sucrose is a substrate for dextran and levan production, fortification of sucrose is required. Thus, production of dextran and levan from a mixture of high reducing sugar juice, such as cashew apple juice, and high sucrose juice, such as sugar cane, beet root and longan juice, should be considered.

CONCLUSION

From the single substrate cashew apple, many products can be prepared through the use of a variety of different microorganisms and processing conditions. Due to its moderate concentration of initial sugar, using cashew apple to produce ethanol and lactic acid may not be appropriate compared with other raw materials. However, cashew apple wine and probiotic beverage contained unique aroma, differentiating it from other juice products. Cashew apple offers a potential source for enzyme production due to the presence of substrates, e.g. lignocellulosic material, pectin and tannin. Screening microorganisms from rotting cashew apples should be investigated to identify microorganisms that can produce mixed enzymes. Biosurfactants produced from underutilized crops such as cashew apple offers an alternative to chemically-synthesized surfactants due to low cost and safety. However, product purification was still lacking in most products and an economic evaluation should be performed before commercialization.

REFERENCES

Alcântara, S.R., F.A.C. de Almeida, and F.L.H. da Silva. 2010. Pectinase production by solid state fermentation with cashew apple bagasse: water activity and influence of nitrogen source. Chemical Engineering Transactions 20: 121-126.

Alcântara, S.R., and F.L.H. da Silva. 2011. Influence of spores concentration, moisture, ammonium sulphate concentration and temperature on polygalacturonase production using cashew apple in the solid state fermentation process. Chemical Engineering Transactions 24: 949-954.

Assunção, R.B., and A.Z. Mercadante. 2003. Carotenoids and ascorbic acid from cashew apple (*Anacardium occidentale* L.): variety and geographic effects. Food Chemistry 81: 495-502. 10.1016/S0308-8146(02)00477-6

Attri, B.L. 2009. Effect of initial sugar concentration on the physico-chemical characteristics and sensory qualities of cashew apple wine. Natural Product Radiance 8: 374-379.

Azevedo, D.C.S., and A. Rodrigues. 2000. Obtainment of high-fructose solutions from cashew (*Anacardium occidentale*) apple juice by simulated moving-bed chromatography. Separation Science and Technology 35: 2561–2581.

Banat, I., A. Franzetti, I. Gandolfi, G. Bestetti, M. Martinotti, L. Fracchia, T. Smyth, and R. Marchant. 2010. Microbial biosurfactants production, applications and future potential. Applied Microbiology and Biotechnology 87: 427-444. 10.1007/S00253-010-2589-0

Banerjee, D., K. Mondal, and B. Pati. 2007. Tannase production by *Aspergillus aculeatus* DBF9 through solid-state fermentation. Acta Microbiologica et Immunologica Hungarica 54: 159-166. 10.1556/AMicr.54.2007.2.6

Bekers, M., J. Laukevics, A. Karsakevich, E. Ventina, E. Kaminska, D. Upite, I. VīNa, R. Linde, and R. Scherbaka. 2001. Levan-ethanol biosynthesis using *Zymomonas mobilis* cells immobilized by attachment and entrapment. Process Biochemistry 36: 979-986. 10.1016/S0032-9592(01)00140-6

Belur, P.D. and G. Mugeraya. 2011. Microbial production of tannase: state of the art. Research Journal of Microbiology 6: 25-40. 10.3923/jm.2011.25.40

Campos, D.C.P., A.S. Santos, D.B. Wolkoff, V.M. Matta, L.M.C. Cabral, and S. Couri. 2002. Cashew apple juice stabilization by microfiltration. Desalination 148: 61-65. 10.1016/S0011-9164(02)00654-9

Carrau, F., K. Medina, L. Fariña, E. Boido, and E. Dellacassa. 2010. Effect of *Saccharomyces cerevisiae* inoculum size on wine fermentation aroma compounds and its relation with assimilable nitrogen content. International Journal of Food Microbiology 143: 81-85. 10.1016/j.ijfoodmicro.2010.07.024

Castilho, L.R., R.A. Medronho, and T.L.M. Alves. 2000. Production and extraction of pectinases obtained by solid state fermentation of agroindustrial residues with *Aspergillus niger.* Bioresource Technology 71: 45-50. 10.1016/S0960-8524(99)00058-9

Castro, G.R., B. Panilaitis, and D.L. Kaplan. 2008. Emulsan, a tailorable biopolymer for controlled release. Bioresource Technology 99: 4566-4571. 10.1016/j.biortech.2007.06.059

Chagas, C., T. Honorato, G. Pinto, G. Maia, and S. Rodrigues. 2007. Dextransucrase production using cashew apple juice as substrate: effect of phosphate and yeast extract addition. Bioprocess and Biosystems Engineering 30: 207-215.

Chokotho, L., and E. Hasselt. 2005. The use of tannins in the local treatment of burn wounds - a pilot study. Malawi Medical Journal 17: 19-20.

Chung, K.T., C.I. Wei, and M.G. Johnson. 1998a. Are tannins a double-edged sword in biology and health? Trends in Food Science & Technology 9: 168-175. 10.1016/S0924-2244(98)00028-4

Chung, K.T., T.Y. Wong, C.I. Wei, Y.W. Huang, and Y. Lin. 1998b. Tannins and human health: a review. Critical Reviews in Food Science and Nutrition 38: 421-464.

Clay, J.W. 2004. World Agriculture, and the Environment: A Commodity-by-commodity Guide to Impacts and Practices. Island Press, Washington, D.C.

Cormier, R. 2008. Clarification of cashew apple juice and commercial applications. available online at: http://anacardium.info/IMG/pdf/Clarification_and_Microfiltration_of_ Cashew_Apple_Juice.pdf (Accessed 23 May 2011).

de Paula, V.C., I.O. Pinheiro, C.E. Lopes, and G.C.M.T. Calazans. 2008. Microwave-assisted hydrolysis of *Zymomonas mobilis* levan envisaging oligofructan production. Bioresource Technology 99: 2466-2470.

Demuth, K., B. Demuth, H.J. Jördening, and K. Buchholz. 2000. Oligosaccharide synthesis with dextransucrase: Kinetics and reaction engineering p.123-135. In S. Bielecki, J. Tramper and J. Polak (eds.) Food Biotechnology. Elsevier Science, Amsterdam. 10.1016/S0921-0423(00)80059-3

Ernandes, F.M.P.G., and C.H. Garcia-Cruz. 2011. Nutritional requirements of *Zymomonas mobilis* CCT 4494 for levan production. Brazilian Archives of Biology and Technology 54: 589-600.

Fernandes, P.A.V., I.R.D. Arruda, A.F.A.B.D. Santos, A.A.D. Araújo, A.M.S. Maior, and E.A. Ximenes. 2007. Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. Brazilian Journal of Microbiology 38: 704-709.

Figueiredo, R.W.D., F.M. Lajolo, R. Elesbo Alves, and H.A.C. Filgueiras. 2002. Physical-chemical changes in early dwarf cashew pseudofruits during development and maturation. Food Chemistry 77: 343-347. 10.1016/S0308-8146(01)00358-2

Fontoin, H., C. Saucier, P.L. Teissedre, and Y. Glories. 2008. Effect of pH, ethanol and acidity on astringency and bitterness of grape seed tannin oligomers in model wine solution. Food Quality and Preference 19: 286-291. 10.1016/j.foodqual.2007.08.004

Garruti, D.S., F.A.P. De Abreu, M.R.B. Franco, M.A.A.P. Da Silva, L.P.B. Wender, and P. Mikael Agerlin. 2006a. The influence of fermentation temperature and sulfur dioxide on the volatile composition and flavour profile of cashew wine. Developments in Food Science 43: 109-112. 10.1016/S0167-4501(06)80026-9

Garruti, D.S., M.R.B. Franco, M.A.A.P. Da Silva, N.S. Janzantti, and G.L. Alves. 2006b. Assessment of aroma impact compounds in a cashew apple-based alcoholic beverage by GC-MS and GC-olfactometry. LWT-Food Science and Technology 39: 373-378. 10.1016/j.lwt.2005.02.006

Giro, M.E.A., J.J.L. Martins, M.V.P. Rocha, V.M.M. Melo, and L.R.B. Gonçalves. 2009. Clarified cashew apple juice as alternative raw material for biosurfactant production by *Bacillus subtilis* in a batch bioreactor. Biotechnology Journal 4: 738-747. 10.1002/biot.200800296

Guilherme, A., M. Silveira, C. Fontes, S. Rodrigues, and F. Fernandes. 2011. Modeling and optimization of lactic acid production using cashew apple juice as substrate. Food and Bioprocess Technology 1-8. 10.1007/S11947-011-0670-Z

Gummadi, S.N., N. Manoj, and D.S. Kumar. 2007. Structural and biochemical properties of pectinases. p. 99–115. In J. Polaina and A. P. MacCabe (eds.) Industrial Enzymes. Springer, Dordrecht. 10.1007/1-4020-5377-0-7

Honorato, T.L., M.C. Rabelo, L.R.B. Gonçalves, G.A.S. Pinto, and S. Rodrigues. 2007. Fermentation of cashew apple juice to produce high added value products. World Journal of Microbiology and Biotechnology 23: 1409-1415. 10.1007/S11274-007-9381-Z

Honorato, T.L., and S. Rodrigues. 2010. Dextransucrase stability in cashew apple juice. Food and Bioprocess Technology 3: 105-110. 10.1007/S11947-008-0053-2

Inyang, U.E., and U.J. Abah. 1997. Chemical composition and organoleptic evaluation of juice from steamed cashew apple blended with orange juice. Plant Foods for Human Nutrition 50: 295-300.

Jayalekshmy, V.G., and P.S. John. 2004. 'Sago' - a natural product for cashew apple juice clarification. Journal of Tropical Agriculture 42: 67-68.

Jayani, R.S., S. Saxena, and R. Gupta. 2005. Microbial pectinolytic enzymes: A review. Process Biochemistry 40: 2931-2944. 10.1016/j.procbio.2005.03.026

Joseph, A.D.O. 2010. Comparative studies of wine produced by spontaneous and controlled fermentation of preserved cashew (*Anacardium occidentale*) juice. Research Journal of Biological Sciences 5: 460-464.

Karuppaiya, M., E. Sasikumar, T. Viruthagiri, and V. Vijayagopal. 2009. Optimization of process conditions using response surface methodology (RSM) for ethanol production from waste cashew apple juice by *Zymomonas mobilis*. Chemical Engineering Communications 196: 1425-1435. 10.1080/00986440902938972

Kumar, R., J. Sharma, and R. Singh. 2007. Production of tannase from *Aspergillus ruber* under solid-state fermentation using jamun (*Syzygium cumini*) leaves. Microbiological Research 162: 384-390. 10.1016/j.micres.2006.06.012

Makkar, R., S. Cameotra, and I. Banat. 2011. Advances in utilization of renewable substrates for biosurfactant production. AMB Express 1: 1-19. 10.1186/2191-0855-1-5

Michodjehoun-Mestres, L., J.M. Souquet, H. Fulcrand, C. Bouchut, M. Reynes, and J.M. Brillouet. 2009. Monomeric phenols of cashew apple (*Anacardium occidentale* L.). Food Chemistry 112: 851-857. 10.1016/j.foodchem.2008.06.056

Molina, A., J. Swiegers, C. Varela, I. Pretorius, and E. Agosin. 2007. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. Applied Microbiology and Biotechnology 77: 675-687. 10.1007/S00253-007-1194-3

Muir-Beckford, M., and N. Badrie. 2000. Consumer acceptance of tropical wines from aloe vera (*Aloe barbadensis*) and cashew apples (*Anacardium occidentale* L.) in the British Virgin Islands. Foodservice Research International 12: 185-196.

Neelakandan, T., G. Usharani, and C. Sekar. 2010. Bioethanol production from cashew apple juice using *Saccharomyces cerevisiae.* International Journal of Current Research 11: 151-153.

Oduwole, O.O., T.O. Akinwale, and O. Olubamiwa. 2001. Economic evaluation of a locally fabricated extraction machine for a cottage cashew juice factory. The Journal of Food Technology in Africa 6: 18-20.

Ogunjobi, M.A.K., and S.O. Ogunwolu. 2010. Development and physicochemical evaluation of wine produced from cashew apple powder. Journal of Food Technology 8: 18-23.

Pereira, A.L.F., T.C. Maciel, and S. Rodrigues. 2011. Probiotic beverage from cashew apple juice fermented with *Lactobacillus casei*. Food Research International 44: 1276-1283. 10.1016/j.foodres.2010.11.035

Pinheiro, Á., M. Rocha, G. Macedo, and L. Gonçalves. 2008. Evaluation of cashew apple juice for the production of fuel ethanol. Applied Biochemistry and Biotechnology 148: 227-234. 10.1007/S12010-007-8118-7

Prado, F.C., J.L. Parada, A. Pandey, and C.R. Soccol. 2008. Trends in non-dairy probiotic beverages. Food Research International 41: 111-123. 10.1016.j.foodres.2007.10.010

Rabelo, M.C., C.P.M.L. Fontes, and S. Rodrigues. 2009. Enzyme synthesis of oligosaccharides using cashew apple juice as substrate. Bioresource Technology 100: 5574-5580. 10.1016/j.biortech.2009.06.060

Rabelo, M., C. Fontes, and S. Rodrigues. 2011. Stability study of crude dextransucrase from *Leuconostoc citreum* NRRL B-742. Indian Journal of Microbiology 51: 164-170. 10.1007/S12088-011-0114-5

Ribéreau-Gayon, P., D. Dubourdieu, B. Donèche, and A. Lonvaud. 2006. Handbook of Enology. John Wiley & Sons, Chichester. 10.1002/0470010398

Rivard, D. 2009. The Ultimate Fruit Winemaker's Guide. Bacchus Enterprises, Ludhiana.

Rocha, M.V., A.H. Oliveira, M.C. Souza, and L.R. Gonçalves. 2006. Natural cashew apple juice as fermentation medium for biosurfactant production by *Acinetobacter calcoaceticus*. World Journal of Microbiology and Biotechnology 22: 1295-1299. 10.1007/S11274-006-9175-8

Rocha, M.V., M.C. Souza, S.C. Benedicto, M.S. Bezerra, G.R. Macedo, G.A. Pinto, and L.R. Gonçalves. 2007. Production of biosurfactant by *Pseudomonas aeruginosa* grown on cashew apple juice. Applied Biochemistry and Biotechnology 136-140: 185-194. 10.1007/S12010-007-9050-6

Rocha, M.V.P., R.V. Gomes, V.M.M. Melo, and L.R.B. Gonçalves. 2008. Evaluation of different carbon sources to surfactin production by *Bacillus subtilis* LAMI007.

Rocha, M., T. Rodrigues, G. De Macedo, and L. Gonçalves. 2009a. Enzymatic hydrolysis and fermentation of pretreated cashew apple bagasse with alkali and diluted sulfuric acid for bioethanol production. Applied Biochemistry and Biotechnology 155: 104-114. 10.1007/S12010-008-8432-8

Rocha, M.V.P., R.V.G. Barreto, V.M.M. Melo, and L.R.B. Goncalves. 2009b. Evaluation of cashew apple juice for surfactin production by *Bacillus subtilis* LAMI008. Applied Biochemistry and Biotechnology 155: 366-378. 10.1007/S12010-080-8459-X

Rocha, M.V.P., T.H.S. Rodrigues, V.M.M. Melo, L.R.B. Gonçalves, and G.R. Macedo. 2011. Cashew apple bagasse as a source of sugars for ethanol production by *Kluyveromyces marxianus* CE025. Journal of Industrial Microbiology & Biotechnology 38: 1099-1107. 10.1007/S10295-010-0889-0

Rodrigues, S., L. Lona, and T. Franco. 2005. The effect of maltose on dextran yield and molecular weight distribution. Bioprocess and Biosystems Engineering 28: 9-14. 10.1007/S00449-005-002-7

Rodrigues, T., M. Dantas, G. Pinto, and L. Gonçalves. 2007. Tannase production by solid state fermentation of cashew apple bagasse. Applied Biochemistry and Biotechnology 137-140: 675-688. 10.1007/S12010-007-9088-5

Rodrigues, T., G. Pinto, and L. Gonçalves. 2008. Effects of inoculum concentration, temperature, and carbon sources on tannase production during solid state fermentation of cashew apple bagasse. Biotechnology and Bioprocess Engineering 13: 571-576. 10.1007/S12257-008-0014-7

Rodrigues, T., M. Rocha, G. De Macedo, and L. Gonçalves. 2011. Ethanol production from cashew apple bagasse: improvement of enzymatic hydrolysis by microwave-assisted alkali pretreatment. Applied Biochemistry and Biotechnology 164: 929-943. 10.1007/S12010-011-9185-3

Rosenberg, E., and E.Z. Ron. 1997. Bioemulsans: microbial polymeric emulsifiers. Current Opinion in Biotechnology 8: 313-316. 10.1016/S0958-1669(97)80009-2

Sabu, A., A. Pandey, M. Jaafar Daud, and G. Szakacs. 2005. Tamarind seed powder and palm kernel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. Bioresource Technology 96: 1223-1228. 10.1016/j.biortech.2004.11.002

Sabu, A., C. Augur, C. Swati, and A. Pandey. 2006. Tannase production by *Lactobacillus* sp. ASR-S1 under solid-state fermentation. Process Biochemistry 41: 575-580. 10.1016/j.procbio.2005.05.11

Sanchez, P.C. 2008. Philippine Fermented Foods: Principles and Technology. The University of the Philippines Press, Quezon.

Sen, R. 2010. Surfactin: Biosynthesis, Genetics and Potential Applications. p. 316-323. In R. Sen (ed.) Biosurfactants. Springer, New York. 10.1007/978-1-4419-5979-9-24

Seydlová, G., and J. Svobodová. 2008. Review of surfactin chemical properties and the potential biomedical applications. Central European Journal of Medicine 3(2): 123-133. 10.2478/S11536-008-0002-5

Silva, D., K. Tokuioshi, E. Da Silva Martins, R. Da Silva, and E. Gomes. 2005. Production of pectinase by solid-state fermentation with *Penicillium viridicatum* RFC3. Process Biochemistry 40: 2885-2889. 10.1016/j.procbio.2005.01.008

Silva, M.E., A.B.T. Neto, W.B. Silva, F.L.H. Silva, and R. Swarnakar. 2007. Cashew wine vinegar production: alcoholic and acetic fermentation. Brazilian Journal of Chemical Engineering 24: 163-169.

Silveira, M., C. Fontes, A. Guilherme, F. Fernandes, and S. Rodrigues. 2012. Cashew apple juice as substrate for lactic acid production. Food and Bioprocess Technology 5: 947-953. 10.1007/S11947-010-0382-9

Singh, S.A., M. Ramakrishna, and A.G. Appu Rao. 1999. Optimisation of downstream processing parameters for the recovery of pectinase from the fermented bran of *Aspergillus carbonarius*. Process Biochemistry 35: 411-417. 10.1016/S0032-9592(99)00089-8

Sudheer Kumar, Y., R.S. Prakasam, and O.V.S. Reddy. 2009. Optimisation of fermentation conditions for mango (*Mangifera indica* L.) wine production by employing response surface methodology. International Journal of Food Science & Technology 44: 2320-2327. 10.1111/j.1365-2621.2009.02076.X

Swennen, K., C.M. Courtin, and J.A. Delcour. 2006. Non-digestible Oligosaccharides with Prebiotic Properties. Critical Reviews in Food Science and Nutrition 46: 459-471. 10.1080/10408390500215746

Tanaka, T., S. Oi, and T. Yamamoto. 1979. Synthesis of levan by levansucrase. Journal of Biochemistry 85: 287-293.

Udeh, P.J. 2004. A Guide to Healthy Drinking Water: All You Need to Know About the Water You Drink. iUniverse, Lincoln.

Uribarri, J., M.S. Oh, and H.J. Carroll. 1998. D-lactic acidosis. A review of clinical presentation, biochemical features, and pathophysiologic mechanisms. Medicine 77: 73-82. 10.1097/00005792-199803000-00001

Venkatesh, M., P.B. Pushpalatha, K.B. Sheela, and D. Girija. 2009. Microbial pectinase from tropical fruit wastes. Journal of Tropical Agriculture 47: 67-69.

Vergara, C.M.D.A.J.C., T.L. Honorato, G.A. Maia, and S. Rodrigues. 2010. Prebiotic effect of fermented cashew apple (*Anacardium occidentale* L) juice. LWT-Food Science and Technology 43: 141-145.

Wu, M.C., C.M. Jiang, P.H. Huang, M.Y. Wu, and Y.T. Wang. 2007. Separation and utilization of pectin lyase from commercial pectic enzyme via highly methoxylated cross-linked alcohol-insoluble solid chromatography for wine methanol reduction. Journal of Agricultural and Food Chemistry 55: 1557-1562. 10.1021/jf062880S

Yoo, S.H., E.J. Yoon, J. Cha, and H.G. Lee. 2004. Antitumor activity of levan polysaccharides from selected microorganisms. International Journal of Biological Macromolecules 34: 37-41. 10.1016/j.ijbiomac.2004.01.002

Yoon, K.Y., E.E. Woodams, and Y.D. Hang. 2005. Fermentation of beet juice by beneficial lactic acid bacteria. LWT - Food Science and Technology 38: 73-75.

Yoon, K.Y., E.E. Woodams, and Y.D. Hang. 2006. Production of probiotic cabbage juice by lactic acid bacteria. Bioresource Technology 97: 1427-1430. 10.1016/j.biortech.2005.06.018

Zepka, L.Q., C.D. Borsarelli, M.A.A.P. Da Silva, and A.Z. Mercadante. 2009. Thermal degradation kinetics of carotenoids in a cashew apple juice model and its impact on the system color. Journal of Agricultural and Food Chemistry 57: 7841-7845. 10.1021/jf900558a

Zhang, H., E.E. Woodams, and Y.D. Hang. 2011. Influence of pectinase treatment on fruit spirits from apple mash, juice and pomace. Process Biochemistry 46: 1909-1913.

Nutritional Requirements of *Aeromonas* sp. EBB-1 for Lipase Production

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ABSTRACT

The demand for the production of lipase, a widely used hydrolytic enzyme in biotechnological applications, has led to research on lipase-producing bacteria and culture strategies. In our previous study, we isolated Aeromonas sp. EBB-1 as a novel thermostable-lipase producer from marine sludge in Angsila, Thailand. Given this bacterium's high production of lipase, we now further investigate the effects of different nutritional supplements on its lipase production. Maximum lipase activity (81-fold) was found after cultivating the strain in a medium containing 2.5% (w/v) yeast extract. Separately, adding 0.1% (w/v) glucose, 1.5% (w/v) proline and 0.5% (w/v) gum arabic enhanced lipase production activity nearly 9-, 10- and 6-fold, respectively. In contrast, adding a combination of nutrients in the same medium inhibited lipase production compared to the control. The result with yeast extract is especially promising, yielding high lipase concentrations from Aeromonas sp. EBB-1 in an inexpensive and simple medium.

Keywords: Nutritional sources, *Aeromonas* sp., Optimization, Lipase production, Thermostable lipase

INTRODUCTION

Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3), one of the most versatile biocatalysts, are used in many biotechnological applications due to the different reactions they are able to catalyze and to their exquisite regio-specificity and chiral selectivity (Arbige and Pitcher, 1989; Jaeger et al., 1994 and Hasan et al., 2006). Most lipases used in industrial applications are generally distributed in plants, animals and microorganisms (Arbige and Pitcher, 1989; Jaeger et al., 1994; Fang et al., 2006). Among them, lipases of microbial origin are some of the most commonly used, since they can catalyze a variety of hydrolytic or

synthetic reactions (Jaeger and Reetz, 1998 and Schmid et al., 2001). The demand for the production of highly-active preparations of lipases has led to research on lipase-producing microorganisms and on culture strategies. Lipase production depends largely on culture practices and medium composition (Kim et al., 1996; Lotti et al., 1998; Dalmau et al., 2000). Finding a low-cost medium for the production of lipases would make even more industrial applications feasible.

In our laboratory, 22 strains of lipase-producing bacteria were previously isolated and screened for the production of thermostable lipase. Among them, *Aeromonas* sp. EBB-1 produced high lipase activity (6.50 ± 0.03 U/ml) and could be grown at high temperatures, of up to 55°C (Charoenpanich et al., 2011), which is attractive for industrial applications. This present study aims to improve lipase production of *Aeromonas* sp. EBB-1 by nutrient source optimization, in an effort to find the simplest and most economical medium.

MATERIALS AND METHODS

Bacterial strain and culture condition

The bacterial strain used in this study was isolated from marine sludge in Angsila, Thailand and identified as *Aeromonas* sp. EBB-1. This strain produces a thermostable lipase that is preferentially active towards long-carbon chain acylesters (Charoenpanich et al., 2011). Throughout the study, the general procedures for cultivation were as follows: 5.0 ml of 15 h bacterial inoculums was inoculated into 100 ml of 0.2 × Luria-Bertani (LB) medium (0.2% Bacto-tryptone, 0.2% NaCl and 0.1% Yeast extract, pH 7.2) containing analyzed supplement. Samples from the culture broths used in this study were taken from the late exponential phase of growth (OD_{600} ~ 0.8). Culture broth was centrifuged at 10,000 × g and 4°C for 20 min. The supernatant obtained was filtered through a 0.45 μm nylon membrane filter (Whatman, England) to collect the cell-free supernatant and use for enzymatic assay. Each experiment was done in triplicate.

Lipase activity assay and protein determination

Lipase activity was measured by a hydrolysis reaction using *p*-nitrophenyl palmitate as substrate (Sigma, Germany) according to the method of Pencreac'h and Baratii (1996). One unit (U) of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions. The amount of *p*-nitrophenol was calculated from the *p*-nitrophenol (Sigma, Germany) standard curve. Protein concentration was determined spectrophotometrically according to the method of Bradford (1976), using the Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard. Statistical analysis was performed using a two-tailed t-test and $P<0.05$ was considered statistically significant.

Nutritional factors affecting lipase production by *Aeromonas* sp. EBB-1

To test the effect of different carbon sources on lipase production, various concentrations (0.05 - 0.2%) of carbon sources were added directly into the medium. All carbon sources were filter-sterilized by 0.2 µm nylon membrane filter (Whatman, England). The following carbon sources were studied: glucose, fructose, galactose, sucrose, glycerol and sugar.

The effect of nitrogen source (0.5-4.0% concentration) was investigated by adding directly into the medium. Organic nitrogen sources used in this study were tryptone, peptone, yeast extract and urea. Inorganic nitrogen sources were ammonium sulfate, potassium nitrate and ammonium nitrate.

For the amino acids experiment, amino acids (0.25-2.5% concentration) were added directly into the medium. The following amino acids were used: glycine, proline, arginine, aspartic acid, phenylalanine and cysteine.

The polysaccharides effect was determined by adding gum arabic, sodium alginate or agar, ranging in concentration from 0.5 to 1.0% (w/v). The hydrocarbon hexadecane was also used for investigation at the same concentrations.

RESULTS

Effect of carbon source on lipase production

In order to evaluate the effect of supplements on lipase production, various carbon sources at 0.1% (w/v) were tested preliminarily. The best carbon source for lipase production by *Aeromonas* sp. EBB-1 was glucose (66.24 ± 0.41 U/ml), with fructose (36.54 ± 1.61 U/ml) and galactose (24.55 ± 3.59 U/ml) yielding less lipase (Figure 1a). No significant difference in activity was found in the presence of sucrose, glycerol and sugar. The lipase yields obtained with either lower or higher concentrations of glucose were considerably less than those observed at 0.1% (w/v), as shown in Figure 1b.

Effect of nitrogen source on lipase production

The ability of *Aeromonas* sp. EBB-1 to produce lipase in medium was examined in different nitrogen sources (0.5% w/v). Maximum lipase production was noted in the yeast extract supplement (163.09 ± 1.94 U/ml). Others, in decreasing order of activity, were tryptone (74.94 ± 1.36 U/ml) and peptone (44.77 ± 1.86 U/ml) (Figure 2a). For inorganic nitrogen sources, urea and ammonium sulfate produced similarly low yields (20.85 ± 1.33 U/ml and 18.48 ± 1.40 U/ml, respectively). Interestingly, the addition of 2.5% (w/v) of yeast extract enhanced lipase production by 81-fold (529.69 ± 3.62 U/ml; Figure 2b).

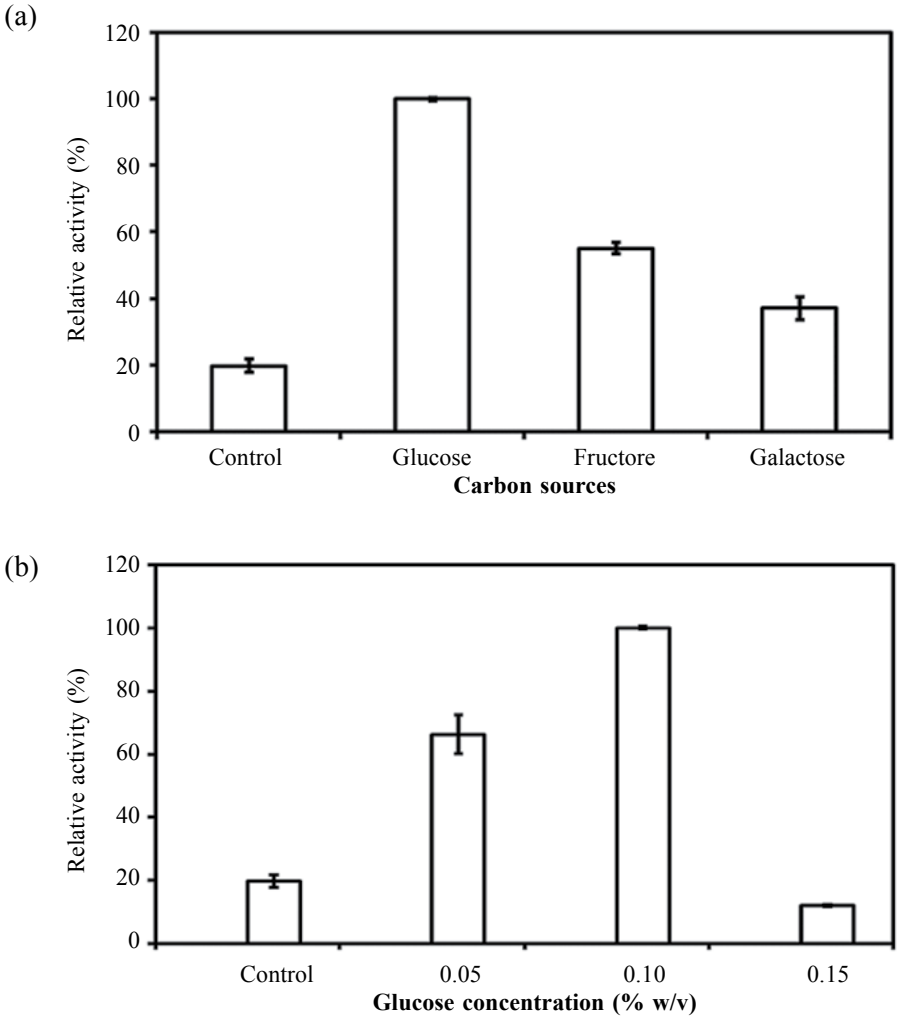


Figure 1. Effect of carbon sources (a) and concentration of glucose (b) on lipase production.

Note: Different carbon sources at 0.1% (w/v) were added to the 0.2 × LB medium and the strain cultivated at 25°C with continuous shaking at 250 rpm until the late exponential phase of growth. The relative activity was based on the highest lipase activity in the experiment and expressed as the mean of three determinations, with the standard deviations (mean ± SD) compared to 0.2 × LB medium (control).

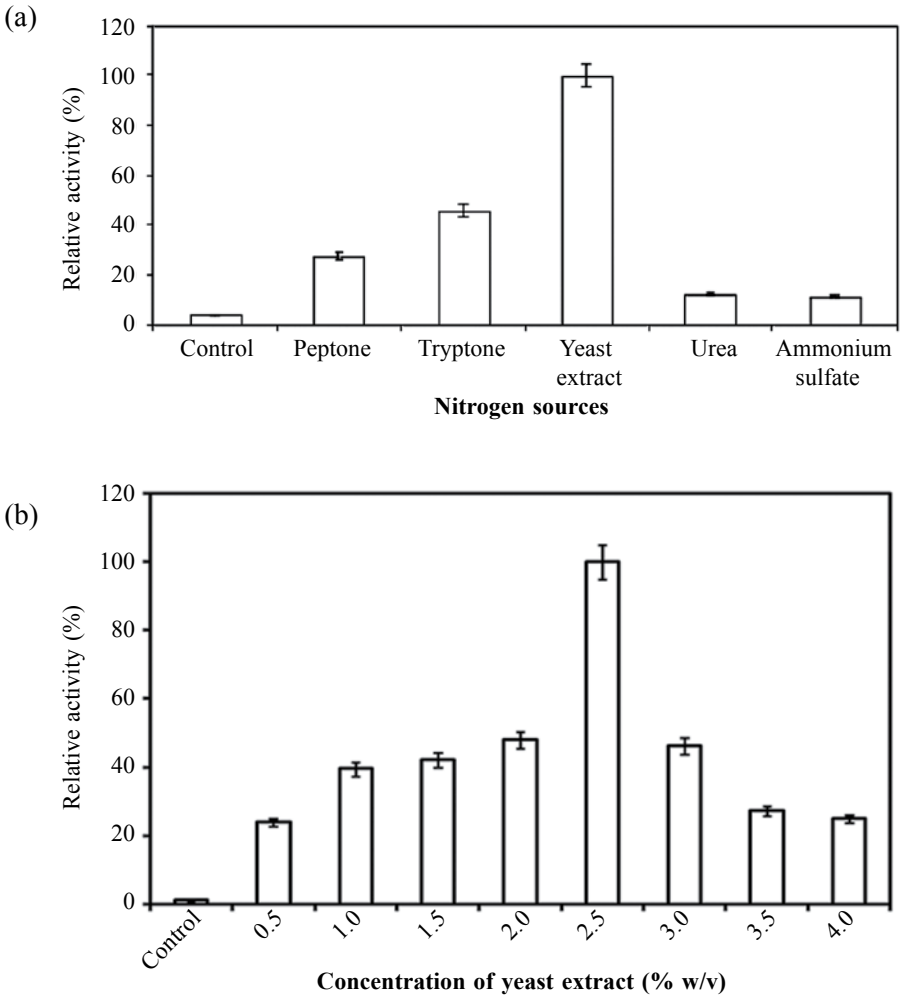


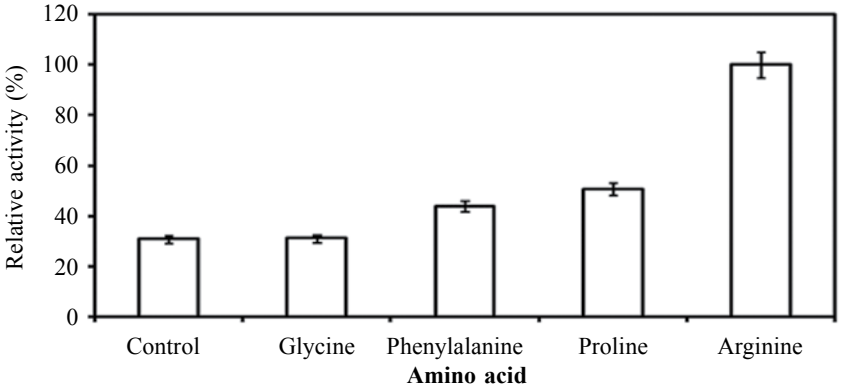
Figure 2. Effect of nitrogen sources (a) and concentration of yeast extract (b) on the production of lipase.

Note: Either organic or inorganic nitrogen source at 0.5% (w/v) was added to the 0.2 × LB medium and the strain cultivated at 25°C with continuous shaking at 250 rpm. The lipase production corresponds to those obtained at the late exponential phase of growth. These values are relative to the highest lipase activity in the experiment and expressed as the means ± SD (n = 3) compared to 0.2 × LB medium (control).

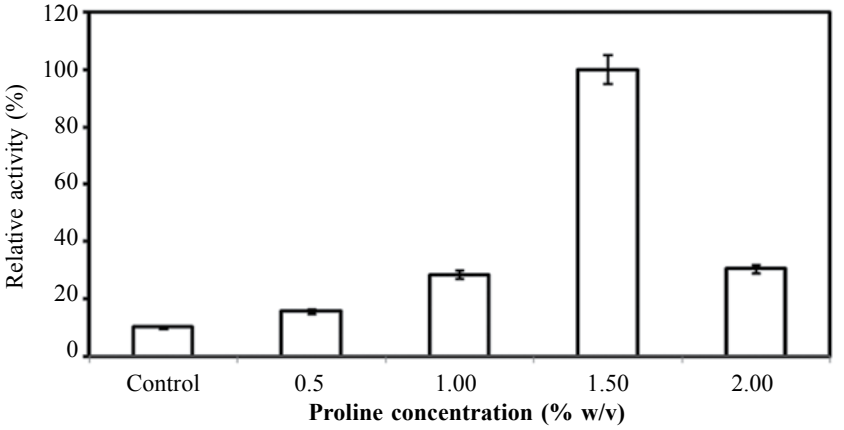
Effect of amino acid on lipase production

Experiments supplementing the medium with 0.5% (w/v) of amino acid were tried. Results indicated that the presence of arginine stimulated the highest lipase production (40.06 ± 2.74 U/ml), while proline (20.31 ± 2.08 U/ml), phenylalanine (17.62 ± 5.30 U/ml) and glycine (12.50 ± 1.11 U/ml) produced low yields, as shown in Figure 3a. Proline at a concentration of 1.5% (w/v, Figure 3b) enhanced lipase production nearly 10-fold, while 0.25% (w/v) arginine (Figure 3c) enhanced production ~6-fold.

(a)



(b)



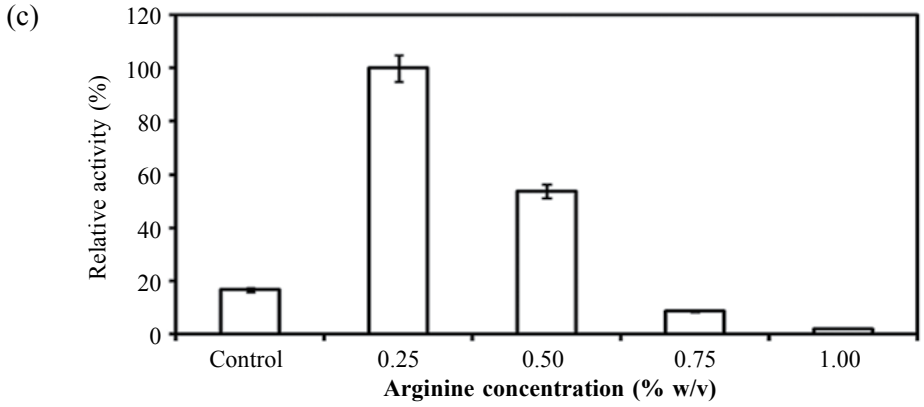


Figure 3. Effect of different amino acids (a) and concentrations of proline (b) and arginine (c) on lipase production.

Note: The samples were taken from the culture at the late exponential phase of growth (OD_{600} ~ 0.8). Experiments were done in triplicate and the activities of lipase were expressed as relative activity compared to the maximum (100% relative activity) observed value.

Effect of additives on lipase production

Three polysaccharides – gum arabic, agar and sodium alginate – were added separately to the medium at different concentrations. Among them, gum arabic produced the highest lipase activity (45.03 ± 5.07 U/ml), with the 0.5% (w/v) concentration promoting the maximum lipase production per unit of the growth of bacterium (Figure 4). On the other hand, sodium alginate and hexadecane showed inhibitory effects for lipase production. No significant production of lipase occurred when *Aeromonas* sp. EBB-1 was grown on the medium containing agar.

To complete these studies, the individual supplement giving the maximum lipase activity was chosen to evaluate its effect on lipase production (Figure 5). As expected, a mixture of yeast extract with other supplements produced higher lipase activity than the control. However, supplementing with yeast extract alone produced the highest activity.

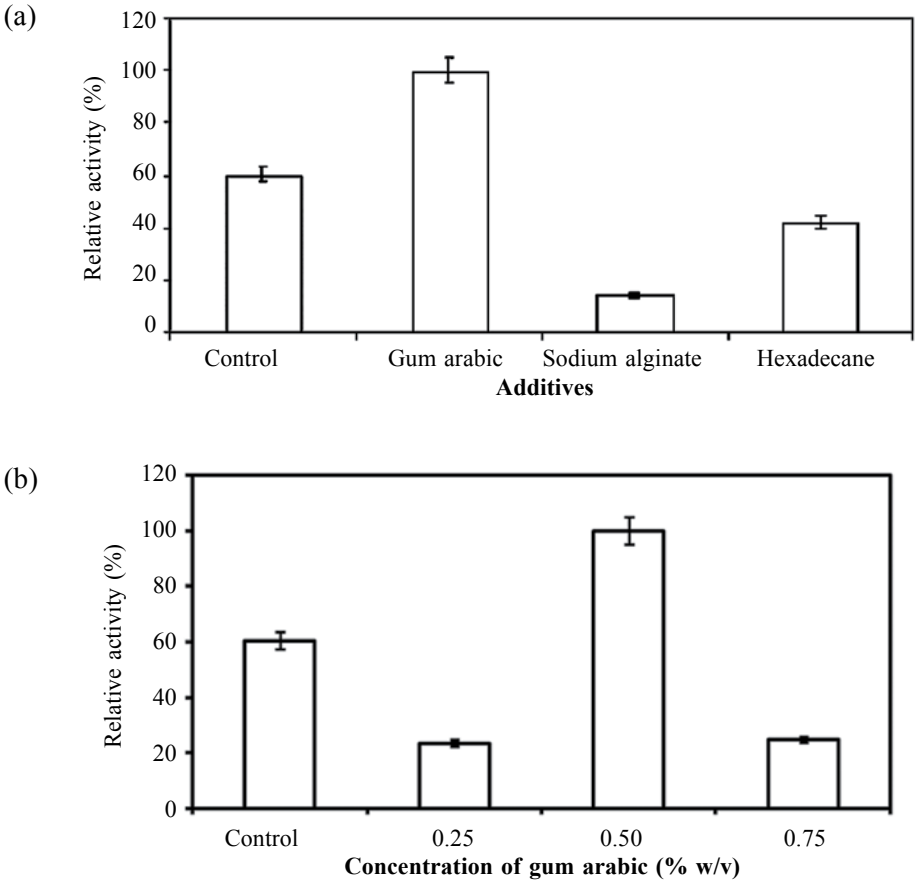


Figure 4. Effect of additives (a) and concentration of gum arabic (b) on lipase production.

Note: Different additives at 0.5% (w/v or v/v) were added to the 0.2 × LB medium and the strain cultivated until the late exponential phase of growth at 25°C with continuous shaking at 250 rpm. Media without the additives were used as control. Lipase activities were determined in triplicate and reported as averages ± standard deviation.

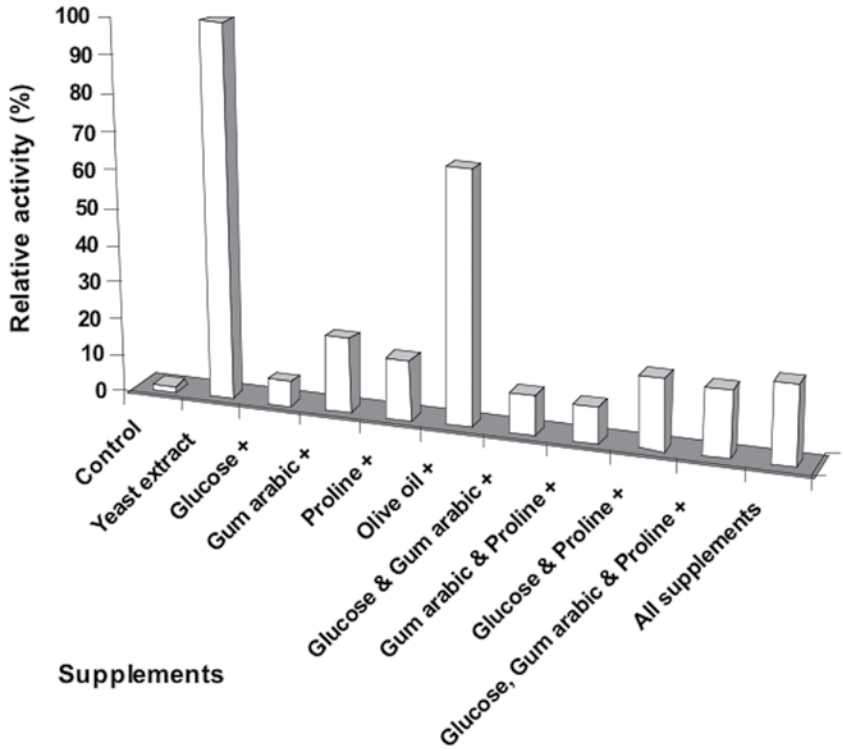


Figure 5. Relative activities among lipase produced by *Aeromonas* sp. EBB-1 using mixtures of nutritional supplements.

Note: Media without the additives were used as control. Lipase activities were determined in triplicates and averages ± SD reported.

DISCUSSION

Bacterial lipases are typically extracellular and influenced greatly by nutritional factors. In this study, we tried to find the optimum medium for lipase production of *Aeromonas* sp. EBB-1, a novel thermostable lipase isolated in a previous study (Charoenpanich et al., 2011). It has been reported that monosaccharides, especially glucose, stimulated both enzyme production in different microorganisms and secretion of lipase accumulated inside the cells (Macfarlane and Macfarlane, 1992; Mehrotra et al., 1999; Dalmau et al., 2000; Boekema et al., 2007 and Uttatree and Charoenpanich, 2011). On the other hand, the repression of enzyme synthesis in the liquid medium by sucrose and other readily-metabolized carbon sources was referred to as catabolite repression, the paradigm of cellular regulation for the low preferential carbon source (Stülke and Hillen, 1999; Brückner and Titgemeyer, 2002; Deutscher, 2008 and Uttatree and Charoenpanich, 2011). In the presence of disaccharides, for example sucrose, catabolite repression in *Aeromonas* sp. EBB-1 might serve as an autoregulatory device to keep sucrose

utilization at a certain level, leading to the lower production of lipase rather than to establish preferential utilization of glucose.

In most microorganisms, either inorganic or organic nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. The results suggest that inorganic nitrogen sources are not essential for growth and lipase production by this strain, although the growth and enzyme productivity were low. The lipase yields obtained with potassium nitrate and ammonium nitrate were considerably lower than the control. This phenomenon might be due to the repression of enzyme synthesis by the rapidly metabolizable ammonium ion concentration in the medium, which interfered with the utilization and metabolism of peptides through catabolite repression (Giesecke et al., 1991 and Snowden et al., 1992). Moreover, both sulfate and nitrate affects calcium availability by precipitation or chelation and, therefore, reduces lipase production (Freire et al., 1997.). Interestingly, the addition of 2.5% (w/v) of yeast extract enhanced lipase production by 81-fold (529.69 ± 3.62 U/ml), even higher than with an olive oil supplement (440 ± 13 U/ml) described previously (Charoenpanich et al., 2011). The simple addition of yeast extract into the medium resulted in higher lipase production of *Aeromonas* sp. EBB-1 than with the oil supplement.

Incorporation of amino acids in the production medium might enhance lipase production (Arbige and Pitcher, 1989; Bornscheuer et al., 2002). At a concentration of 0.5% (w/v), arginine stimulated the highest lipase production, but others produced low yields. Proline at a concentration of 1.5% (w/v) enhanced lipase production nearly 10-fold, while 0.25% (w/v) arginine enhanced production approximately 6-fold. The inhibitory effect of glycine has been reported previously (Ikura and Horikoshi, 1987; Uttatree and Charoenpanich, 2011).

The increase in lipase production by adding gum arabic might be due to this compound enhancing the mechanical liberation of the enzyme at the cell surface (Winkler and Stuckmann, 1979; Mahler et al., 2000; Martinez and Nudel, 2002). A combination of yeast extract with other supplements produced higher lipase activity than the control, but still lower than supplementing with yeast extract alone. This is important; proving that yeast extract alone provided a simple and convenient nitrogen source for industrial-scale lipase production.

By optimization of medium components, the production of an extracellular lipase from *Aeromonas* sp. EBB-1 was improved. After addition of yeast extract (2.5% w/v), an 81-fold increase of lipolytic activity was found compared to that obtained using 0.2 × LB medium. Adding 0.1% (w/v) glucose, 1.5% (w/v) proline and 0.5% (w/v) gum arabic also improved lipase production, although to a lesser extent (6-10 fold). However, adding a combination of nutrients in the same medium inhibited lipase production compared to the control. These results are promising because this strain produces lipases in a simple medium (0.2% Bacto-tryptone, 0.2% NaCl and 2.6% Yeast extract, pH 7.2).

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REFERENCES

Arbige, M.V., and W.H. Pitcher. 1989. Industrial enzymology: a look towards the future. Trends in Biotechnology 7: 330-335.

Boekema, B.K.H.L., A. Beselin, M. Breuer, B. Hauer, M. Koster, F. Rosenau, K.E. Jaeger, and J. Tommassen. 2007. Hexadecane and Tween 80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. Applied and Environmental Microbiology 73: 3838-3844. 10.1128/AEM.00097-07

Bornscheuer, U.T., C. Bessler, R. Srinivas, and S.H. Krishna. 2002. Optimizing lipase and related enzymes for efficient application. Trends in Biotechno-logy 20: 433-437. 10.1016/S0167-7799(02)02046-2

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing, the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Brückner, R., and F. Titgemeyer. 2002. Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiology Letters 209: 141-148. 10.1016/S0378-1097(02)00559-1

Charoenpanich, J., S. Suktanarag, and N. Toobbucha. 2011. Production of a thermostable lipase by *Aeromonas* sp. EBB-1 isolated from marine sludge in Angsila, Thailand. ScienceAsia 37: 105-114. 10.2306/scienceasia1513-1874.2011.37.105

Dalmau, E., J.L. Montesinos, M. Lotti, and C. Casas. 2000. Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme and Microbial Technology 26: 657-663. 10.1016/S0141-0229(00)000156-3

Deutscher, J. 2008. The mechanisms of carbon catabolite repression in bacteria. Current Opinion in Microbiology 11: 87-93. 10.1016/j.mib.2008.02.007

Fang, Y., Z. Lu, F. Lv, X. Bie, S. Liu, Z. Ding, and W. Xu. 2006. A newly isolated organic solvent tolerant *Staphylococcus saprophyticus* M36 produced organic solvent-stable lipase. Current Microbiology 53: 510-515. 10.1007/S00284-006-0260-X

Freire, D.M.G., E.M.F. Teles, E.P.S. Bon, and G.L. San't Anna Jr. 1997. Lipase production by *Penicillium restrictum* in a bench-scale fermenter: Effect of carbon and nitrogen nutrition, agitation and aeration. Applied Biochemistry and Biotechnology 63: 409-421.

Giesecke, U.E., G. Bierbaum, H. Rudde, U. Spohn, and C. Wandrey. 1991. Production of alkaline protease with *Bacillus licheniformis* in controlled fed-batch process. Applied Microbiology and Biotechnology 35: 720-724.

Hasan, F., A.A. Shah, and A. Hameed. 2006. Industrial applications of microbial lipases. Enzyme and Microbial Technology 39: 235-251. 10.1016/j.enzmicte.2005.10.016

Ikura, Y., and K. Horikoshi. 1987. Effect of amino compounds on alkaline amylase production by alkaliphilic *Bacillus* sp. Journal of Fermentation Technology 65: 707-709.

Jaeger, K.E., S. Ransac, B.W. Dijkstra, C.C. van Henrel, and O. Misset. 1994. Bacterial lipases. FEMS Microbiology Reviews 15: 29-63. 10.1016/0168-6445(94)90025-6

Jaeger, K.E., and M.T. Reetz. 1998. Microbial lipases from versatile tools for biotechnology. Trends in Biotechnology 16: 396-403. 10.1016/S0167-7799(98)01195-0

Kim, S.S., E.K. Kim, and J.S. Rhee. 1996. Effects of growth rate on the production of *Pseudomonas fluorescens* lipase during the fed-batch cultivation of Escherichia coli. Biotechnology Progress 12(5): 718-722.

Lotti, M., S. Monticelli, J.L. Montesinos, S. Brocca, F. Valero, and J. Lafuente. 1998. Physiological control on the expression and secretion of *Candida rugosa* lipase. Chemical and Physical Lipids 93: 143-148. 10.1016/S0009-3084(98)00038-3

Macfarlane, G.T., and S. Macfarlane. 1992. Physiological and nutritional factors affecting synthesis of extracellular metalloproteinase by *Clostridium bifermentans*. Applied and Environmental Microbiology 58: 1973-1976.

Mahler, G.F., R.G. Kok, A. Cordenons, K.J. Hellingwerf, and B.C. Nudel. 2000. Effects of carbon sources on extracellular lipase production and lipA transcription in *Acinetobacter calcoaceticus*. Journal of Industrial Microbiology and Biotechnology 24: 25-30.

Martinez, D.A., and B.C. Nudel. 2002. The improvement of lipase secretion and stability by addition of inert compounds into *Acinetobacter calcoaceticus* cultures. Canadian Journal of Microbiology 48: 1056-1061. 10.1139/W02-108

Mehrotra, S., P.K. Pandey, R. Gaur, and N.S. Darmwal. 1999. The production of alkaline protease by a *Bacillus* species isolate. Bioresource Technology 67: 201-203. 10.1016/S0960-8524(98)00107-2

Pencreac'h, G., and J.C. Baratti. 1996. Hydrolysis of p-nitrophenyl palmitate in *n*-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. Enzyme and Microbial Technology 18: 417-422. 10.1016/0141-0229(95)00120-4

Schmid, A., J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, and B. Witholt. 2001. Industrial biocatalysis today and tomorrow. Nature 409: 258-268. 10.1038/35051736

Snowden, L.Y., I.I. Blumentals, and R.M. Kelly. 1992. Regulation of proteolytic activity in the hyperthermophiles *Pyrococcus furisosus*. Applied and Environmental Microbiology 58: 1134-1141.

Stülke, J., and W. Hillen. 1999. Carbon catabolite repression in bacteria. Current Opinion in Microbiology 2: 195-201. 10.1016/S1369-5274(99)80034-4

Uttatree, S., and J. Charoenpanich. 2011. Nutritional requirements and physical factors affecting the production of organic solvent-stable lipase by *Acinetobacter baylyi*. Chiang Mai University Journal of Natural Sciences 10(1): 115-132.

Winkler, U.K., and M. Stuckmann. 1979. Glycogen, hyaluronidate and some other polysaccharides greatly enhance the formation of exocellular lipase by *Serratia marcescens*. Journal of Bacteriology 138: 663-670.

Antioxidant and Anticancer Activities from Leaf Extracts of Four *Combretum* Species from Northern Thailand

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ABSTRACT

Four Combretum species (Combretaceae) from northern Thailand (Combretum deciduum, Combretum griffithii, Combretum latifolium and Combretum quadrangulare) were tested for antioxidant and anticancer activities. Antioxidant activities were assessed by ABTS and DPPH radical scavenging capacity methods. Anticancer activity was tested against three cancerous human cell lines (KB, MCF7 and NCI-H187). All methanolic leaf extracts showed antioxidant activities with the ABTS and DPPH methods. The methanolic leaf extracts of C. deciduum inhibited KB-oral cavity and MCF7-breast cancer cell lines, C. latifolium inhibited MCF7-breast cancer cell line and C. quadrangulare inhibited KB-oral cavity and NCI-H187-small cell lung cancer cell lines. However, the methanolic leaf extracts of C. griffithii were inactive against all three cell lines. All methanolic leaf extracts exhibited non-cytotoxicity to Vero cell lines.

Keywords: *Combretum deciduum, Combretum griffithii, Combretum latifolium, Combretum quadrangulare*, Antioxidant activity, Anticancer activity

INTRODUCTION

The genus *Combretum* belongs to the family Combretaceae. This genus of trees, woody climbers and shrubs is distributed in the tropics, including southern Africa, Asia and America. The genus is well known in folk medicine for its medicinal value. In southern Africa, *Combretum* is used to treat abdominal disorders, backaches, bacterial infections, bilharzia, cancer, coughing, the urinary system, colds, conjunctivitis, constipation, diarrhea, dysentery, dysmenorrhea, earaches, fever, gastric ulcers, general weakness, gonorrhea, headaches, heart disease, hookworm, hypertension, jaundice, leprosy, nose bleeds, pneumonia, skin diseases, sore throats, swelling caused by mumps, syphilis, toothaches, malaria and diabetes (Clarke, 1878; Banskota et al., 2003; Eloff et al., 2008 and Lima et al., 2012).

Previous research on the genus includes the antioxidant activities of *C. decandrum* Roxb. (DC) and *C. duarteanum* Cambess. and the anticancer activities and cytotoxicity of *C. duarteanum, C. collinum* Fresen., *C. apiculatum* Sond. subsp

apiculatum, C. fragrans F. Hoffm., *C. micranthum* G. Don, *C. padoides* Engl. & Diels, *C. hereroense* Schinz, *C. psidioides* Welw. and *C. zeyheri* Sond. (Lima et al., 2012).

According to the Thai Forest Bulletin, 19 species of the genus *Combretum* are found in Thailand (Nanakorn, 1986). Four *Combretum* species grow commonly in northern Thailand: *C. deciduum* Coll. & Hemsl., *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz. The leaves, stem bark, root and seeds of *C. quadrangulare* have been used in Thailand and other countries as traditional medicine as antihepatitis, antipyretic, antidysenteric and anthelmintic agents (Banskota et al., 2003). Several studies in Thailand on the root and seeds of *C. quadrangulare* reported anthelmintic activity (Somanabandhu, 1984 and Euswas et al., 1988), antibacterial activity (Nantachit et al., 2006) and toxicity (Nakornchai et al., 1987; 1994). *C. latifolium* has not been reported on, but its stem and fruits have been used by rural people as an astringent and for dysentery, dysmenorrhea and nourishing the blood and body. Locals have used a water decoction of the stem of *C. griffithii* as a traditional medicine for hepatitis (Moosophon et al., 2011). The use of *C. deciduum* as a traditional medicine has not been reported, and no study on *C. deciduum* has been found.

Given the leaves can be harvested easily without killing the plants and several prior studies of *Combretum* species also used the leaves (Pettit et al., 1987; Banskota et al., 1998; 2003; McGaw et al., 2001; Fyhrquist et al., 2002; Inngjerdingen et al., 2004; Karou et al., 2005; Eldeen et al., 2007; Maregesi et al., 2007; Eloff et al., 2008; Gronhaug et al., 2008 and Coulidiati et al., 2009), this study has selected the leaves for extractions.

No report has tested the antioxidant activity of the leaves of *C. deciduum, C. griffithii, C. latifolium* and *C. quadrangulare*. Only one of the four, *C. griffithii*, has been tested for anticancer activity. The purpose of this study, therefore, was to investigate these four species, focusing on validating their antioxidant and anticancer activities using leaf extracts from specimens growing in northern Thailand.

MATERIALS AND METHODS

Plant material

The plants were collected from convenient areas of several provinces in northern Thailand. *C. latifolium* specimens were collected in Wang Nua District, Lampang Province in December 2009. *C. quadrangulare* specimens were collected in Doi Saket District, Chiang Mai Province in June 2009. *C. deciduum* and *C. griffithii* specimens were collected in Mae Rim District, Chiang Mai Province in July 2010. The specimens were identified at the Chiang Mai University Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The voucher specimens were deposited in the Herbaria at the Faculty of Pharmacy and the Department of Biology, Faculty of Sciences, Chiang Mai University.

Plant extraction

The dried, powdered leaves of *Combretum deciduum* 264.35 g, *C. griffithii* 302.91 g, *C. latifolium* 611.29 g and *C. quadrangulare* 930.00 g were macerated in methanol for approximately 24 hours at room temperature and filtered. The marc was macerated again using the same procedure twice. The filtrates were then pooled and concentrated under reduced pressure until dry. In the same methods, n-hexane and dichloromethane extracts were prepared.

Antioxidant activity tests

The antioxidant activity of the crude plant extracts obtained from the above procedure was determined by the ABTS and DPPH methods.

ABTS method

The antioxidant activity of the crude extracts was investigated using the ABTS radical cation ($ABTS^{\bullet+}$) scavenging method following the methodology of Roberta et al. (1999) and compared with Trolox standards (concentration range 0.5-2.5 mM). For the ABTS method, 20 µl of crude plant extracts (0.1 g/ml) were mixed with 2.0 ml of diluted ABTS solution (A_{734nm} = 0.700 ± 0.020) and the absorbance was determined at 734 nm after 5 minutes incubation at room temperature. A solvent blank was run in each assay. All determinations were carried out at least three times, and in triplicate. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (I%) was calculated by the following equation:

$$I\ (\%) = [\ (A_{blank} - A_{sample}) / A_{blank}\ ]\ \times 100$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a function of the concentration of antioxidants and of Trolox and vitamin C for standard reference data.

DPPH method

A stock solution (5.0×10^{-4} mol/l) of DPPH was prepared by dissolving 10.0 mg in 50 ml 95% ethanol. This solution was stored at 4°C away from light, and was stable after a week. The DPPH working solution containing 1.0×10^{-4} mol/l was prepared by pipetting 50 ml of the stock solution into a 200 ml volumetric flask and diluting with 95% ethanol to volume. This working solution was prepared fresh daily and protected from light (Thongchai et al., 2009). The test sample (20 µl) was added to 180 µl of ethanolic DPPH solution in a 96-well microtiter plate. The reaction mixture was incubated at 37°C for 30 minutes, and then the absorbance of each well was measured at 540 nm. The DPPH solution was used as negative control. Trolox, vitamin C and quercetin were used as reference standards. For 50% inhibitory concentration (IC_{50}) evaluation of the crude extracts, a graph showing concentration versus % DPPH reduction was plotted. The IC_{50} was calculated from the calibration curve and activity was expressed as the percentage DPPH scavenging relative to the control using the following equation:

$$\text{DPPH scavenging activity (\%)} = [\ (A_{control} - A_{sample}) / A_{control}\ ] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound.

Anticancer activity tests

Anticancer method. The anticancer activity of the crude extracts was tested using three cancerous human-cell lines: KB cell line (epidermoid carcinoma of the oral cavity, ATCC CCL-17), MCF7 cell line (breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (small cell lung carcinoma, ATCC CRL-5804). This test was determined by resazurin microplate assay (REMA) following a modified method using fluorescent dye for mammalian cell cytotoxicity according to O'Brien et al. (2000). Ellipticine, Doxorubicin and Tamoxifen were used as positive controls. 0.5% DMSO and sterile water were used as negative controls. In brief, cells at a logarithmic growth phase were harvested and diluted to 2.2×10^4 cells/ml for KB and 3.3×10^4 cells/ml for MCF7 and NCI-H187 in fresh medium. Successively, 5 µl of the methanolic extract was diluted in 5% DMSO and 45 µl of the cell suspension was added to 384-well plates and incubated at 37°C in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF7; 5 days for NCI-H187), 12.5 µl of 62.5 µg/ml resazurin solution was added to each well and the plates were then incubated at 37°C for 4 hours. Fluorescence signal was measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. The inhibitory concentration (IC_{50}) represented the concentration that caused a 50% reduction in cancer cell line growth.

Cytotoxicity method. The cytotoxicity method conformed to published standard methods (BS-EN30993-5 and ISO10993-5) using Vero cell lines (African green monkey kidney; ATCC Cat. No. CCL-81) by the MTT cytotoxicity method. The cells were exposed to the sample for 24 hours over the concentration ranges of 1000-7.8 µg/ml. The results were shown by percent survival of cells at each concentration compared to control and IC_{50} values. The method was a modified version of conventional direct and indirect contact tests. The MTT method (Plumb et al., 1989) is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a microplate reader. It is assumed that metabolically deficient cells will not survive, thus the MTT method is also an indirect measurement of cell viability. The cells were seeded in a 96-well plate at a density of 3,000 cells/well, and incubated for 48 hours. The samples at various concentrations were added to the cells and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested by the MTT method. Briefly, 50 µl of MTT in phosphate buffer saline (PBS) at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the wells and formazan solubilized

with 200 µl of DMSO and 25 µl of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of four wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from eight concentrations in the test range using four wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to the controls.

RESULTS

Among the three extracts, the methanolic extracts of the four *Combretum* species showed the most potent antioxidant activity using three conventional standards (Trolox, vitamin C and quercetin) (Table 1 and 2). These methanolic extracts were subsequently investigated for anticancer activity (Table 3).

Antioxidant activity

The antioxidant activities of the extracts are shown in Table 1 and 2. Methanolic extracts had the most potent antioxidant activity, followed by n-hexane extracts and dichloromethane extracts.

For the ABTS method (Table 1), the highest antioxidant activity was obtained with methanolic extracts (9.85-22.61 mM Trolox/mg of extracts and 2.15-4.96 mM vitamin C/mg of extracts), followed by dichloromethane extracts (5.75-9.82. mM Trolox/mg of extracts and 1.25-2.14 mM vitamin C/mg of extracts) and n-hexane extracts (3.87-8.74. mM Trolox/mg of extracts and 0.83-1.90 mM vitamin C/mg of extracts).

Table 1. Antioxidant capacities of extracts from leaves of four *Combretum* species.

Extracts and standards	ABTS % inhibition/mg of extracts	ABTS mM Trolox/mg of extracts	ABTS mM vitamin C/mg of extracts
C. deciduum			
Hexane	30.37 ± 0.0015	3.87	0.83
Dichloromethane	44.97 ± 0.0076	5.75	1.25
Methanol	78.41 ± 0.0006	10.05	2.19
C. griffithii			
Hexane	57.70 ± 0.0029	7.38	1.61
Dichloromethane	54.35 ± 0.0015	6.95	1.51
Methanol	76.86 ± 0.0012	9.85	2.15
C. latifolium			
Hexane	62.61 ± 0.0015	8.02	1.75
Dichloromethane	53.11 ± 0.0108	6.79	1.48
Methanol	176.06 ± 0	22.61	4.96
C. quadrangulare			
Hexane	68.20 ± 0.0021	8.74	1.90
Dichloromethane	76.62 ± 0.0040	9.82	2.14
Methanol	154.59 ± 0.0006	19.85	4.35

Note: NT = not tested. Values are given as mean ± S.D. of triplicate experiments. Values represent the significantly different results ($p \leq 0.05$).

For the DPPH method (Table 2), the IC_{50} values of the n-hexane extracts were 0.52-6.53 µg/ml, the dichloromethane extracts were 1.52-5.93 µg/ml and the methanolic extracts were 0.13-0.81 µg/ml. The three standards showed antioxidant activity with the DPPH method with IC_{50} values of 0.06, 0.07 and 0.05 µg/ml, respectively.

Table 2. DPPH radical scavenging activity of extracts from leaves of four *Combretum* species.

Extracts and standards	DPPH IC_{50} (µg/ml)
C. deciduum	
Hexane	1.76 ± 0.0201
Dichloromethane	1.52 ± 0.0273
Methanol	0.81 ± 0.0202
C. griffithii	
Hexane	0.52 ± 0.0220
Dichloromethane	5.93 ± 0.0283
Methanol	0.23 ± 0.0100
C. latifolium	
Hexane	2.79 ± 0.0218
Dichloromethane	3.57 ± 0.0218
Methanol	0.13 ± 0.0224
C. quadrangulare	
Hexane	6.53 ± 0.0215
Dichloromethane	5.16 ± 0.0169
Methanol	0.23 ± 0.0219
Standards	
Trolox	0.06 ± 0.0233
Vitamin C	0.07 ± 0.0170
Quercetin	0.05 ± 0.0260

Note: NT = not tested. Values are given as mean ± S.D. of triplicate experiments. Values represent the significantly different results ($p \leq 0.05$).

Anticancer Activity

The methanolic extracts of all four *Combretum* species exhibited significant anticancer activity against KB, MCF7 and NCI-H187 cell lines with IC_{50} (Table 3). The methanolic extract of *C. deciduum* inhibited KB and MCF7 cell lines with an IC_{50} value of 34.34 µg/ml and 28.84 µg/ml, respectively. The methanolic extract of *C. latifolium* inhibited MCF7 cell line with an IC_{50} value of 26.63 µg/ml. Methanolic extract of *C. quadrangulare* inhibited KB and NCI-H187 cell lines with IC_{50} values of 26.76 µg/ml and 46.88 µg/ml, respectively. However, methanolic extract of *C. griffithii* was inactive against all three cell lines. Ellipticine, Doxorubicin and Tamoxifen were used as standard compounds. All extracts were non-cytotoxic against Vero cell lines.

Table 3. Anticancer activity of methanolic extracts from leaves of four *Combretum species*.

Extracts and standards	IC_{50} (µg/ml)			
	Human cancer cell lines			Vero cell lines
	KB	MCF7	NCI-H187	
C. deciduum	34.34	28.84	Inactive	Non-cytotoxic
C. griffithii	Inactive	Inactive	Inactive	Non-cytotoxic
C. latifolium	Inactive	26.63	Inactive	Non-cytotoxic
C. quadrangulare	26.76	Inactive	46.88	Non-cytotoxic
Ellipticine	1.19	NT	1.11	NT
Doxorubicin	0.39	9.04	0.07	NT
Tamoxifen	NT	9.61	NT	NT

Note: 0.5% DMSO was used as negative control. IC_{50} > 50 µg/ml = inactive. NT = not tested.

DISCUSSION

The leaf extracts of four *Combretum* species showed antioxidant and anticancer activities, which suggests the presence of antioxidant and anticancer compounds in the leaves. The antioxidant activity of these four species is newly reported here. Anticancer activity on KB, MCF-7 and NCI-H187 cell lines of *C. deciduum* and *C. latifolium* are initially reported here.

Methanolic extracts of the four species displayed the most potent antioxidant activity against both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals. Methanolic extracts of *C. latifolium* leaves showed the highest antioxidant activity, suggesting that this extract is a rich source of antioxidants. The antioxidant activity of *C. quadrangulare, C. griffithii* and *C. deciduum* followed. To study antioxidant activity, it is recommended to use at least two methods, as we did here. Our results showed that extracts with different polar compounds exhibit different antioxidant activities.

As traditional medicinal plants associated with anticancer uses might be potential sources of potent natural antioxidant, we also investigated these extracts for anticancer activity. The methanolic extracts from the leaves of *C. deciduum, C. latifolium* and *C. quadrangulare* exhibited anticancer activity against cancer cell lines (KB, MCF7 NCI-H187). However, the methanolic extract from the leaves of *C. griffithii* was inactive against all three cell lines. All methanolic leaf extracts were non-cytotoxic against Vero cell lines. It is generally accepted that free radicals react with biological molecules, leading to the possible development of cancer. Considerable laboratory evidence from chemical, cell culture and animal studies indicates that antioxidants may slow or possibly prevent cancer by stabilizing biological molecules and preventing damage to cells. The study confirmed that the methanolic extracts are the most potent in terms of their antioxidant, suggesting that the polar compounds residing in these extracts may be responsible. However, the anticancer activity of the plants could not be associated with these polar extracts, which have shown strong radical scavenging activity.

These results suggest that free radical scavenging activity may not be the only mechanism preventing development of cancer.

Lima et al. (2012) reviewed the bioactivities of the genus *Combretum*, in which the authors stated that ethanolic leaf extract of *C. decandrum* showed antioxidant activity with IC_{50} value of 0.75 g/kg by ferrous ion oxidation-xylenol orange method in rats. And ethanolic leaf extracts of *C. duarteanum* possess a strong antioxidant potential by using thiobarbituric acid reactive species (TBARS), hydroxyl radical–scavenging and scavenging activity of nitric oxide assays. In the review, Lima et al. (2012) also stated that the ethanolic extracts of the leaves, root and stem of *C. duarteanum* showed anticancer activity against KB cells. Methanolic and ethanolic extracts of dried air parts of *C. collinum* exhibited anticancer activity against squamous carcinoma KB with IC_{50} value of 20.00 µg/ml and methanolic extracts of leaves and root exhibited anticancer activity against MCF7 breast cancer with IC_{50} value of 25.00 µg/ml. Methanolic extracts of leaves and root of *C. apiculatum* subsp *apiculatum*, *C. fragrans, C. micranthum*; methanolic extracts of stem bark and root of *C. padoides*; methanolic extracts of stem bark of *C. hereroense* and *C. psidioides*; and methanolic extracts of root and fruits of *C. zeyheri* inhibited MCF7 breast cancer cells with IC_{50} value of 25.00 µg/ml.

Our findings support the above research, having found similar antioxidant and anticancer properties for extracts from the genus *Combretum*. These results indicate that properties of *Combretum* species might be further explored in the search for new antioxidant and anticancer compounds. This work could be extended by testing other parts of the four species studied here, or expanding to additional species.

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REFERENCES

Banskota, A.H., Y. Tezuka, L.K. Phung, K.Q. Tran, I. Saiki, Y. Miwa, T. Taga, and S. Kadota. 1998. Cytotoxic cycloartane-type triterpenes from *Combretum quadrangulare*. Bioorganic and Medicinal Chemistry Letters 8: 3519-3524. 10.1016/S0960-894X(98)00644-1

Banskota, A.H., Y. Tezuka, Q.L. Tran, and S. Kadota. 2003. Chemical constituents and biological activities of Vietnamese medicinal plants. Current Topics in Medicinal Chemistry 3: 227-248.

Clarke, C.B. 1878. Combretaceae, In Flora of British India Vol. p. 452-459. Brothers LTD, England.

Coulidiati, T.H., H. Millogo-Kone, A. Lamien-Meda, C.E. Lamien, M. Lompo, M. Kiendrebeogo, S. Bakasso, M. Yougbare-Ziebrou, J. Millogo-Rasolodimby, and O.G. Nacoulma. 2009. Antioxidant and antibacterial activities of Combretum nioroense Aubrev. ex Keay (Combretaceae). Pakistan Journal of Biological Sciences 12(3): 264-269. 10.3923/pjbs.2009.264.269

Eldeen, I.M.S., and J. van Staden. 2007. In vitro pharmacological investigation of extracts from some trees used in Sudanese traditional medicine. South African Journal of Botany 73: 435-440. 10.1016/j.sajb.2007.03.009

Eloff, J.N., D.R. Katerere, and L.J. McGaw. 2008. The biological activity and chemistry of the southern African Combretaceae. Journal of Ethnopharmacology 119: 686-699. 10.1016/j.jep.2008.07.051

Euswas, P., S. Srirod, P. Chunthanon, and T. Chompoochant. 1988. Studies on anthelmintic activity of Sakae naa (*Combretum quadrangulare* Kurz) seeds on roundworms of buffalo calves. Kasetsart University Journal 22: 201-206.

Fyhrquist, F., L. Mwasumbi, C.-A. Haeggström, H. Vuorela, R. Hiltunen, and P. Vuorela, 2002. Ethnobotanical and antimicrobial investigation on some species of *Terminalia* and *Combretum* (Combretaceae) in Tanzania. Journal of Ethnopharmacology 79: 169-177. 10.1016/S0378-8741(01)00375-0

Gronhaug, T.E., S. Glaeserud, M. Skogsrud, N. Ballo, S. Bah, D. Diallo, and B.S. Paulsen. 2008. Ethnopharmacological survey of six medicinal plants from Mali, West-Africa. Journal of Ethnobiology and Ethnomedicine 4(26): 1-11. 10.1186/1746-4269-4-26

Inngjerdingen, K., C.S. Nergard, D. Diallo, P.P. Mounkoro, and B.S. Paulsen. 2004. An ethnopharmacological survey of plants used for wound healing in Dogonland, Mali, West Africa. Journal of Ethnopharmacology 92: 233-244. 10.1016/j.jep.2004.02.021

Karou, D., M.H. Dicko, J. Simpore, and A.S. Traore. 2005. Antioxidant and antibacterial activities of polyphenols from ethomedicinal plants of Burkina Faso. African Journal of Biotechnology 4(8): 823-828.

Lima, G.R.M., I.R.P. Sales, M.R.D.C. Filho, N.Z.T. Jesus, H.S. Falcão, J.M. Barbosa-Filho, A.G.S. Cabral, A.L. Souto, J.F. Tavares, and L.M. Batista. 2012. Bioactivities of genus *Combretum* (Combretaceae): A review. Molecules 17: 9142-9206. 10.3390/molecules17089142

Maregesi, S.M., O.D. Ngassapa, L. Pieters, and A.J. Vlietinck. 2007. Ethnopharmacological survey of the Bunda district, Tanzania: Plants used to treat infectious diseases. Journal of Ethnopharmacology 113: 457-470. 10.1016/j.jep.2007.07.006

McGaw, L.J., T. Rabe, S.G. Sparg, A.K. Jager, J.N. Eloff, and J. van Staden. 2001. An investigation on the biological activity of *Combretum* species. Journal of Ethnopharmacology 75: 45-50. 10.1016/S0378-8741(00)0045-0

Moosophon, P., S. Kanokmedhakul, and K. Kanokmedhakul. 2011. Diarylpropanes and an arylpropyl quinone from *Combretum griffithii.* Journal of Natural Products 74: 2216-2218. 10.1021/np200593d

Nakornchai, S., R. Temsiririrkkul, Y. Wongkrajang, and K. Atisook. 1994. Toxicity study of *Combretum quadrangulare* Kurz (part II). Mahidol University Journal of Pharmaceutical Science 21(4): 118-125.

Nakornchai, S., R. Temsiririrkkul, Y. Wongkrajang, P. Jongtaveesuk, and P. Junjamjang. 1987. Toxicity study of *Combretum quadrangulare* Kurz I. Mahidol University Journal of Pharmaceutical Science 14(4): 69-74.

Nanakorn, W. 1986. The genus *Combretum* (Combretaceae) in Thailand. Thai Forest Bulletin 16: 154-204.

Nantachit, K., D. Santiarvorn, and B. Khantawa. 2006. Antibacterial activity of the seeds of *Combretum quadrangulare* Kurz (Combretaceae). Chiang Mai University Journal 5: 333-339.

O'Brien, J., I. Wilson, T. Orton, and F. Pognan. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry 267: 5421-5426.

Pettit, G.R., G.M. Cragg, and S.B. Singh. 1987. Antineoplastic agent, 122. constituents of *Combretum caffrum*. Journal of Natural Products 50(3): 386-391.

Plumb, J.A., R. Milroy, and S.B. Kaye. 1989. Effect of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium base assay. Cancer Research 49: 4435-4440.

Premjet, D., S. Premjet, R.A. Ario Lelono, and S. Tachibana. 2010. Callus induction and determination of iridoid glycosides from *Barleria prionitis* Linn leaf explants. Australian Journal of Basic and Applied Sciences 4(9): 4461-4467.

Roberta, R., P. Nicoletta, P. Anna, P. Ananth, Y. Min, and R.-E. Catherine. 1999. Antioxidant activity applying an improved ABTS radical cation decororization assay. Free Radical Biology and Medicine 26: 1231-1237.

Somanabandhu, A. 1984. Thai crude drugs their preparations and specifications (*Combretum quadrangulare* Kurz, Sakae naa). Mahidol University Journal of Pharmaceutical Science 11(1): 1-7.

Thongchai, W., B. Liawruangrath, and S. Liawruangrath. 2009. Flow injection analysis of total curcuminoids in turmeric and total antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl assay. Food Chemistry 122: 494-499. 10.1016/j.foodchem.2008.05.083

Ueda, J., Y. Tezuka, A.H. Banskota, Q.L. Tran, Q.K. Tran, Y. Harimaya, I. Saiki, and S. Kadota. 2002. Antiproliferative activity of Vietnamese medicinal plants. Biological and Pharmaceutical Bulletin 25(96): 753-760. 10.1248/bpb.25.753

none

Freshwater Fish Diversity at Greater Noakhali, Bangladesh

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ABSTRACT

This study assessed the spatial-temporal diversity of fish at greater Noakhali, an aquatic ecosystem that supports the most diverse fish communities in Bangladesh. Fish samples were collected from eight locations from July 2010 to June 2011 and diversity analyzed using PAST software. Findings showed that greater Noakhali is the habitat for 128 fish species. For the whole sampling area, the Shannon diversity index, evenness, Margalef richness and dominance index values were 4.501, 0.889, 15.763 and 0.012, respectively. Oreochromis mossambicus, Mastacembelus armatus and Tenualosa toli were the major contributory species in temporal terms and Tenualosa ilisha, Somileptes gongota and Mystus vittatus in spatial terms.

Keywords: Freshwater, Fish biodiversity, Noakhali, Bangladesh

INTRODUCTION

The extensive freshwater resources in Bangladesh (5,433,900 ha, covering 37% of the country) are the third most bio-diverse aquatic fishery in Asia, after China and India, with about 800 species in fresh, brackish and marine waters (Hussain and Mazid, 2001). This species diversity has been attributed to the diverse aquatic ecosystems that are scattered across the country in the form of rivers, ponds, ditches, lakes, beels/haors/baors (saucer shaped water bodies with monsoon expansion and winter contraction), floodplains and canals. Total fish production from the inland/freshwater area in 2003-04 was 914,752 MT, representing 78.3% of total fisheries production, accounting for 4.92% of GDP, 23% of the gross value added to agricultural products, more than 11% of export earnings, and employment for over 2 million people (DoF, 2005). Although fish provide 63% of Bangladesh's animal protein intake, fisheries production is not keeping pace with population growth (Hussain, 2010). To address this issue, the fisheries sector needs to maximize fish production in parallel with conserving its biological diversity.

Fisheries populations are very dynamic, both temporally and spatially (Chowdhury et al., 2010). Greater Noakhali possesses an extensive aquatic ecosystem, which supports multitudes of species of plants, fish, prawn and other organisms. Of these, fish are the most important element and the major source of dietary protein for the rural poor. This sector also generates employment opportunities that form the lifeline for the rural economy. Only a few years back, greater Noakhali contained a huge number of fish. However, over-exploitation, habitat alteration and indiscriminate use of agro-chemicals has led to drastic declines in their numbers, threatening people's livelihood. Considering the lack of baseline information on the fish species of greater Noakhali, this study explores the existing fish faunal composition, including their temporal and spatial diversity, of greater Noakhali.

Study area

This study was conducted at greater Noakhali (Figure 1), located at the central coastal zone of Bangladesh between latitude 22°30′ and 23°15′ N and longitude 89°45′ and 91°30′ E. The Noakhali River and small Feni River join many canals, tributaries, creeks and stream corridors. The tidal range at the Noakhali coast is large, ranging from 0.48 m at neap tide to 3.79 m at spring tide (Das and Hossain, 2005). Average temperatures vary between 12°C during December-February to 34°C during April-June. The monsoon or rainy season (June-October) is characterized by southeast monsoon winds with high rainfall, humidity and cloud cover. The greater Noakhali possesses different types of aquatic ecosystems, supporting a multitude of aquatic flora and fauna (Hossain, 2009).

Fisheries in this area support livelihood options for a significant proportion of the rural population, who primarily grow rice and fish, and to a lesser extent are engaged in fisheries aquaculture (Hossain and Das, 2010). The fishermen of the Noakhali coast fish for goby from early November to late March and for Bombay duck with estuarine set bag net and small-engine boats at the Meghna estuary from mid November to late March (Hossain, 2011). From late May to early November, they fish Hilsha. Besides fishing in rivers and estuaries, fishermen also use seine nets in local ponds along with hand nets, push nets, lift nets and traps in ponds, canals, rivers, creeks and flood plains.

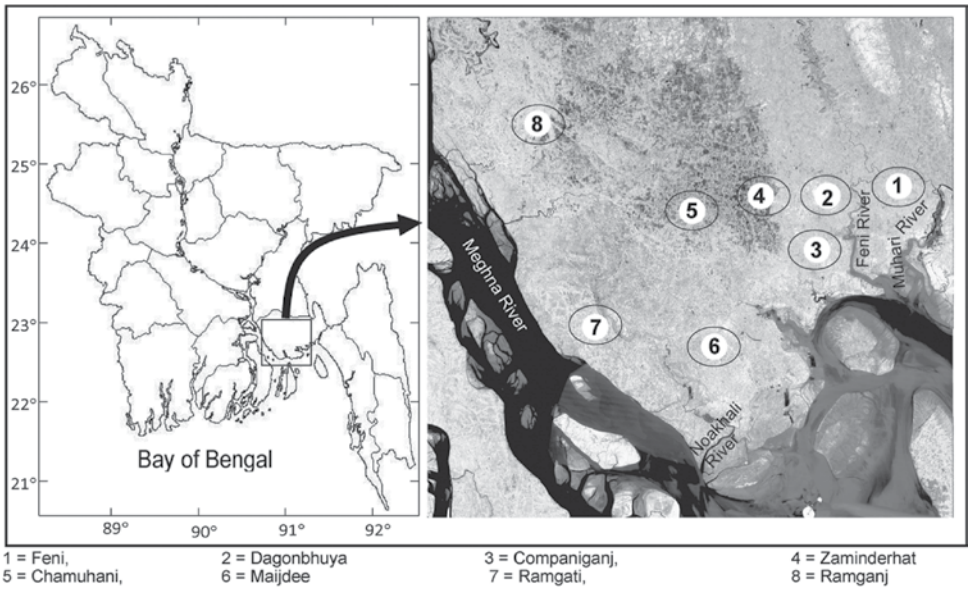

Figure 1. Geographical location of greater Noakhali, Bangladesh.

MATERIALS AND METHODS

Data collection

To collect fish species specimens, the study area is divided into eight sampling stations: Feni (sampling station #1, hereafter St 1), Dagonbhuya (St 2), Companiganj (St 3), Zaminderhat (St 4), Chamuhani (St 5), Maijdee (St 6), Ramgati (St 7) and Ramjanj (St 8). Fish samples were collected from July-October 2011 through extensive field visits. The fish were collected with seine nets, hand nets, push nets, lift nets, traps and hooks. Generally, fishermen throw away non-target fish, whether alive or dead, that they catch as a byproduct. Local fishermen were requested to keep all fish, target and non-target, for our research purposes. Samples were collected through personal visits to fishing and landing centers as well as fish markets in the area. Fishermen, fish traders and fish farmers were consulted for sampling purposes. Samples were collected, photographed and refrigerated. Chemicals were not added to preserve the fish. The samples were then transferred to the lab for taxonomic identification. The specimens were identified using the keys of Hamilton, 1822; Bhuiyan, 1964; Fischer & Whitehead, 1974; Shafi & Quddus, 1982; Rahman, 1989; Talwar & Jhingran, 1991; Bhuiyan et al., 1992; DeBruin et al., 1995; Siddiqui et al., 2007 and Hossain et al., 2007.

Data analysis

In the first stage of data analysis, the diversity of the fish assemblage was quantified and compared statistically. Paleontological Statistics (PAST) version 2.15, a software package for paleontological data analysis written by P.D. Ryan, D.A.T. Harper and J.S. Whalley, was used to run the analysis. PAST has grown

into a comprehensive statistics package that is used not only by paleontologists, but also in many fields of life science, earth science, and even engineering and economics. Species diversity was assessed using four different indices: Shannon–Wiener, richness, evenness and dominance indices in both the spatial and temporal spectrum. The pre-monsoon, monsoon and post-monsoon diversity indices were calculated from the spatial raw data by combining the fish communities of all study sites. All indices –Shannon-Weiner, Margalef, evenness and dominance – were calculated from the raw data for each temporal assemblage of fish.

Shannon-Weiner diversity index (Shannon, 1949; Shannon & Weaver, 1963; Ramos et al., 2006) considers both the number of species and the distribution of individuals among species. Shannon-Weiner diversity was calculated by the following formula:

$$H' = \sum_{i-1}^{S} P_i * \log P_i$$

Where, S is the total number of species and P_i is the relative cover of i^{th} species.

Margalef index (d) (Margalef, 1968) measured species richness according to the following formula:

$$d = (S-1)/\log N$$

Where S is total species and N is total individuals.

Buzas and Gibson's evenness (Harper, 1999) was measured by the following formula:

$$E = e^H/S$$

The dominance index (Harper, 1999) determines whether particular fisheries species dominate in a particular aquatic system. It is a useful index of resource monopolization by a superior competitor, particularly in communities that have been invaded by exotic species. This index was determined by the following formula:

$$D = \sum_i \left(\frac{n_i}{n} \right)^2$$

Where n_i is number of individuals of species i.

One-way analysis of variance (ANOVA) was used for diversity indices to calculate any difference among the months and stations. In the event of significance, a post-hoc Tukey HSD test determined which means were significantly different at a 0.05 level of probability (Spjotvoll & Stoline, 1973). Similarity percentages analysis (SIMPER) (Clarke, 1993) determined the percentage of similarity among

months and stations. In addition, SIMPER also estimated the percentage of major contributing species, for both months and stations. Hierarchical clustering (Clarke & Warwick, 1994) produced a dendrogram for investigating similarities among months and stations.

RESULTS

Species abundance and distribution

A total of 5,146 individual specimens were enumerated, comprising 128 species of finfish (Table 1). *Oreichthys cosuatis* represented the most individuals counted (82, or 1.6% of total individuals) and *Puntius mahmoodi* the least (9, or 0.17%). St 2 had the most individuals (799, 15.5%) counted throughout the study period and St 1 the least (491, 9.5%). Seasonal variation in abundance was significant in all sampling zones. The monsoon season recorded the highest number of individuals (2,069, 40.2%) and post-monsoon the least (1,403, 27.3%).

Table 1. Temporal and spatial fish species abundance and distribution in Noakhali, Bangladesh.

Species	Total	%	St 1	St 2	St 3	St 4	St 5	St 6	St 7	St 8	Pre-monsoon	monsoon	Post-monsoon
Chitala chitala	43	0.84	5	6	7	3	5	7	5	5	16	18	9
Chitala latifi	49	0.95	3	3	6	3	6	8	11	9	16	19	14
Notopterus notopterus	41	0.80	4	6	3	7	5	6	3	7	14	17	10
Pisodonophis boro	33	0.64	1	1	4	6	3	8	6	4	8	14	11
Corica soborna	51	0.99	6	4	6	4	5	13	7	6	10	19	22
Gonialosa manmina	29	0.56	4	4	3	3	6	6	1	2	11	9	9
Anodontostoma chacunda	18	0.35	4	4	9	0	1	0	0	0	9	6	3
Tenualosa ilisha	54	1.05	14	13	14	6	6	0	1	0	19	21	14
Tenualosa toli	31	0.60	11	10	6	0	4	0	0	0	11	14	6
Ilisha megaloptera	51	0.99	5	7	9	4	5	8	7	6	15	23	13
Gudusia chapra	41	0.80	3	3	14	0	12	0	9	0	14	16	11
Channa marulius	66	1.29	6	6	7	10	11	9	7	10	18	29	19
Channa orientalis	41	0.79	4	5	4	4	3	8	5	8	15	14	12
Channa punctatus	56	1.09	6	5	5	14	6	9	6	5	17	25	14
Channa striatus	41	0.79	4	4	4	7	5	5	6	6	13	17	11
Amblypharyngodon microlepis	48	0.93	6	4	4	10	4	5	10	5	18	19	11
Amblypharyngodon mola	45	0.88	4	1	6	9	4	6	10	5	17	16	12
Aristichthys nobilis	46	0.90	7	8	4	0	4	8	7	8	15	19	12
Barbonymus gonionotus	49	0.95	7	5	5	9	1	10	7	5	18	18	13
Catla catla	48	0.93	9	4	5	7	6	8	3	6	15	20	13
Chela cachius	49	0.95	5	14	4	8	4	7	0	7	18	21	10
Chela laubuca	25	0.49	5	6	4	0	5	0	5	0	8	10	7
Cirrhinus cirrhosus	61	1.19	6	5	7	3	14	14	8	4	18	28	15

Table 1. Temporal and spatial fish species abundance and distribution in Noakhali, Bangladesh (Cont.).

Cirrhinus reba	51	0.98	8	3	3	10	11	11	5	0	15	21	15
Ctenopharyngodon idella	52	1.01	10	6	10	8	3	3	4	8	18	17	17
Cyprinus carpio	57	1.11	8	5	8	5	6	6	11	8	18	22	17
Danio dangila	47	0.91	7	4	5	6	6	7	5	7	15	18	14
Esomus danricus	42	0.81	6	4	5	7	4	4	5	7	16	15	11
Esomus lineatus	39	0.75	8	4	7	5	4	4	3	4	16	12	11
Hypophthalmichthys molitrix	40	0.78	8	1	5	4	4	4	6	8	13	15	12
Labeo calbasu	37	0.72	7	6	4	8	1	2	4	5	10	14	13
Labeo gonius	41	0.80	8	4	8	5	6	6	4	0	13	16	12
Labeo rohita	53	1.02	9	5	5	9	4	8	4	9	24	17	12
Oreichthys cosuatis	82	1.59	10	14	9	15	5	10	4	15	35	29	18
Osteobrama cotio	38	0.74	6	11	15	6	0	0	0	0	13	17	8
Osteochilus hasseltii	50	0.98	7	5	6	4	11	4	4	9	17	20	13
Puntius chola	53	1.02	8	4	4	8	7	4	3	15	18	17	18
Puntius conchonius	37	0.71	7	6	8	8	4	0	4	0	9	15	13
Puntius guganio	59	1.15	6	4	5	12	14	9	5	4	24	21	14
Puntius phutunio	45	0.88	8	14	9	3	11	0	0	0	16	20	9
Puntius sarana	59	1.14	8	9	15	5	5	4	4	9	21	22	16
Puntius sophore	44	0.86	3	5	6	4	3	4	0	19	14	17	13
Puntius terio	30	0.58	3	7	11	3	6	0	0	0	9	15	6
Puntius ticto	51	0.98	5	7	9	4	5	7	5	9	20	17	14
Puntius yusufi	48	0.94	4	6	5	5	4	4	5	15	18	16	14
Puntius mahmoodi	9	0.17	5	0	0	0	0	0	4	0	5	2	2
Puntius kaderi	14	0.27	0	0	0	0	3	0	11	0	5	6	3
Puntius matini	67	1.30	4	4	11	8	11	10	7	12	19	29	19
Rasbora daniconius	44	0.86	3	6	9	4	9	5	4	4	13	18	13

Table 1. Temporal and spatial fish species abundance and distribution in Noakhali, Bangladesh (Cont.).

Salmostoma bacaila	30	0.59	1	4	5	5	5	4	3	3	12	9	9
Acanthocobitis botia	63	1.22	5	14	4	6	4	14	6	10	22	24	17
Schistura beavani	40	0.78	3	9	5	5	5	4	5	4	14	16	10
Botia dario	11	0.21	2	4	5	0	0	0	0	0	3	4	4
Botia lohachata	19	0.37	2	6	11	0	0	0	0	0	4	10	5
Lepidocephalus berdmorei	37	0.71	2	4	9	8	6	3	5	0	12	15	10
Lepidocephalichthys annandalei	62	1.21	2	14	5	7	10	5	5	14	17	25	20
Lepidocephalichthys guntea	33	0.64	2	9	7	11	0	4	0	0	17	10	6
Somileptes gongota	67	1.30	2	11	8	11	11	14	5	5	19	29	19
Pygocentrus nattereri	67	1.30	6	10	5	11	9	11	7	8	21	27	19
Batasio batasio	64	1.24	6	7	10	10	5	5	11	10	20	26	18
Batasio tengana	57	1.11	2	12	13	8	4	3	7	8	13	25	19
Hemibagrus menoda	17	0.33	2	0	0	0	5	0	9	1	4	10	3
Mystus bleekeri	59	1.15	2	11	1	11	8	5	11	10	17	22	20
Mystus cavasius	52	1.02	2	10	5	9	5	4	11	6	12	22	18
Mystus gulio	52	1.01	2	4	0	5	11	11	7	12	17	20	15
Mystus tengara	40	0.78	2	14	9	4	7	0	4	0	12	13	15
Mystus vittatus	66	1.28	6	7	13	5	11	2	10	12	19	25	22
Sperata aor	51	0.98	4	10	6	8	10	2	4	7	18	15	18
Sperata oblongata	52	1.01	3	9	3	5	10	7	7	8	18	16	18
Ompok bimaculatus	16	0.31	0	0	3	0	5	6	2	0	5	6	5
Ompok pabda	34	0.66	6	9	7	8	4	0	0	0	9	15	10
Ompok pabo	21	0.41	0	7	9	5	0	0	0	0	5	8	8
Wallago attu	49	0.95	4	8	11	4	5	1	8	8	18	22	9
Wallago sudharami	29	0.56	0	0	6	14	5	0	4	0	11	10	8

Table 1. Temporal and spatial fish species abundance and distribution in Noakhali, Bangladesh (Cont.).

Silonia silondia	38	0.74	4	7	2	11	5	0	4	5	13	20	5
Pseudeutropius atherinoides	26	0.51	0	10	1	5	10	0	0	0	6	14	6
Pangasius pangasius	53	1.03	2	9	3	3	13	2	9	12	12	26	15
Clarias batrachus	39	0.75	2	7	3	6	6	2	4	9	10	19	10
Clarias gariepinus	52	1.02	2	10	9	5	5	12	5	4	16	23	13
Heteropneustes noakhaliensis	45	0.87	2	9	4	4	9	6	7	4	15	16	14
Aplocheilus panchax	42	0.82	2	7	1	8	5	7	7	5	11	25	6
Monopterus cuchia	36	0.70	2	10	2	5	10	7	0	0	9	19	8
Platycephalus indicus	29	0.56	2	9	1	4	13	0	0	0	7	12	10
Lates calcarifer	34	0.66	2	9	3	14	6	0	0	0	11	16	7
Chanda nama	57	1.11	2	5	9	11	5	10	4	11	16	26	15
Pseudambasis baculis	46	0.89	2	7	3	5	4	8	5	12	16	20	10
Pseudambasis lala	50	0.98	2	7	9	3	14	5	9	1	21	16	13
Pseudambasis ranga	50	0.97	2	6	7	6	11	5	13	0	15	22	13
Parambasis thomassi	58	1.13	2	9	10	5	5	7	12	8	22	24	12
Nandus nandus	40	0.78	2	5	9	4	3	5	5	7	13	17	10
Nandus meni	43	0.84	2	7	7	6	6	5	5	5	12	21	10
Badis badis	23	0.45	0	7	10	1	5	0	0	0	10	7	6
Oreochromis mossambicus	37	0.72	9	6	9	3	4	1	3	2	12	14	11
Oreochromis niloticus	46	0.89	2	9	9	7	10	5	3	1	15	16	15
Liza parsia	24	0.47	2	5	5	4	8	0	0	0	5	12	7
Mugil cephalus	34	0.66	2	7	2	8	5	10	0	0	8	17	9
Rhinomugil corsula	29	0.57	2	7	2	5	5	8	0	0	11	10	8
Polynemus paradiseus	21	0.41	2	6	2	4	7	0	0	0	10	6	5
Acentrogobius caninus	38	0.74	2	9	2	15	5	1	2	2	14	17	7

Table 1. Temporal and spatial fish species abundance and distribution in Noakhali, Bangladesh (Cont.).

Acentrogobius viridipunctatus	30	0.58	2	5	2	11	1	5	3	1	8	15	7
Apocryptes bato	19	0.37	2	7	2	5	3	0	0	0	5	12	2
Pseudapocryptes elongatus	21	0.41	2	7	2	3	7	0	0	0	11	5	5
Awaous guamensis	20	0.39	2	6	2	6	4	0	0	0	5	8	7
Glossogobius giuris	26	0.51	2	9	2	5	8	0	0	0	7	13	6
Oxyurichthys microlepis	23	0.45	2	5	7	4	5	0	0	0	10	8	5
Parapocryptes batoides	23	0.45	2	7	6	4	4	0	0	0	8	10	5
Stigmatogobius sadanundio	33	0.64	2	7	9	1	14	0	0	0	13	13	7
Odontamblyopus rubicundus	31	0.60	6	6	5	3	11	0	0	0	9	12	10
Taenioides buchanani	30	0.58	4	7	7	7	5	0	0	0	8	15	7
Eleotris fusca	24	0.47	3	7	7	4	3	0	0	0	13	5	6
Eleotris lutea	32	0.62	4	6	6	10	6	0	0	0	8	11	13
Anabas testudineus	49	0.96	6	7	9	3	5	5	9	5	15	20	14
Anabas oligolepis	42	0.82	0	7	5	7	4	4	11	4	15	15	12
Pseudosphromenus cupanus	29	0.56	4	6	9	4	6	0	0	0	9	11	9
Malpulutta kretseri	11	0.21	0	0	0	0	0	0	6	5	3	5	3
Colisa fasciatus	50	0.96	4	2	2	11	4	11	7	9	15	19	16
Colisa lalia	38	0.74	0	6	2	8	2	9	8	3	12	13	13
Ctenops nobilis	35	0.68	2	2	2	5	4	5	11	4	15	12	8
Macrognathus aculeatus	49	0.95	6	2	7	5	9	4	11	5	17	17	15
Macrognathus pancalus	52	1.00	2	7	6	7	9	5	10	6	20	19	13
Mastacembelus armatus	55	1.06	4	2	9	5	11	5	10	9	15	25	15
Xenentodon cancila	27	0.52	3	8	3	3	5	0	5	0	6	13	8
Oryzias dancena	63	1.23	6	12	7	9	9	11	4	5	18	32	13
Tetraodon cutcutia	51	0.98	3	5	9	5	11	9	4	5	13	22	16
Total	5146	100	491	799	733	720	746	545	562	550	1674	2069	1403

Diversity status

After polling whole samples (48), total H′ value was 4.5005 (Table 2). The maximum H′ value by station was 4.675 at St 2 and the minimum was 4.226 at St 8. In terms of temporal distribution, the maximum H′ value by season was 4.62 during monsoon at St 1 and the minimum was 4.009 during pre-monsoon at St 8. The average H′ value was 4.368 for pre-monsoon, 4.437 for monsoon and 4.365 for post-monsoon. Significant difference (Table 6) was observed between samples and within samples (F=17.58 and P=0.001).

Table 2. Shannon-Weiner (H′) diversity value in eight sampling stations.

Sampling station	Sampling season			Pooled (H′) value	
	Pre-monsoon	Monsoon	Post-monsoon	Each station	Whole sampling area
St1	4.353	4.620	4.083	4.581	4.5005
St2	4.599	4.575	4.542	4.675	
St3	4.491	4.608	4.424	4.640	
St4	4.490	4.497	4.581	4.610	
St5	4.536	4.515	4.570	4.625	
St6	4.202	4.254	4.229	4.309	
St7	4.264	4.222	4.356	4.338	
St8	4.009	4.207	4.138	4.226	
Each season	4.368	4.437	4.365		

Total evenness value for the whole sampling area was 0.888 (Table 3). The maximum evenness value by station was 0.911 at St 7 and the minimum was 0.849 at St 1. In terms of temporal distribution, the maximum evenness value was 0.928 during post monsoon at St 7 and the minimum was 0.811 during pre monsoon at St 8. The average evenness value was 0.848 for pre-monsoon, 0.841 for monsoon and 0.902 for post-monsoon. Similar to H′ value, significant difference was also observed (Table 6) between and within the samples for evenness value (F=16.63 and P=0.0004).

Table 3. Evenness index (E) value in eight sampling stations.

Sampling station	Sampling season			Pooled evenness	
	Pre-monsoon	Monsoon	Post-monsoon	Each station	Whole sampling area
St1	0.863	0.882	0.927	0.849	0.8885
St2	0.864	0.822	0.912	0.909	
St3	0.850	0.850	0.878	0.870	
St4	0.841	0.816	0.895	0.897	
St5	0.840	0.809	0.894	0.887	
St6	0.868	0.838	0.915	0.885	
St7	0.847	0.832	0.928	0.911	
St8	0.811	0.883	0.871	0.901	
Each season	0.848	0.841	0.902		

Total dominance index value for the whole sampling area was 0.0124 (Table 4). The maximum dominance index value by station was 0.016 at St 8 and the minimum was 0.010 at St 2. In terms of temporal distribution, the maximum dominance index value was 0.018 during post-monsoon at St 1 and the minimum was 0.011 during monsoon at St 1. The average dominance index value was 0.015 for pre-monsoon, 0.014 for monsoon and 0.014 for post-monsoon. No significant difference (Table 6) was found between and within samples for dominance index value (F=1.03 and P=0.3825).

Table 4. Dominance index (D) value in eight sampling stations.

Sampling station	Sampling season			Pooled dominance	
	Pre-monsoon	Monsoon	Post-monsoon	Each station	Whole sampling area
St1	0.015	0.011	0.018	0.012	0.0124
St2	0.011	0.012	0.012	0.010	
St3	0.013	0.011	0.014	0.011	
St4	0.013	0.013	0.011	0.011	
St5	0.013	0.013	0.012	0.011	
St6	0.017	0.016	0.016	0.015	
St7	0.016	0.017	0.014	0.015	
St8	0.021	0.017	0.018	0.016	
Each season	0.015	0.014	0.014		

Total Margalef richness value for the whole sampling area was 15.763 (Table 5). The maximum Margalef richness value by station was 18.40 at St 1 and the minimum was 11.96 at St 8. In terms of temporal distribution, the maximum Margalef richness value was 20.96 during monsoon at St 1 and the minimum was 13.09 during pre-monsoon at St 8. The average Margalef richness value was 17.464 for pre-monsoon, 18.139 for monsoon and 16.904 for post- monsoon.

No significant difference (Table 6) was found between and within samples for Margalef richness value (F=1.431 and P=0.2719).

Table 5. Margalef richness (d) value in eight sampling stations.

Sampling station	Sampling season			Pooled richness	
	Pre-monsoon	Monsoon	Post-monsoon	Each station	Whole sampling area
St1	17.67	20.96	13.40	18.40	15.763
St2	20.39	19.96	19.56	17.49	
St3	18.78	20.65	17.76	17.89	
St4	19.29	19.57	19.94	16.91	
St5	20.20	19.68	19.91	17.26	
St6	14.80	15.49	14.62	13.25	
St7	15.49	14.67	16.31	12.95	
St8	13.09	14.13	13.73	11.96	
Each season	17.464	18.139	16.904		

Table 6. Factorial analysis of variance for fisheries diversity indices.

Indices	Source of Variation	Sum of Squares	df	Mean squares	F-ratio	P
Shannon-Wiener index	Between samples	0.026583	2	0.013291	17.58	0.0001
	Within samples	0.776267	21	0.036965		
	Total	0.80285	23			
Evenness diversity index	Between samples	0.01793	2	0.008965	16.63	0.0004
	Within samples	0.011318	21	0.000539		
	Total	0.029248	23			
Dominance diversity index	Between samples	0.0006	2	0.00003	1.03	0.3825
	Within samples	0.00017	21	0.0036		
	Total	0.000176	23			
Margalef richness index	Between samples	6.11853	2	3.05927	1.431	0.2719
	Within samples	162.368	21	7.73182		
	Total	168.487	23			

Spatial and temporal relation of fisheries bio-diversity

According to SIMPER (Table 7), 60.9% similarity was found among the seasons and the major contributing species were *Oreochromis mossambicus* (2.5%), *Mastacembelus armatus* (2.5%), *Tenualosa toil* (2.5%), *Oryzias dancena* (2.0%), *Chanda nama* (2.0%) and *Ctenopharyngodon idella* (2.0%). Among the stations, 56.01% similarity was observed and the major contributing species were *Tenu-*

alosa ilisha (1.4%), *Somileptes gongota* (1.2%), *Mystus vittatus* (1.2%), *Puntius phutunio* (1.2%), *Pangasius pangasius* (1.2%) and *Gudusia chapra* (1.2%). At the similarity level, 45% separation, either for month or station, was identified by cluster analysis (Figure 2).

The cluster analysis represents two groups of fish that divided the fish community structure into two major groups between 0.48 and 0.54 similarity levels. The first cluster consists of: St 1 with monsoon; St 2 with pre-monsoon, monsoon and post-monsoon; St 3 with pre- monsoon, monsoon and post-monsoon; St 4 with pre-monsoon, monsoon and post-monsoon; St 5 with pre-monsoon, monsoon and post-monsoon; St 6 with pre-monsoon, monsoon and post-monsoon; St 7 with pre-monsoon, monsoon and post-monsoon; and St 8 with pre-monsoon, monsoon and post-monsoon seasons. The second cluster consists of St 1 with pre-monsoon and post-monsoon seasons.

Table 7. Average similarity and discriminating fish species in all stations and seasons.

Average Similarity			
Temporal (60.9%)		Spatial (56.0%)	
Species	Contribution %	Species	Contribution %
Oreochromis mossambicus	2.5	*Tenualosa ilisha*	1.4
Mastacembelus armatus	2.5	*Somileptes gongota*	1.2
Tenualosa toli	2.5	*Mystus vittatus*	1.2
Oryzias dancena	2.0	*Puntius phutunio*	1.2
Chanda nama	2.0	*Pangasius pangasius*	1.2
Ctenopharyngodon idella	2.0	*Gudusia chapra*	1.2

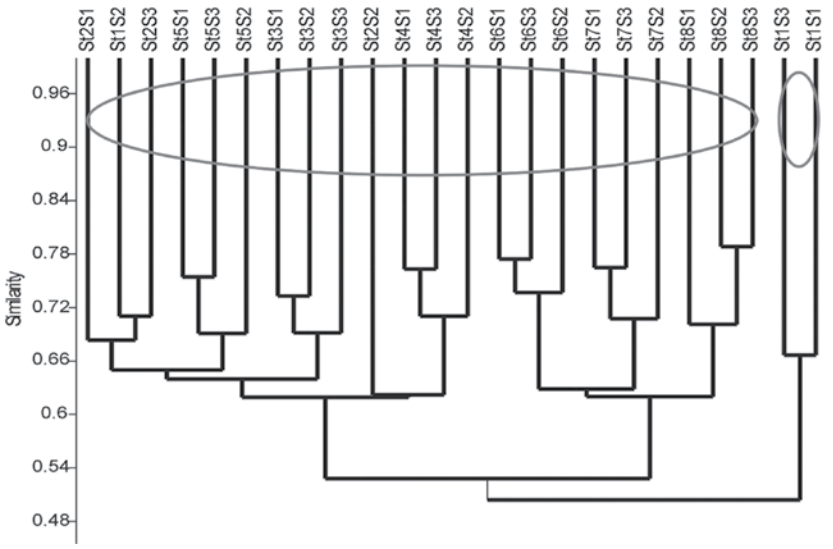

Figure 2. Spatial and temporal cluster of fish assemblage based on Bray-Curtis similarity matrix.

DISCUSSION

This study recorded 128 species of freshwater fishes from the greater Noakhali District. The following species contributed more than 1% of the total composition: *Tenualosa ilisha, Channa marulius, Channa punctatus, Cirrhinus cirrhosus, Ctenopharyngodon idella, Cyprinus carpio, Labeo rohita, Oreichthys cosuatis, Puntius chola, Puntius guganio, Puntius sarana, Puntius matini, Acanthocobitis botia, Lepidocephalichthys annandalei, Somileptes gongota, Pygocentrus nattereri, Batasio batasio, Batasio tengana, Mystus bleekeri, Mystus cavasius, Mystus gulio, Mystus vittatus, Sperata oblongata, Pangasius pangasius, Clarias gariepinus, Chanda nama, Parambasis thomassi, Macrognathus pancalus, Mastacembelus armatus* and *Oryzias dancena.*

Rahman (2005) identified 265 freshwater fish species in Bangladesh. From our survey, Noakhali represents 48% of the country's total fish species (Table 8). Mymensingh and Rajshai, regions with similar environmental characteristics as Noakhali, also have a similar number of species, 139 and 133, respectively, and share considerable species overlap with Noakhali.

Table 8. Studies on freshwater fish species of Bangladesh in the past 50 years.

Number of species	Number of family	Study area	References
128	34	Noakhali	Present study (2013)
139	34	Mymensingh	Chandra (2009)
251	61	Bangladesh	Siddiqui et al. (2007)
265	55	Bangladesh	Rahman (2005)
133	32	Rajshahi	Bhuiyan et al. (1992)
106	34	Mymensingh and Tangail	Doha (1973)
71	25	Dhaka	Bhuiyan (1964)

Variation in species composition was observed at different locations in the Noakhali study area due to the different environmental characteristics of the aquatic ecosystem. The number of order, families and species of fish represented in greater Noakhali is a rich and diverse resource, providing a significant contribution to both the national economy and protein demand for Bangladesh.

However, human interaction is continuously reducing the water body of the area. This, coupled with increased fishing pressure, is reducing fisheries diversity in greater Noakhali. St 2 has the highest number of individuals (803). This is an area that is subject to relatively low human interference and thus is under-fished and retains an optimum environmental condition. In contrast, St 1, which is subject to extreme human interference, had the lowest number of individuals (491).

Major dominant species were observed in the present study area, similar to several studies that reported the dominance of the resident species (Doha, 1973; Bhuiyan et al., 1992; Rahman, 2005 and Chandra, 2009).

A biodiversity index seeks to characterize the diversity of a sample or community by a single number (Magurran, 1988). The concept of "species diversity" involves two components: the number of species or richness and the distribution

of individuals among species. However, the formal treatment of the concept and its measurement is complex (Williamson, 1973). The Shannon-Wiener diversity index considers the richness and proportion of each species, while the evenness and dominance indices represent the relative number of individuals in the sample and the fraction of common species, respectively. The biodiversity index values (H') obtained from the present study are not high according to the Shannon-Weaver biodiversity index values and they do not show differences among the stations, either. According to Keskin and Ünsal (1998), lower species biodiversity can reflect the high selectivity effect of fishing gear. This study ignored the fishing gear effect. The maximum Shannon diversity index was during the monsoon at St 1 and the minimum during the pre-monsoon at St 8. In each case, the high Shannon diversity index value indicates low individuals and low diversity involved with a high number of individuals. The main causes of the differences occurring in the biodiversity indexes are seasonal variations in nutrients of the sea grass beds, affecting the coexistence of many fish species (Huh and Kitting, 1985); atmospheric air currents and environmental conditions (Keskin and Unsal, 1998); and seasonal fish migrations (Ryer and Orth, 1987). The maximum evenness value was at St 7 and the minimum at St 1. In terms of temporal distribution, maximum evenness value was during the post-monsoon at St 7 and the minimum during pre-monsoon at St 8. A number of fish species reproduce in the monsoon water bodies of Bangladesh, which may be the reason why the number of individuals increased during and after the monsoon period, as new individuals joined the fish stocks. In addition to this, ecological conditions also have an effect on the distribution of the fish species. The maximum dominance index value was at St 8 and the minimum at St 2. In terms of temporal distribution, the maximum dominance index value was during the post-monsoon and the minimum during the monsoon. If we compare the temporal variation of dominance status among the all sampling zones and months, it did not fluctuate much. The maximum Margalef richness value was at St 1 and the minimum at St 8. In terms of temporal distribution, maximum Margalef richness value was during the monsoon and the minimum during the pre-monsoon.

In terms of the spatial and temporal assemblage structure of fish, this study found two major groups using cluster analysis. Group 1 and 2 showed 45% similarity with each other. This study also found virtually the same similarity of the fish assemblage among the stations and months. The major contributing species for both stations and months are also similar, although their percentage contribution differs from each other. The fluctuating hydrological and meteorological parameters of seasonality are the primary factor affecting this similarity and dissimilarity (Whitfield, 1989; Loneragan & Potter, 1990; Young & Potter, 2003). Seasonality also affects the spawning activity of fish, which ultimately influences the catch composition (McErlean et al., 1973).

REFERENCES

Bhuiyan, A.L. 1964. Fishes of Dacca. Asiatic Society of Pakistan, Dacca. 148 pp.

Bhuiyan, A.S., M.N. Islam, and M.T. Hossain. 1992. A checklist of the fishes of Rajshahi. Rajshahi Univ. Studies, Part-B (20): 287-306

Chandra, K. J. 2009. Availability of fish fauna in some selected districts in Bangladesh. Bang. J. Anim. Sci., 38(1&2): 151-163

Chowdhury, M.S.N., M.S. Hossain, S.R. Chowdhury, and N.G. Das. 2010. Fisheries Species Composition and Water Quality of the Naaf River Estuary. Bangladesh Journal of Marine Sciences and Fisheries, 1 (1): 1-20.

Clarke K.R. 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18:11743.

Clarke K.R., and R.M. Warwick. 1994. Change in Marine Communities. An Approach to Statistical Analysis and Interpretation. Plymouth: Natural Environment Research Council. 144 pages.

Das, N.G., and M.S. Hossain. 2005. Livelihood and resource assessment for aquaculture development in waterlogged paddy lands: Remote sensing, GIS and participatory approach. DOF/GNAEP/Chittagong University, Bangladesh, 122p.

DeBruin, G.H.P., B.C. Russell, and A. Bogusch. 1995. FAO Species Identification Field Guide for Fishery Purposes. The Marine Fishery Resources of Sri Lanka. Rome: Food and Agricultural Organisation. 400 pages.

Doha, S. 1973. Fishes of the districts of Mymensingh and Tangail. Bangladesh J. Zool. 1: 1-10.

DoF, 2005. Fish Fortnight Souvenir 2005. Department of Fisheries (DoF), Ministry of Fisheries and Livestock, Dhaka, Bangladesh. 152 p.

Fischer, W., and P.J.P. Whitehead. 1974. FAO Species Identification Sheets for Fishery Purposes. Eastern Indian Ocean (fishing area 57) and Western Central Pacific (fishing area 71). Vols. 14. Rome: FAO.

Hamilton, F. 1822. An Account of the Fishes Found in the River Ganges and its Branches. Archibald Constable and Company, Edinburgh. 405. pp.

Harper, D.A.T. (ed.). 1999. Numerical Palaeobiology. John Wiley & Sons.

Hossain, M.S., N.G. Das, and M.S.N. Chowdhury. 2007. Fisheries Management of the Naaf River. Coastal and Ocean Research Group of Bangladesh, 257pp.

Hossain, M.S. 2009. Floodplain aquaculture in Begumgonj: New horizon for rural livelihoods in Bangladesh, Aquaculture Asia XIV (3): 7-10

Hossain, M.S., and N.G. Das. 2010. GIS-based multi-criteria evaluation to land suitability modelling for giant prawn (*Macrobrachium rosenbergii*) farming in Companigonj Upazila of Noakhali, Bangladesh. Computers and Electronics in Agriculture, 70 (1): 172-186. 10.1016/j.compag.2009.10.003

Hossain, M.S. 2011. Fisheries for society: Way to poverty alleviation and nutritional security at Noakhali coast, Fisheries and Aquaculture News, 1: 25-28.

Huh, S.H., and C.L. Kitting. 1985. Trophic relationships among concentrated populations of small fishes in seagrass meadows. J. Exp. Mar. Biol. Ecol. 92: 29-43.

Hussain, M.G., and M.A. Mazid. 2001. Genetic inprovement and conservation of carp species in Bangladesh. Bangladesh Fisheries Research Institute and International Center for Living Aquatic Resources Management.

Hussain, M.G. 2010. Freshwater fishes of Bangladesh: Fisheries, biodiversity and habitat, Aquatic Ecosystem Health & Management, 13(1): 85-93. 10.1080/14634980903578233

Keskin, Ç., and N. Ünsal. 1998. The Fish Fauna of Gökçeada Island, NE Aegean Sea, Turkey. Ital. J. Zoo., 65: 299-302.

Loneragan, N.R, and I.C. Potter. 1990. Factors influencing community structure and distribution of different life-cycle categories of fishes in shallow waters of a large Australian estuary. Marine Biology 16:25-37. 10.1007/BF2114671

McErlean, A.J., S.G. O'Connor, J.A. Mihursky, and C.I. Gibson. 1973. Abundance, diversity and seasonal patterns of estuarine fish populations. Estuarine, Coastal and Marine Science 1:1936.

Magurran, A.E. 1988. Ecological diversity and its measurement. Princeton University Press, Princeton.

Margalef, R. 1968. Perspectives in Ecological Theory. Chicago, IL: University of Chicago Press. 111 pages.

Rahman, A.K.A. 1989. Freshwater Fishes of Bangladesh. 1st ed. Zoological Society of Bangladesh, Dhaka. 364 pp.

Rahman, A.K.A. 2005. Freshwater Fishes of Bangladesh. 2nd ed. Zoological Society of Bangladesh, Dhaka. 394 pp.

Ramos, S., R.K. Cowen, P. Re, A.A. Bordalo. 2006. Temporal and spatial distribution of larval fish assemblages in the Lima estuary (Portugal). Estuarine, Coastal and Shelf Science, 66: 303-314. 10.1016/j.ecss.2005.09.012

Ryer, C.H., and R.J. Orth. 1987. Feeding Ecology of the Northern Pipefish, Syngnathus fuscus, in a Seagrass Community of the Lower Chesapeake Bay. Estuaries, 10(4): 330-336. 10.1007/BF02689864

Shafi, M., and M.M.A. Quddus. 1982. Fisheries resources of Bay of Bengal (Bonggoposagarer Matshaya Sampad, In Bengali). Bangla Academy, Dhaka.

Shannon, C.E. 1949. Communication in the presence of noise. Proceedings of the Institute of Radio Engineers 37:1021. 10.1109/PROC.1984.12998

Shannon, C.E., and W. Weaver. 1963. The Mathematical Theory of Communications. Urbana, IL: University of Illinois Press. 125 pages.

Siddiqui, K.U., M.A. Islam, S.M.H. Kabir, M. Ahmad, A.T.A. Ahmed, A.K.A. Rahman, E.U. Haque, Z.U. Ahmed, Z.N.T. Begum, M.A. Hasan, M. Khondker, and M.M. Rahman, (eds.). 2007. Encyclopedia of Flora and Fauna of Bangladesh. Vol. 23. Freshwater Fishes. Asiatic Society of Bangladesh, Dhaka 300 pp.

Spjotvoll, E., and M.R. Stoline. 1973. An extension of the T-method of multiple comparisons to include the cases with unequal sample sizes. Journal of the American Statistical Association 68:97678.

Talwar, P.K., and A.G. Jhingran. 1991. Inland fishes of India and adjacent countries. Vol.1&2, Oxford & IBH Publishing Company Pvt. Ltd, New Delhi, pp.1-1158.

Whitfield, A.K. 1989. Ichthyoplankton in a Douthern African surf zone: Nursery area for the postlarvae of estuarine associated fish species? Estuarine, Coastal and Shelf Science 29:533-547.

Williamson, M. 1973. Species diversity in ecological communities. In: Bartlett MS, Horns RW (eds), The mathematical theory of the dynamics of biological populations. Academic, London, pp 325-336.

Young, G.C., and I.C. Potter. 2003. Do the characteristics of the ichthyoplankton in an artificial and a natural entrance channel of a large estuary differ? Estuarine, Coastal and Shelf Science, 56:765-779. 10.1016/S0272-7714(02)00300-1

none

Effectiveness of an Alcohol Relapse Prevention Program Based on the Satir Model in Alcohol-dependent Women

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ABSTRACT

This study aimed to determine the effectiveness of an alcohol relapse prevention program based on the Satir Model on self-esteem, self-efficacy, life congruence and drinking behaviors by measuring heavy drinking days, abstinence days and levels of serum gamma-glutamyl transferase (GGT) in alcohol-dependent women. A randomized controlled trial was designed. Thirty-nine alcohol-dependent women hospitalized at either Suan Prung Psychiatric Hospital or Thanyarak Chiangmai Hospital, in Chiang Mai, Thailand, were randomly assigned into an experimental group of 18 women or a control group of 21 women. Results revealed that immediately following, and at 12 and 16 weeks after completing the alcohol relapse prevention program, participants in the experimental group demonstrated statistically significant increased self-esteem, self-efficacy and life congruence; increased abstinence days; and decreased heavy drinking days compared to the control group. In addition, at 16 weeks after completing the program, the experimental group had statistically significant lower levels of serum GGT than the control group. The alcohol relapse prevention program based on the Satir Model improved psychological health and prevented alcohol relapse among alcohol-dependent women.

Keywords: Alcohol-dependent women, Effectiveness, Relapse prevention, Satir Model

INTRODUCTION

Studies in Thailand and other countries indicate that significant risk factors for alcohol relapse in women include: (1) depression (Gjestad et al., 2011; Snow & Anderson, 2000; Zywiak et al., 2006); (2) stress from marriage and stress caused by an alcoholic spouse (Chansantor, 1998; Kamkan, 2005; Chowwilai, 2006; Walitzer & Dearing, 2006 and Jongchokdee, 2010); (3) low self-esteem (Angove & Fothergill, 2003; Silverstone & Salsali, 2003 and Jakobsson et al., 2008); (4) low self-efficacy (Scott, Foss, & Dennis, 2005; Moos & Moos, 2006; Mensinger

et al., 2007 and Maisto et al., 2008); (5) disconnectedness (Masters & Carlson, 2006); and (6) psychological trauma caused by sexual/physical/emotional abuse (Chansantor, 1998; Knopik et al., 2004; Chowwilai, 2006).

Existing relapse prevention programs (from 2001 to 2010) were identified through literature reviews using Cochrane, Joannabrigs, PubMed, Science Direct and CINAHL database records, including database records from the Thai University Library Digital Collection and Thai journals. Using systematic review methods, five types of interventions were identified as significantly effective in stopping drinking when compared to control groups. However, none of these relapse prevention programs has been adopted in Thailand for a variety of reasons. Up to now, no intervention program has addressed factors specifically related to alcohol relapse in women.

Evidence indicates that women have difficulty coping with their internal feelings and emotions. Alcohol consumption is one way in which they try to cope with unpleasant emotions. Understanding that alcohol consumption is a coping phenomenon correlates well with the therapeutic belief about coping as established by the Satir Model, which states that the problem is not the problem; coping is the problem (Satir, 1983 and Satir et al., 1991). The Satir Model views symptoms of intra-psychic conflict – stress, depression, pain, trauma, conflict, suffering, low self-esteem and alcohol consumption – as a person's way to cope with emotional pain or negative feelings. According to the Satir Model, alcohol-dependent women drink and relapse to drinking in order to cope with their intra-psychic conflict.

The Satir Model focuses on internal change. As literature reviews indicate, the Satir Model is appropriate for helping alcohol-dependent patients change their intra-psychic world. Using the iceberg as a metaphor for a person's intra-psychic world, the Satir Model addresses a person's yearnings, expectations, perceptions, feelings and feelings about feelings, as well as their survival coping patterns and behavior. Alcohol-dependent women continue to consume alcohol because they have unfulfilled yearnings, and drinking alcohol is their dysfunctional response to those feelings. However, if their yearnings are fulfilled, and a functional coping response can be established, then alcohol-dependent women can become positive choice makers. They are able to take responsibility for their internal and external world. They are more congruent, experience higher self-esteem and can relate empathetically with others (Satir, 1983 and Satir et al., 1991).

Five previous studies indicated that nursing intervention based on the Satir Model could effect intra-psychic change in alcohol-dependent patients. However, these studies also showed gaps of knowledge about the prevention of relapse among alcohol-dependent women. None of the programs had been designed to manage factors of alcohol relapse among women, the internal validity of the studies was also limited – there was no random allocation and none of the studies used a control group. Three studies did not address the variables of drinking behavior or alcohol relapse prevention. Furthermore, none focused on the inability to cope with psychological problems faced by alcohol-dependent women.

The state-of-the-art thinking about factors related to alcohol relapse among women indicate that alcohol relapse prevention programs need to be tailored to

women by using a randomized controlled trial. In addition, currently existing alcohol relapse prevention programs do not specifically address factors of alcohol relapse in women that may lead to rapid relapse and more frequent re-admission. This study examines the effectiveness of an alcohol relapse prevention program based on the Satir Model on self-esteem, self-efficacy, life congruence, heavy drinking days, abstinence days and serum gamma-glutamyl transferase levels in alcohol-dependent women.

MATERIALS AND METHODS

Study design and sample

A randomized controlled trial (RCT) design, double-blind clinical trial compares an alcohol relapse prevention program based on the Satir Model with regular care. First, the research assistants at each of the two research sites who interviewed participants to obtain data were blind to treatment allocation. Second, the participants in both groups did not know, or were blind to, which group they participated in.

Ethical Consideration. Data were collected after the research proposal had been approved by the Research Ethics Committee of the Faculty of Nursing, Chiang Mai University; Suan Prung Psychiatric Hospital; and Thanyarak Chiangmai Hospital.

Sample and setting. The sample was obtained from Suan Prung Psychiatric Hospital and Thanyarak Chiangmai Hospital, in Chiang Mai, Thailand.

Fifty-one subjects agreed and 45 met inclusion criteria in this study. Thirty-nine subjects participated in this study and were interviewed at the baseline. Final analysis included only 27 subjects.

Inclusion included women dependent on alcohol at the mild to moderate level as assessed by the Severity of Alcohol Dependence Questionnaire (SADQ), 25 years old or older, having almost low to almost high self-esteem, and almost low to almost high life congruence as measured by the Coopersmith Self-Esteem Inventory Adult Form and the Life Congruence Scale, respectively. Other inclusion criteria were having moderate to severe depressive symptoms as assessed by the PHQ-9, completing regular inpatient psychosocial care, having a psychiatrist or physician sign for discharge from the hospital, and having a spouse or family members who could participate in the program twice.

Participants were not recruited for the study if they received a diagnosis of bipolar disorder or schizophrenia, or received any of the following anti-relapse medication: Disulfiram, Topiramate or anti-craving medications.

Random allocation. Eighteen participants were randomly assigned to the experimental group and 21 participants to the control group by simple random sampling with replacement.

Instrument for data collection. The Timeline Follow-back interview (TLFB) was used to collect retrospective drinking information on alcohol use 30 days prior to hospitalization. The TLFB, developed by Sobell and Sobell (Gmel & Rehm, 2004), has been found to have high test-retest reliability and concurrent validity across multiple populations of drinkers (Klein et al., 2007). Heavy drinking for women in this study is defined as ≥ 4 drinks during one occasion (Gmel & Rehm, 2004; Sommers 2005; WHO, 2001).

Laboratory testing was used to evaluate the level of serum GGT. The GGT is a biochemical measurement that is not vulnerable to problems of inaccurate recall or reluctance of individuals to give candid reports of their drinking behaviors or attitudes (Gmel & Rehm, 2004). In this study, the GGT blood test was conducted twice: before being discharged from the hospital, which averaged 4-6 weeks of inpatient treatment; and at 16 weeks after program completion.

The Coopersmith Self-Esteem Inventory Adult Form is a rating scale of 25 items developed by Coopersmith (1984). This scale is based on the definition of 'self-esteem' as defined by Coopersmith, which is similar to Satir's definition (Satir & Baldwin, 1983). The SEI-Adult Form consists of four components of the self – specifically, social, academic, family and personal areas of experience. The total scale scores range from 25 to 150, with higher scores indicating higher self-esteem. This scale was translated into Thai and tested for reliability by Wongleakpai (1989). The researcher evaluated the validity of the Thai version of the SEI-Adult form by calculating the content validity index (CVI) from opinions of six experts. The mean of item-level CVI was 0.75 and the scale-level CVI was 0.75. The reliability of this scale was determined by a one-week test-retest, and the internal consistency was estimated with 10 alcohol-dependent women. The correlation coefficient was 0.88 ($p = 0.01$); the Cronbach's alpha coefficient was 0.81.

The General Perceived Self-Efficacy Scale is a rating scale developed by Matthias Jerusalem and Ralf Schwarzer in 1981. It is a 4-point rating scale with 10 items to assess perceived self-efficacy regarding coping and adaptation abilities in both daily activities and isolated stressful events. The scale is designed for adults and adolescents ≥ 12 years of age. The total scale scores range from 10 to 40, with higher scores indicating stronger belief in self-efficacy. This scale was translated into a Thai version by Sukmak et al. (2002) and it was administered to 103 amphetamine-dependent patients for components and confirmatory factor analyses. The researcher tested the internal consistency reliability of this scale among 10 alcohol-dependent women before using it in this study. The Chronbach's alpha coefficient was 0.86.

The Life Congruence Scale is a rating scale developed by Lee (2002), a 7-point rating scale with 21 items. This scale is based on the 'congruence concept' of the Satir Model. The original, 75-item congruence scale developed by Lee was translated into a Thai version and the psychometric properties of the scale were determined by Srikosai and Taweewattanaprecha (2011). The scale has five dimensions: intrapsychic, interpersonal, spiritual, creative and communal. The total scores range from 21 to 147, with higher scores indicating higher life con-

gruence. The validity and reliability of the Thai version of the Life Congruence Scale were established and high (Srikosai & Taweewattanaprecha, 2011). The reliability of the Thai version of the Life Congruence Scale was determined by an internal consistency estimate with 10 alcohol-dependent women from Suan Prung Psychiatric and Thanyarak Chiangmai Hospitals. The Cronbach's alpha coefficient was 0.81.

Data analysis: Descriptive statistics, t-test, Two-Way Repeated-Measures ANOVA (Mix Model) and Mann-Whitney U test.

Procedure for developing the program

(1) Review Satir Model concepts, the therapeutic beliefs of the Satir Model and related literature. (2) Develop the first draft in English. This step was required to get the input a non-Thai speaking expert who was essential to developing an effective protocol. (3) Submit the English version and a research proposal to six experts for process evaluation in the areas of language, feasibility and appropriateness. (4) Revise the English program based on the recommendations and suggestions of the experts. (5) Test with four alcohol-dependent women for feasibility and appropriateness. (6) Develop a Thai version from the English version and from the outcomes of testing in order to get a protocol intended for women with alcohol dependence who are Thai, speak Thai and come from a Thai cultural context. (7) Submit the Thai version to six Thai experts to examine the process for language, feasibility and appropriateness. (8) Revise the Thai version based on the suggestions of experts. (9) Test again with four alcohol-dependent women for feasibility and appropriateness.

RESULTS

Thirty-nine participants ranged in age from 25 to 60 years, with an average age of starting to drink of 18 years. Nineteen participants were married. A similar number were divorced/widowed and single. Twenty-six participants completed primary school and most participants worked in agriculture. A majority had income lower than THB 3,000 (USD 93) per month and never smoked. Most of participants stated that the cause of their drinking or re-drinking was stress, followed by persuasion from friends or spouse. The number of one-time and multiple admissions were similar, and the diagnoses were alcohol dependence (F10.2) and alcohol dependence and alcohol-induced psychotic disorder (F10.2 and F10.5) (61.5% and 25.6%, respectively). No aspects of the demographic characteristic of participants in the experimental group were different from participants in the control group.

The scores of self-esteem, self-efficacy and life congruence in the experimental group were significantly higher than the control group at the post-test immediately after completing the program, and at 12 and 16 weeks after program completion (Figure 1, 2, 3).

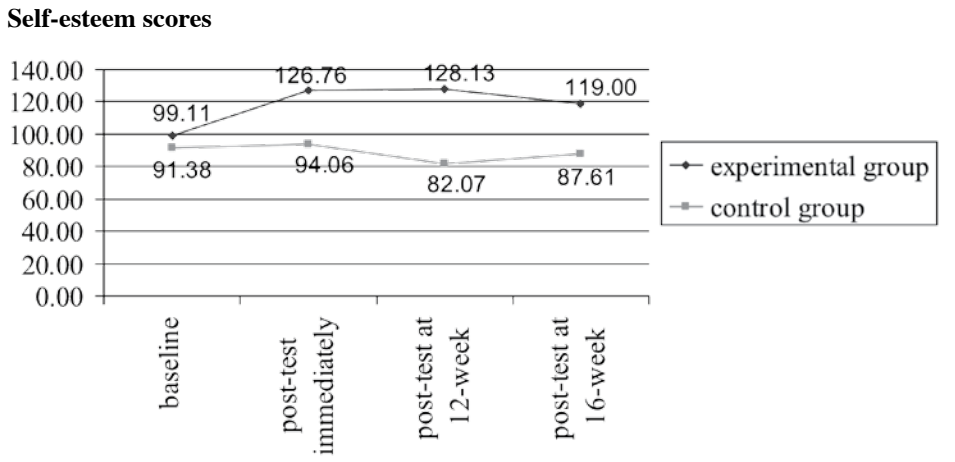

Figure 1. Change in overall self-esteem scores of the experimental and control groups over time.

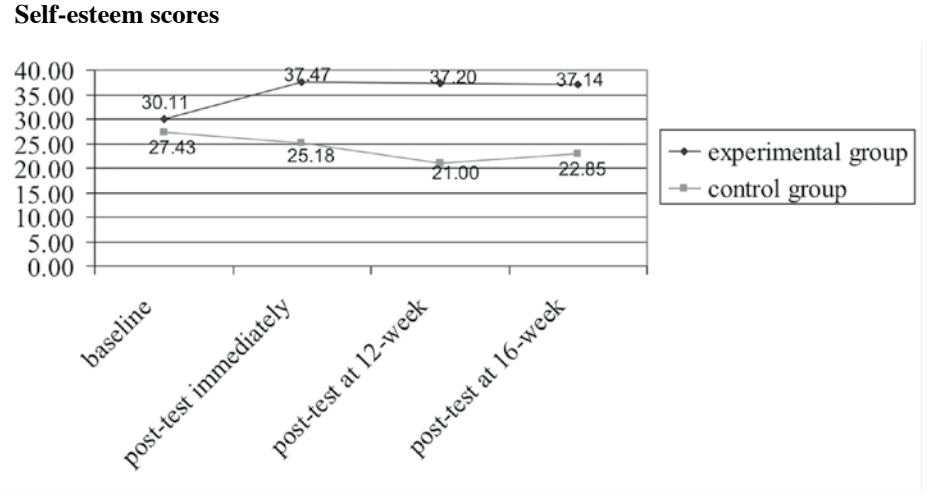

Figure 2. Change in overall self-efficacy scores of the experimental and control groups over time.

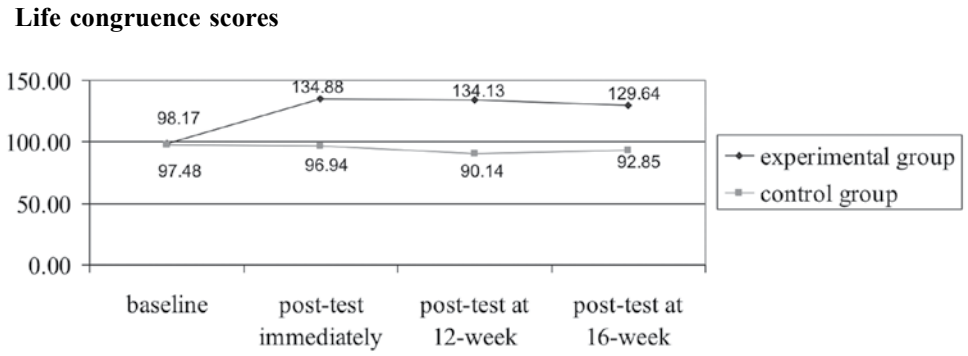

Figure 3. Change in overall life congruence scores of the experimental and control groups over time.

The scores of percent of heavy drinking days in the experimental group were significantly lower than the control group at post-test immediately after completing the program, and at 12 and 16 weeks after program completion ($p < 0.01$) (Figure 4).

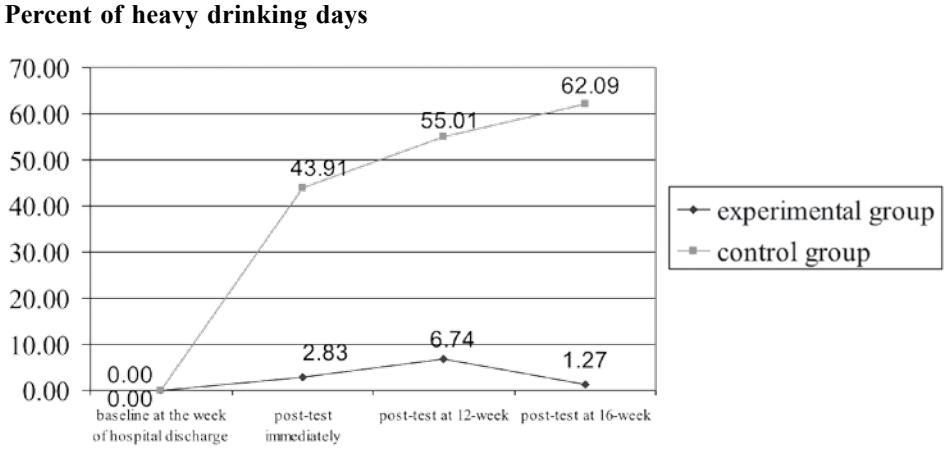

Figure 4. Change in overall percent of heavy drinking days of the experimental and control groups over time.

The scores of percent of abstinence days in the experimental group were significantly higher than the control group at post-test immediately after completing the program, and at 12 and 16 weeks after program completion ($p < 0.01$) (Figure 5).

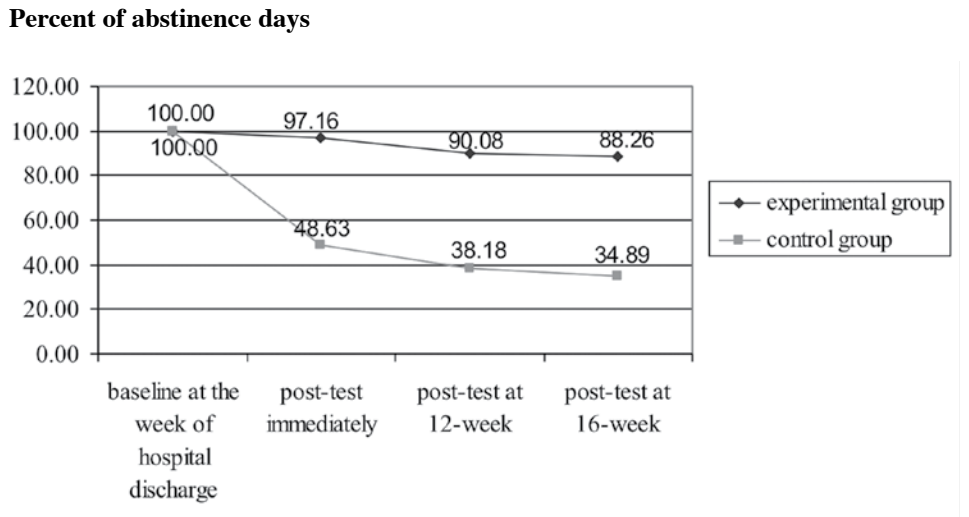

Figure 5. Change in overall percent of abstinence days of the experimental and control groups over time.

The level of serum GGT in the experimental group was significantly lower than the control group at 16 weeks after program completion ($p < 0.001$) (Figure 6).

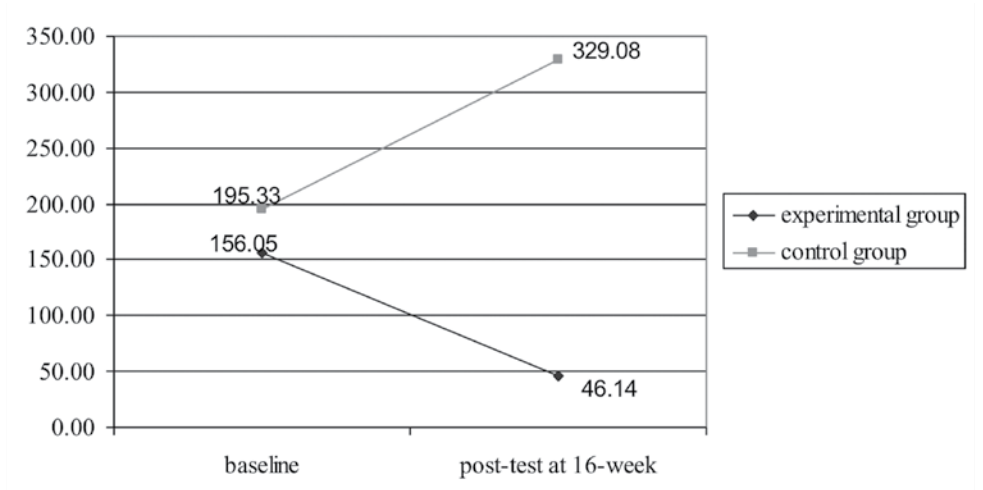

Figure 6. Change in overall level of serum gamma-glutamyl transferase of the experimental and control groups over time.

DISCUSSION

Participants in the experimental group had significantly higher self-esteem, self-efficacy and life congruence at immediate post-test, and at 12 and 16 weeks after program completion, than participants in the control group, which indicates that an alcohol relapse prevention program based on the Satir Model is effective over time on increasing self-esteem, self-efficacy and life congruence, when compared to regular care. A possible explanation is that the participants continued to experience their own positive life energy. They were able to cope well with intra-psychic conflict and utilize their improved coping styles because their yearnings were fulfilled. This was achieved by the therapist using five essential elements in each therapy session, including 'positive directional goals', 'systematic approach', 'internal experiential', 'change focused' and 'the use of self'. The therapist facilitated the participants' increased self-esteem and self-acceptance as a step for the patients to learn to fulfill their other yearnings by themselves. When an individual's yearnings are fulfilled, then realistic and positive expectations to the self, to others and from others are present. These changed expectations can affect perceptions of reality, and positive directional feelings and positive feelings about feelings can eventually be achieved (Satir, 1983; Satir et al., 1991).

These findings support previous studies that found that five alcohol-dependent women who received two to three therapy sessions based on the Satir Model had higher mean scores of self-esteem compared to baseline scores (73.40 versus 91.40) (Srikosai & Taweewattanaprecha, 2012) and five patients with alcohol problems who received five sessions of couples therapy based on the Satir Model increased self-esteem from relatively low scores to relatively high scores (Bangsaeng, 2010). A woman with mixed anxiety/depressive disorder, after being treated with the Satir Model, could accept herself and feel more confidence, pride and independence. These changes decreased her depression and anxiety (Jainkitjapaiboon, 2009). Alcohol-dependent men and their family members, after completing family therapy based on the Satir Model, had better self-esteem scores and perceptions about family function (Patmanee, 2009). Furthermore, Taiwanese women who experienced an educational program based on the Satir Model achieved internal change or positive self-growth through non-defensive acceptance of their family of origin. They could recognize and accept themselves, and accept their family members as they were. They became aware of change and could make positive changes towards internal growth (Pei, 2008).

Participants in the experimental group had significantly less heavy drinking days and increased abstinence days at an immediate post-test, and at 12 weeks and 16 weeks after program completion than participants in the control group. A possible explanation is that higher self-esteem, self-efficacy and life congruence are associated with changes in drinking behaviors. Based on the Satir Model (Satir, 1983; Satir et al., 1991), if yearnings are fulfilled, then positive expectations are present and perception of reality increase. Positive directional feelings and feelings about feelings will be achieved and congruent survival coping stance will be used, and these lead to healthy behavior – choosing not to drink. The interaction of each level of the iceberg also leads to becoming a positive choice-maker, taking

responsibility for one's internal and external world, being more congruent and experiencing higher self-esteem. Participants in the experimental group showed the ability to control drinking during the program.

Participants in the experimental group had significantly lower levels of serum gamma-glutamyl transferase (GGT) 16 weeks after program completion ($p < 0.001$) than participants in the control group. This finding is the result of increased alcohol abstinence and decreased heavy drinking, consistent with the literature, which states that after 2-6 weeks of abstinence, levels of GGT generally decrease to within the normal reference range (Gmel & Rehm, 2004; Kranzler & Tennen, 2005). A systematic review of studies found that the GGT level is a more sensitive marker for heavy drinking in women than carbohydrate-deficient transferring (CDT). In addition, the sensitivity of GGT in women is 60 percent versus 52 percent sensitivity for the macrocytic volume (MCV). The GGT test, therefore, appears to be the most appropriate single biological marker to assess mid-term and long-term drinking in women (Sommers, 2005). If participants have increased alcohol abstinence days or decreased heavy drinking days, less serum GGT leaks across the cell membrane into the blood (Allen et al., 2003). Therefore, the effects of an alcohol relapse prevention program based on the Satir Model on drinking behaviors have strong reliability because lower GGT levels were present.

CONCLUSION

These research results add new knowledge that an alcohol relapse prevention program based on the Satir Model leads to higher self-esteem, self-efficacy and life congruence; fewer heavy drinking days; more abstinence days and a decrease in GGT levels among alcohol-dependent women. This program should be used as a nursing intervention for female patients with alcohol dependence hospitalized in psychiatric hospitals or hospitals for alcohol and drug dependence treatment.

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REFERENCES

Allen, J. P., P. Sillanaukee, N. Strid, and R.Z. Litten. 2003. Biomarkers of heavy drinking. In J.P. Allen and V.B. Wilson (Eds.), Assessing alcohol problems: A guide for clinicians and researchers (pp. 37-53). (2nd ed.). National Institute on Alcohol Abuse and Alcoholism. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health.

Angove, R., and A. Fothergill. 2003. Women and alcohol: Misrepresented and misunderstood. Journal of Psychiatry and Mental Health Nursing, 10(2), 213-219.

Banmen, J. 2007. If depression is the solution, what are the problems? The Satir Journal, 1(2), 61-81.

Bangsaeng, S. 2010. Couple therapy for patient with alcohol user admitted in Khon Kaen Rajanakarind Psychiatric Hospital based on Satir's Model. Master's independent study, Khon Kaen University, Thailand.

Banmen, J., and K. Maki-Benmen. 2006. Application of the Satir Growth Model introduction. Retrieved May 12, 2011, from http://www.satir.

Chansantor, A. 1998. Contributing factors to and effects of alcohol dependence among women. Master's thesis, Chiang Mai University, Thailand.

Chouwilai, J. 2006. The alcohol consumption: as a factor of domestic violence. In Center for Alcohol Studies (Ed.), The Second National Alcohol Conference: Alcohol Consumption and Related problems in Thailand (pp. 48-49), 13-14 December, 2006. Rama Garden Hotel, Bangkok, Thailand.

Coopersmith, S. 1984. SEI: Self-esteem inventory. California: Consulting Psychologists Press.

Ford, S. 2007. Satir in the Sand Tray: facilitating peace within. The Satir Journal, 1(3), 2-26.

Gjestad, R., J. Franck, K.A. Hagtvet, and B. Haver. 2011. Level and change in alcohol consumption, depression and dysfunctional attitudes among females treated for alcohol addiction. Alcohol and Alcoholism, 46(3), 292-300. 10.1093/alcalcagr018

Gmel, G., and J. Rehm. 2004. Measuring alcohol consumption. In contemporary drug problems (pp. 467-474), 31(3). New York: Fall.

Jakobsson, A., G. Hensing, and F. Spak. 2008. The role of gendered conceptions in treatment seeking for alcohol problems. Scandinavian Journal of Caring Sciences, 22(2), 196-202. 10.1111/j.1471-6712.2007.00513.X

Jainkitjapaiboon, P. 2009. Individual Psychotherapy with Satir Model: Case study of the patient with mixed anxiety depressive disorder. Bulletin of Suan Prung, 25(1), 90-104.

Jongchokdee, P. 2010. The effectiveness of stress inoculation training program among wives of patients with alcohol dependence. Master's thesis, Burapa University, Thailand.

Kamkan, J. 2005. The socio-cultural context affecting on alcohol drinking behavior in the worker of Dongkilek village, Chachang Sub-district, San Kamphaeng District, Chiang Mai Province. Master's independent study, Chiang Mai University, Thailand.

Klein, A.A., P.R. Stasiewicz, J.R. Koutsky, C.M. Bradizza, and S.F. Coffey. 2007. A psychometric evaluation of the Approach and Avoidance of Alcohol Questionnaire (AAAQ) in alcohol dependent outpatients. Journal of Psychopathology and Behavioral Assessment, 29, 231-240. 10.1007/S10862-007-9044-2

Knopik, V.S., A.C. Heath, P.A.F. Madden, K.K. Bucholz, W.S. Slutske, E.C. Nelson, and et al. 2004. Genetic effects on alcohol dependence risk: re-evaluating the importance of psychiatric and other heritable risk factors. Psychological Medicine, 34(8), 1519-1530. 10.1017/S0033291704002922

Kranzler, H. R., and H. Tennen. 2005. How to measure relapse in humans. In R. Spanagel & K.F. Mann (Eds.), Drugs for relapse prevention of alcoholism (pp. 23-39). Basel: Birkhauser Verlag.

Lee, B.K. 2002. Development of a congruence scale based on the Satir Model. Contemporary Family Therapy, 24(1), 217-239. 10.1023/A:1014390009534

Maisto, S.A., P.R. Clifford, R.L. Stout, and C.M. Davis. 2008. Factor mediating the association between drinking in the first year after alcohol treatment and drinking at three years. Journal of Studies on Alcohol and Drugs, 52(1), 728-737.

Masters, C., and D.S. Carlson. 2006. The process of reconnecting: recovery from the perspective of addicted women. Journal of Addictions Nursing, 17(4), 205-210. 10.1080/10884600600995200

Mensinger, J.L., K.G. Lynch, T.R. TenHave, and J.R. McKay. 2007. Mediators of telephone-based continuing care for alcohol and cocaine abuse. Journal of Consulting and Clinical Psychology, 75(5), 775-784. Retrieved July 23, 2010, rom NIH Public Assess: Author Manuscript. 10.1037/0022-006X.75.5.775

Moos, R.H., and B.S. Moos. 2006. Rates and predictors of relapse after natural and treated remission from alcohol use disorders. Addiction, 101(2), 212-222. 10.1111/j.1360-0443.2006.01310.X

Patmanee, K. 2009. Family therapy in family with alcohol dependence patient based on Satir Model. Master's independent study, Khon Kaen University, Thailand.

Pei, Y. 2008. From caterpillar to butterfly: Action research study of a Satir-based women's program in Taiwan. The Satir Journal, 2(1), 55-107.

Satir, V. 1983. Conjoint family therapy. (3rd ed.). Palo: Science and Behavior Books.

Satir, V., and S.M. Baldwin. 1983. Satir step by step: A guide to creating change in families. Palo Alto: Science and behavior books.

Satir, V., Banmen, J., Gerber, J., & Gomori, M. 1991. The Satir Model: Family therapy and beyond. California: Science and behavior book.

Scott, C.K., M.A. Foss, and M.L. Dennis. 2005. Pathways in the relapse-treatment-recovery cycle over 3 years. Journal of Substance Abuse Treatment, 28, S63-S72. 10.1016/j.jsat.2004.09.006

Silverstone, P. H., and M. Salsali. 2003. Low self-esteem and psychiatric patients: Part I The relationship between low self-esteem and psychiatric diagnosis. Annals of General Hospital Psychiatry, 2(2), doi:10.1186/1475-2832-2-2

Snow, D., and C. Anderson. 2000. Exploring the factors influencing relapse and recovery among drug and alcohol addicted women. Journal of Psychosocial Nursing & Mental Health Services, 38(7), 8-19.

Sommers, M.S. 2005. Measurement of alcohol consumption: Issues and challenges. In J.J. Fitzpatrick (Series Ed.), Annual review of nursing research (Volume 23): Alcohol use, Misuse, Abuse, and Dependence (pp. 27-64). New York: Springer.

Srikosai, S., and S. Taweewattanaprecha. 2012. Internal change among alcohol-dependent persons receiving Satir Transformational Systemic Therapy: a case study of inpatients. Suan Prung Psychiatric Hospital, Chiang Mai, Thailand.

Srikosai, S., and S. Taweewattanaprecha. 2011. Psychometric evaluation of the Life-Congruence Scale based on the Satir Model: Thai version. Journal of the Psychiatric Association of Thailand, 56(3), 21-30.

Sukmak, V., A. Sirisoonthon, and P. Meena. 2002. Validity of the general perceived self-efficacy scale. Journal of the Psychiatric Association of Thailand, 47(1), 31-37.

Walitzer, K.S., and R.L. Dearing. 2006. Gender differences in alcohol and substance use relapse. Clinical Psychology Review, 26, 128-148. 10.1016/j.cpr.2005.11.003

Wongleakpai, N. 1989. Effects of the relationship group on self-esteem among youth. Master's thesis, Chulalongkorn University, Thailand.

WHO. 2001. Why screen for alcohol use?. In B. F. Thomas, H-B. C. John, S. B. John, & N. G. Maristela (Eds.), AUDIT, The Alcohol Use Disorders Identification Test: Guidelines for Use in Primary Care (pp. 5-9). (2nd ed.). Department of Mental Health and Substance Dependence.

Zywiak, W.H., R.L. Stout, W.B. Trefry, I. Glasser, G.J. Connors, and S.A. Maisto, et al. 2006. Alcohol relapse repetition, gender, and predictive validity. Journal of Substance Abuse Treatment, 30(4), 349-353. 10.1016/j.jsat.2006.03.004

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Book

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Chapter in book

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Edited proceeding, symposia etc.

Pratt, A., R.J. Gilkes, S.C. Ward, and D.A. Jasper. 2000. Variations in the properties of regolith materials affect the performance of tree growth in rehabilitated bauxite mine-pits in the Darling Range, SW-Australia. p.87-88. In A. Brion and R.W. Bell (eds) Proceeding of Remade Land 2000, the International Conference on Remediation and Management of Degraded Lands. Fremantle, 30 Nov-2 Dec 2000. Promaco Conventions, Canning Bridge.

Dissertation

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