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Evaluation of Digestion Procedures on Heavy Metals in Soil of a Dumpsite in Ibadan, South-western Nigeria

Segun Michael Abegunde1*, Adedayo Olamide Oyebanji2, Oladele Osibanjo3

1Department of Science Technology, Federal Polytechnic, Ado-Ekiti, Ekiti State
2Department of Chemical Sciences, Joseph Ayo Babalola University, Ijero Ekiti, Ekiti State
3Department of Chemistry, University of Ibadan, Nigeria

Correspondence author e-mail: *abegundes@gmail.com

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Abstract

Acid digestion is important in solubilizing metal ions in heavy metals determination. A number of procedures exist and the one used is dependent on different factors. This work is aimed at investigating the performance of three different acid digestion procedures (Aqua Regia, 2 M HNO3 and HNO3/HClO4) for the extraction of Pb, Cd and Zn in soil sample from an abandoned dumpsite of a lead-acid producing industry in Lalupon, Oyo State. Heavy metals in soil extracts were determined using atomic absorption spectrophotometer. Results revealed that Aqua Regia gave the highest content level of Pb and Cd, HNO3/HClO4 showed best performance for Zn while 2 M HNO3 had the least performance for the metals considered. Result of 2 M HNO3 was less than the other two and could not represent total metal content of the soil. Without losing focus of the objectives of research, variation in the effectiveness of acid digestion procedures should be borne in mind when making a choice.

Keywords: Digestion procedure, Extractants, Heavy metals, Dumpsite, Soil

1. Introduction

Metals are found naturally on earth in rocks, soils and sediment trapped in different forms. Anthropogenic input of heavy metals in soil results in contamination. Chemicals are a major source of contamination introduced during washing of agricultural wastes like fertilizer and pesticide from farmland or effluent generated from industrial activities (Abegunde, 2017). The chemical behaviours of these contaminants are controlled by soil composition, properties such as pH and a number of processes such as metal cation release from contaminated source, cation exchange and specific adsorption onto surface of minerals (Guveni & Akinci, 2011; Hlavay, Prohaska, Weisz, Wenzel, & Stingeder 2004). Their impacts depend on the total metal concentrations in soil, mobility and bioavailability (Kaasalainen & Yli-Halla, 2003; Roundhill, Slangi, Memon, Bhanger & Yilmaz, 2009; Szakova, Miholova, Tlustos, Sestakova, & Frkova, 2010).

Several methods have been used by researchers in the digestion of soil samples for the determination of metallic levels. Such methods have been through the use of fluxes or inorganic acids such as HCl, H3PO4, HNO3, HClO4, HF, H2SO4 or their combination. These acids (extractants) exhibit various properties which enable them to perform specific functions during extraction (Tam & Yao, 1999; Alam & Tokunaga, 2006; Kislik, 2002). The choice of extractants depend on the aim of the study, type of contaminants, properties of the extractant, experimental conditions and need for minimum interference by contaminants (Kaasalainen & Yli-Halla, 2003; Roundhill, Slangi, Memon, Bhanger & Yilmaz, 2009; Guveni & Akinci, 2011; Wilson et al., 2005; Twyman, 2005). Improper selection of extractant could cause effects such as partial dissolution of soil sample resulting in decreased metal content levels in soil samples (Hlavay et al., 2004; Twyman, 2005). Extractants may be acidic or basic depending on the mode of action. This depends on interacting mechanisms such as the metal-ion extractant affinity, metal ion concentration, extraction temperature and acidity of the medium (Tam & Yao, 1999; Nogoles et al., 1995; Szakova et al., 2010). Successful extraction, determination and isolation of biologically active components from a material are largely dependent on the type of solvent used in the extraction procedure (Abegunde & Ayodele-Oduola, 2015). The extent to which ions of extractant show affinity

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for metals also depends on concentration of the metal in soil and inter metal interference in heterogeneous contaminated soil during extraction (Tam & Yao, 1999; Nogales et al., 1995). Extractants are specific in action; for example, H$_2$SO$_4$ is not used to digest samples containing Barium (Ba) and neither is HCl used for samples containing Silver (Ag) and Lead (Pb). Besides, Arsenic compounds form volatile compounds with HCl and H$_2$SO$_4$ is not suitable as extractant to analyze some metals (especially alkaline earth metals) simultaneously because of the possibility of forming insoluble sulphate salts (Twyman, 2005; Nogales, 1995). Their specificity can be improved by combining a series of acids during extraction (Guveni & Akinci, 2011; Kislik, 2002; Wilson et al., 2005; Tam & Yao, 1999; Szakova et al., 2010). Mixtures of HCl, HNO$_3$, HClO$_4$, and HF dissolve most metals in soils and this is reported to have great accuracy in analysis (Twyman, 2005). Report is given on the use of Aqua regia in the dissolution of sulfides, phosphates, many metals and alloys including Gold (Au), Platinum (Pt) and Palladium (Pd) (Tam & Yao, 1999). The use of acids in sequence has been reported to give good results (Argon Lab Systems, 2007). Use of HNO$_3$ and HClO$_4$ premixed in 3:1 ratio followed by the addition of HF has been used with high extraction yield (Twyman, 2005). Consequently, the aim of this research was to identify the most reliable and best digestion procedure among those used for the heavy metals under consideration. Specific objective was to compare the efficiency of the three procedures being major wet digestion methods for evaluating heavy metals in soil. The three heavy metals were selected because they are major metal pollutants from lead acid battery. The results of the research were analyzed for statistical significance by analysis of variance.

2. Experimental
2.1. Sample Collection and Preparation
Soil from a dumpsite in Lalupon, Lagelu Local Government Area of Oyo State, south-western Nigeria, was used for this work. The soil samples were randomly and extensively collected using a soil auger, transferred into black polythene bags and transported to the laboratory. The samples were air-dried, gently crushed with pestle in agate mortar, sieved through 2 mm fraction, homogenized and stored in polythene bags. The homogenized samples were digested using different acid digestion procedures, filtered and kept in plastic bottles for heavy metals analysis using AAS.

2.2. Digestion with Aqua Regia (concentrated HCl and HNO$_3$ in the 3:1)
Soil sample (1.0 g) was weighed and transferred into digestion flask. Aqua regia (20 mL) was added to it and digestion carried out on a heating mantle in a fume cupboard. The temperature was gradually increased, and agitation occasionally done until volume of content was reduced to about 5 mL. Excessive evaporation of extractant was prevented by covering each flask with watch glass. The resulting solution was filtered, washed with de-ionized and double-distilled water and transferred quantitatively into a 50 mL volumetric flask and made up to the mark with distilled water.

2.3. Digestion with Nitric–Perchloric Acid (HNO$_3$/HClO$_4$)
1.0 g of the soil sample was weighed and transferred into a 250 mL digestion tube and 10 mL of concentrated HNO$_3$ was added. The mixture was boiled gently for 30–45 minutes to oxidize all easily oxidizable matter. After cooling, 5 mL of 70% HClO$_4$ was added and the mixture boiled gently until dense white fumes appeared. After cooling, 20 mL of distilled water was added, and the mixture was boiled further to release any fumes. The solution was cooled, filtered through Whatman No. 42 filter paper and <0.45 μm Millipore filter paper and transferred quantitatively to a 50 mL volumetric flask and made up to the mark with distilled water.

2.4. Digestion with concentrated Nitric Acid
A separate 1.0 g soil sample was weighed and transferred into a digestion tube. A 20 mL solution of 2M HNO$_3$ was added. The tube containing the mixture was placed in a beaker of water and heated for 2 hours with gradual increase in temperature to 100°C. At intervals, the tube was opened, the suspension shaken and the tube covered again. The suspension was thereafter cooled, filtered into 50 mL volumetric flask and made up to the mark with distilled water. The digested samples were analyzed for heavy metals using atomic absorption spectrometer (AAS) Buck Scientific model 210VGP.
3. Results and Discussion Result

Table 1. Heavy metal contents (mg/kg) of soil samples by three digestion procedures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Digestion Procedure</th>
<th>Pb (mg/kg)</th>
<th>Cd (mg/kg)</th>
<th>Zn (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1</td>
<td>HNO3/HClO3</td>
<td>2233.33±57.74a</td>
<td>1.90±0.00a</td>
<td>270.33±0.58a</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
<td>43200.00±173.21a</td>
<td>2.33±0.29b</td>
<td>260.50±0.00a</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>834.67±04.15c</td>
<td>0.05±0.00b</td>
<td>5.17±0.00b</td>
</tr>
<tr>
<td>AB2</td>
<td>HNO3/HClO3</td>
<td>3500.00±173.21b</td>
<td>1.50±0.00b</td>
<td>45.33±0.00b</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
<td>4443.33±208.17b</td>
<td>3.00±0.00b</td>
<td>267.83±0.29b</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>499.33±208.17c</td>
<td>0.04±0.00c</td>
<td>5.22±0.50c</td>
</tr>
<tr>
<td>AB3</td>
<td>HNO3/HClO3</td>
<td>8500.00±173.21a</td>
<td>1.50±0.00b</td>
<td>245.33±0.29a</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
<td>900.00±173.21a</td>
<td>0.83±0.00a</td>
<td>112.67±0.58a</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>26.67±57.74b</td>
<td>0.01±0.00b</td>
<td>2.22±0.29a</td>
</tr>
<tr>
<td>AB4</td>
<td>HNO3/HClO3</td>
<td>2486.67±115.47a</td>
<td>1.33±0.00a</td>
<td>249.83±0.00a</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
<td>4856.67±208.17b</td>
<td>3.00±0.00b</td>
<td>275.50±0.50a</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>899.33±208.17c</td>
<td>0.04±0.00c</td>
<td>5.22±0.87a</td>
</tr>
<tr>
<td>AB5</td>
<td>HNO3/HClO3</td>
<td>885.00±115.47a</td>
<td>1.50±0.00b</td>
<td>103.67±0.29a</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
<td>1046.67±208.17b</td>
<td>0.50±0.00a</td>
<td>67.17±0.29a</td>
</tr>
<tr>
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<td>2M HNO3</td>
<td>774.67±57.74b</td>
<td>0.01±0.00b</td>
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<tr>
<td>AB6</td>
<td>HNO3/HClO3</td>
<td>1073.33±115.47a</td>
<td>1.00±0.00a</td>
<td>327.67±0.76a</td>
</tr>
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<td>Agua Regia</td>
<td>333.33±152.75a</td>
<td>0.50±0.00a</td>
<td>67.17±0.29a</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>46.00±115.47a</td>
<td>0.01±0.00b</td>
<td>2.14±0.76c</td>
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<tr>
<td>AB7</td>
<td>HNO3/HClO3</td>
<td>1666.67±115.47a</td>
<td>1.50±0.00a</td>
<td>103.67±0.29a</td>
</tr>
<tr>
<td></td>
<td>Agua Regia</td>
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<td>0.83±0.00a</td>
<td>95.67±1.04b</td>
</tr>
<tr>
<td></td>
<td>2M HNO3</td>
<td>74.00±115.47a</td>
<td>0.01±0.00b</td>
<td>2.14±0.76c</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation for three determinations. DL = Detection limit. (DL are 0.08, 0.01 and 0.005 ppm for Pb, Cd and Zn respectively).

Within each column for each sample, means that do not share a letter are significantly different.

Figure 1. Performance of solvents for the extraction of Pb

Figure 2. Performance of solvents for the extraction of Cd

Figure 3. Performance of solvents for the extraction of Zn

4. Discussion

Results of concentrations of the three selected heavy metals in seven soil samples using three different digestion procedures are tabulated in Table 1. Performance of the three procedures for the extraction of each metal was also evaluated and presented in Figures 1, 2 and 3. Aqua regia performed best among the three procedures for the extraction of Pb in all the samples except for sample AB5 and AB6 where the performance of HNO3/HClO4 was the highest. Also, extraction with aqua regia proved most effective among the three procedures for the extraction of Cd. Aqua regia was able to extract at least 0.5 mg from every 1 kg of the soil samples. However, the level of Cd concentration in sample AB3 in the extract by HNO3/HClO4 and in the extracts by HNO3/HClO4 and 2M HNO3 for sample AB7 were found below the detection limit of the machine used. HNO3/HClO4 showed better
performance than aqua regia in the extraction of Zn for soil samples AB1, AB5 and AB7.

Aqua regia showed to be most effective for extraction of Pb, Cd and Zn while 2 M HNO₃ was the least. The latter also performed extremely poor in extracting Zn when compared with aqua regia and HNO₃/HClO₄. Effectiveness of aqua regia and HNO₃/HClO₄ could be attributed to the fact that both are mixtures of different acids. The variations in extractability showed in this research work further confirmed the reports that some extractants have more affinity for some contaminant metals than others due to the soft nature of such metal ions, the type, size and the geometry of the extractant ions and also probably due to inter-metal interference in heterogeneous contaminated soils during extraction (Kislik, 2002; Guveni & Akinci, 2011). The relatively high Pb levels of some of the soil samples were expected as the samples were taken from an abandoned dump site of a defunct lead-acid battery producing plant.

On the other hand, the results of the analysis of variance and Tukey pairwise comparisons of the experimental results as presented in table 1 shows significant difference in the performance of the three digestion procedures for the three metals considered in all the samples except for the extraction of Zn in samples AB3 and for the extraction of Cd in sample AB5.

5. Conclusion
The variation in the level of metals extracted by each digestion procedure shows that the acids or the digestion procedures have different extractive ability. Understanding the behaviour of each metal in relation to the acids will help to choose the best extractant or digestion procedure at any instance of metal extraction from soils.

6. References


Tam, N. F. Y. & Yao, M. M. (1999). Three digestion methods to determine concentrations of Cu, Zn, Cd, Ni, Pb, Cr, Mn, and Fe in mangrove sediments from SaiKeng, ChekKeng and Sha Tau Kok, Hong Kong. Environmental Contamination and Toxicology, 62(6), 708-716.


β-glucosidase enzyme screening from various parts of Tabebuia argentea
Chariwat Pitsanuwong1*, Kanokorn Wechakorn2
1Faculty of Science and Technology, Suan Sunandha Rajabhat University, U-thong Nok Road, Dusit, Bangkok 10300, Thailand
2Department of Chemistry, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathumthani, 12110, Thailand
Corresponding author e-mail: *chariwat.ph@ssru.ac.th
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Abstract
The aim of this work was to search for the novel β-glucosidase enzyme from various parts of Tabebuia argentea; flower, flower bud, shoot, seed shank and seed. The enzyme was extracted from the samples by using appropriate buffer. The ammonium sulfate [(NH4)2SO4] salt precipitation in the different salt concentrations was used to initial fractionated purification steps. The monitoring of enzyme activity was carried out by using hydrolysis reaction of glycosidic bond using p-nitrophenyl-D-glucopyranoside (pNPG) as an enzyme substrate. The UV-vis spectroscopy was used to detect the corresponding p-nitrophenylate product under basic condition at 405 nm. The enzyme activity via pNPG hydrolysis of seeds extract was around 15-fold over the other plant part extracts of Tabebuia argentea. This was followed by selected enzyme fraction of the seeds extract subjected to optimum temperature study and temperature for the best enzyme activity was 30-40 °C. The highest enzyme activity fraction will be used for further purification and enzymatic properties test before application as a biocatalyst in biological process.

Keywords: β–glucosidase, Tabebuia argentea, p-nitrophenyl-D-glucopyranoside

1. Introduction
The glycoside hydrolases (EC 3.2) assist in hydrolysis reaction of glycosidic linkage in complex sugars (i.e., the disaccharide, oligosaccharide, polysaccharides, cellulose, and other carbohydrates) (Andrade Pinto, De Souza, & Oliveira, 2010) with release of glucose molecules and the corresponding products. The β-glucosidase enzymes are a group of enzymes that catalyze specific bonds within naturally occurring biopolymers composed of beta-1, 4-linked glucosyl residues. Normally, β-glycosidases are found in several sources; many parts of plant, fungi, bacteria, animal, and human. They appear to differ in their specificity for β-glycosidic bond of glucosyl group and aryl-or alkyl-group. The β-glucosidases (3.2.1.21) play important roles in many of biological processes, such as growth regulation and development, lignification, phytohormone activation, cell wall degradations, defense mechanisms, and release aromatic compounds such as saponin, coumarin, quinones, stilbenoid, flavonoid etc. in plants. (Cheung, & Anderson, 1997; Dharmawardhana, Ellis, & Carson, 1995; Duroux, Delmotte, Lancelin, Keravis, and Jay-Allemand, 1998; Girio, Fonseca, Carvalheiro, Marques and Bøgel-Lukasik, 2010; Guo-Yong, Xiaonan, Binbin, Rui and Minjian, 2014)

The novel β-glucosidase enzymes from Thai plants were revealed, such as rice β-glucosidase (Oryza sativa), dalcochinase from Thai rosewood (Dalbergia cochinchinensis), cassava linamarase isolated from Cassava (Manihot esculenta Crantz) [7-11] and the β-glucosidase isolated from hard seed coat of Prunes (Prunus domestica) which is a glucose tolerance enzyme (Morant, Jorgensen, Jorgensen, & Paquette, 2008). The advantages of β-glycosidase enzymes are that they are used as catalysts in many biological processes for ethanol production via hydrolysis of lignocellulosic to sugar, followed by fermentation together with other enzymes (Nisius, 1988; Opasiri et al., 2003; Opasiri et al., 2004) The conventional method to screen is by the detection of p-nitrophenolate quantity released from the substrate p-nitrophenyl-β-D-glucopyranoside (pNPG) hydrolysis from β-glucosidase activity measurement under basic condition (Figure 1) by using UV-vis spectroscopic technique.
As we know, many reports of plants from *Tabebuia* genus show that the most abundant chemical constituents are saponin, coumarin, quinones, stilbenoid, and flavonoid connected to glucopyranoside. Some of the plant extracts are used for parasite control (Hari Babu et al. 2010; Hemamalini, & Sambasiva Rao, 2014; Hommalai, Chaiyen, & Svasti, 2005; Zechel & Wihers, 2000). In this report, we screened the enzyme from various parts of *Tabebuia argentea*: flowers, flower buds, shoots, seed shanks and seeds by using p-nitrophenyl-β-D-glucopyranoside (pNPG) as an enzyme substrate. The appropriated fraction was selected for further study.

### 2. Materials and Methods

#### 2.1. Materials

The chemicals used for this study were obtained from commercial suppliers and used without further purification. Double-distilled water was used in all experiments. Various parts of *Tabebuia argentea*: flowers, flower buds, shoots, seed shanks and seeds (Figure 2) were collected in March-April 2016 from Wangnoi district, Phra NakhonSi Ayutthaya province, Thailand. UV-vis absorption spectra were recorded on an Agilant89090A spectrophotometer.

#### 2.2. Enzyme extraction

50 g each of *Tabebuia argentea* parts were collected and washed before the enzyme extraction, excluding seeds that had to be soaked for 24 hours before the extracts were done. For β-glucosidase enzyme, extraction was determined as follows: 50 g of each sample was blended with 400 ml of 0.1 M cold sodium acetate buffer (pH 5.5) in the presence of 0.5 μM phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor. The homogenizing solution was kept on ice before centrifugation at 7500 rpm. Supernatant of each fraction was collected for ammonium sulfate precipitation at 4 °C before use.

#### 2.3. Ammonium sulfate precipitation

Ammonium sulfate salt was ground before gently adding to supernatant of each part extract using concentration 0-30 % w/w (NH₄)₂SO₄ salt. Precipitation was allowed to form for 45 min at 4°C with stirring, the completely precipitated solution was centrifuged at 7500 rpm at 4°C for 30 min. The supernatant was further added to the (NH₄)₂SO₄ salt powder slowly but steadily with thorough mixing until total concentration of 60 % w/w. The mixed solution was also stirred and left on ice for 45 min. Then solution was centrifuged at the same speed at 4°C for 30 min. The 2 desalted fractions of each sample were dissolved in 0.1 M cold sodium acetate buffer (pH 5.5) and collected at 4°C until used for activity assay and protein determination.

#### 2.4. Enzyme activity assay

The 10 fractions were exchanged to the appropriated buffer. β-glucosidase activity assay is the reaction of the release of p-nitrophenolate by hydrolysis of p-nitrophenyl glucopyranoside (p-NPG) substrate under basic condition of 2 M Na₂CO₃ solution. The reaction mixture (total volume 1 ml) for activity assay containing enzyme solution (50 μl) and phosphate buffer pH 6.5 (650 μl) was pre-incubated at 37°C. The enzyme activity of each fraction occurred after adding 300 μl of 50 mM p-NPG as substrate, follow by further incubation for 10 min. The enzymatic reaction was stopped by adding 2 ml of 2 M Na₂CO₃ solution. The absorbance (A) of each activity assay fraction was monitored at λ = 405 nm, followed by the comparison to p-nitrophenolate calibration curve. The highest active fraction was selected for optimum temperature study (25- 50°C) of the enzyme by using the same condition.

#### 2.5. Protein determination

The protein concentration determination; 200 μl biuret reagent and 800 μl protein solution were mixed, followed by incubation at room temperature for 25 min and the absorbance at 540 nm was measured against the blank reagent, which contained the same volume of distilled water instead of protein solution. The protein concentrations were determined from a calibration curve generated using 0-10 mg bovine serum
albumin (BSA) as a protein standard.

Figure 2. Various parts of *Tabebuia argentea* a) flower buds b) flowers c) shoots d) seed shanks and e) seeds

3. Result and Discussion

The protein solution was initial purified by the steps of ammonium sulfate precipitation. The fractions of 5 parts were as follows; concentration 0-30 and 30-60 %w/w (NH$_4$)$_2$SO$_4$. The methods separated according to the ionic strength of the solution and salt concentration, the results as shown in Table 1 and Figure 3 The β-glucosidase activity was determined using activity assay as explained in the above. All fractions from various parts showed higher activity in the present of salt concentration 0-30 than 30-60 %w/w (NH$_4$)$_2$SO$_4$, except in the fraction of 30-60 %w/w (NH$_4$)$_2$SO$_4$ precipitation of seeds extract showed the highest activity assay and protein determination (data not show).

Table 1. The enzyme activity from various parts of *Tabebuia argentea* 30-60 % (red) w/w

<table>
<thead>
<tr>
<th>Parts of <em>Tabebuia argentea</em></th>
<th>Abs</th>
<th>β-glucosidase (µmol/50µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shoots</td>
<td>0.12</td>
<td>16.7±0.20</td>
</tr>
<tr>
<td>flower buds</td>
<td>0.02</td>
<td>4.3±0.10</td>
</tr>
<tr>
<td>flowers</td>
<td>0.01</td>
<td>3.0±0.10</td>
</tr>
<tr>
<td>seed shanks</td>
<td>0.01</td>
<td>3.3±0.08</td>
</tr>
<tr>
<td>seeds</td>
<td>0.40</td>
<td>51.5±0.81</td>
</tr>
</tbody>
</table>

The protein solution showed higher of the activity assay about 15-fold over other fractions, which displayed medium level of the protein concentration. It is indicated that there are more enzymes active to pNPG substrate in seed part than others like most β-glucosidase enzymes extracted from plant. The shoot part of *Tabebuia argentea* showed medium of the activity in both concentrations, it can be studied by adjusting the salt precipitation process before future purification.

Figure 3. The enzyme activity from various parts of *Tabebuia argentea* salt 0-30 % (deep blue) and 30-60 % (red) w/w (NH$_4$)$_2$SO$_4$. Insert; change of 4-nitrophenol absorption intensity at 405 nm.

To further demonstrate its activity, the highest active fraction of 30-60% (NH$_4$)$_2$SO$_4$ salt precipitation from seeds extract was selected for optimum temperature study. The appropriated reaction of enzyme activity assay by using pNPG as substrate was carried out. The enzyme activity in different temperature 25, 30, 35, 40, 45, and 50°C was revealed. The results showed that at low temperature there was a weak UV-vis signal with little activity, similar to reactions at temperatures above 40 °C. The enhanced signal was observed in a temperature range of 30-35°C (Figure 4), the optimum temperature for this enzyme activity on pNPG is 30 °C. It is obvious that the appropriated condition for enzyme activity can be used in the range 30-35°C.

![Figure 2](http://www.ssstj.sci.ssu.ac.th/suan.sunandha.science-tech.journal/images/fig2.jpg)

![Figure 3](http://www.ssstj.sci.ssu.ac.th/suan.sunandha.science-tech.journal/images/fig3.jpg)
4. Conclusions
In this study, we have demonstrated the activity assay and protein concentration diagram at different (NH₄)₂SO₄ salt concentrations: 0-30% and 30-60% extract from various parts of *Tabebuia argentea*. We selected the fractions from 30-60% (NH₄)₂SO₄ salt precipitation from seeds extract that showed the highest activity consistent to the protein concentration, with optimum temperature at which this enzyme responds to p-NPG is 30-35°C. This fraction was selected for future study; purification steps by using membrane cut-off, follow by column chromatographic techniques for further purification and characterization.

5. Acknowledgement
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6. References


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Abstract
The objective of this research was to (1) study mental health status of diabetic patients at Health Promoting Hospital in Tha Rae subdistrict, Mueang district, Sakon Nakhon province and to (2) analyze the positive development of the diabetic patient’s mental health. This research was done in the form of surveys and interview. The sample population includes 238 diabetic patients. The research methodology includes interviews, general information questionnaire, and mental health assessment’s findings from the Department of Mental Health. The data of this mental health research findings is analyzed by utilizing quantitative research method which includes percentage, mean and standard deviation.

The research findings revealed that the majority of research population or 54.20% falls under the group of healthier (high) mental health than average. Furthermore, the survey showed 87.00% of diabetic patients possess emotional stability, 90.40% of diabetic patients receives emotional support from others and 95% of diabetic patients can handle their daily life problems.

Keywords: mental health, diabetic patients, promoting mental health program, diabetic patients’ mental health

1. Introduction
According to World Health Organization (WHO) report in 2012, there’s 1 out of every 10 adults had diabetes which is the most problematic chronic disease in the 21st century. Additionally, there were 387 million diabetic patients in 2014 and a predicted population of 600 million diabetic patients worldwide in 2030 (Diabetes Association of Thailand, 2013). Data showed that the prevalence of diabetes was 9.00% and this disease caused around 1.5 million deaths in 2012. For Thailand in 2014, there were 11,389 deaths from diabetes or 32 deaths daily. Out of every 100,000 Thai citizen, there was 17.53 diabetes-related deaths. Thai diabetic patients entered hospitals belongs to the Thai Ministry of Public Health a total of 698,720 visits yearly or 1,081.25 visits out of every 100,000 Thai citizens. Furthermore, there were only 37.90% of diabetic patients can control their glucose blood level well (Apsuwan, 2015). Department of Mental Health announced that besides taking care of their physical health, diabetic patients also need to take care of their mental health (Sanimklam, 2008; Ministry of Public Health, 2009). Diabetic patients can have an emotional reaction to the news in many ways: some people might feel resentment, in denial, anger, moody, or annoyed towards the changes in their lifestyle after being diagnosed with diabetes. Also, some patients underestimate the significance, neglect changing their lifestyle, and don’t realize the importance and fail of diabetic treatment or unable to manage their diabetic disease (Folk Doctor Foundation, 2009; 2010). Next, diabetes-diagnosed patients will face the risk of falling into depression up to 30%. Understanding the necessity to screen and identify diabetes-diagnosed patients with depression, public hospitals must host events to identify depressed patients with chronic diseases (Thai Health Promotion Foundation, 2015; National Cancer Institute, 2017). It is necessary for diabetic patients to change their health behaviors and ideal for them to change their mentality about the situation first to motivate themselves when managing this disease. When you feel discouraged and don’t recognize the problem and start helping yourself by making treatment decision, it is very difficult to find happiness. Also, during the tough
time, their family members and close ones need to give the patient emotional support. This will aid the patient to continue fighting against his/her disease. In addition, everyone has a different level of mental strength when dealing with lifestyle changes, adjusting their mindset when facing tough times, and able to continue your daily life after experiencing tragedy. Mental strength includes resilience, optimism and determination. And those possess mental health can overcome difficulties and obstacles in their life well without any following mental disorders. General people should also educate themselves on mental health and always improve their mental health. When facing tough times or sad moments in their lives, this will help them to pass through these moments without any negative impacts on them (Ministry of Public Health, 2009).

Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province, provided medical clinics for diabetic patients living in Tha Rae subdistrict to come and use their service. There was a total of 238 patients used the clinic’s service. Also, data showed that there will be continuous upward growth in the number of patients entering the clinic in the next upcoming years (Health Promoting Hospital at Tha Rae subdistrict, 2017). When comparing to the previous years statistics, a major problem that diabetic patients face is undergoing a lot of distress, which can result in depression. This further causes difficulties in living their daily life and numerous additional obstacles that these patients must face. If the patients have a strong mental health, it can help them to overcome these difficulties and obstacles in their lives. Also, if they can practice having a strong mindset toward changes in their lifestyle and habit, it will become very useful for them when undergoing tough times during their life with diabetes (Wamalun, 2012). Consequently, researchers are now recognizing the importance of the diabetic patient’s mental health and to identify if the patient will fall under bad mental health group, normal mental health group or healthier mental health group according to the mental health assessment criteria from Department of Mental Health in 2008. This mental health assessment includes 3 mental health criteria: the ability to maintain emotional stability (resilience), the ability to maintain positive morale (optimism) and the ability to manage life problems (determination) (Ministry of Public Health, 2009).

In addition, researchers are also examining the development in patient’s mental health. These scholars will study from 238 patients under treatment at Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province. Data and information from this research will be used as foundation information for future involving organizations to use for the applicable benefit and for improving the mental health of these current 238 patient (Health Promoting Hospital at Tha Rae subdistrict, 2017).

2. Objective
2.1 To study the mental health of diabetic patients under treatment at Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province.
2.2 To investigate the mental health development of diabetic patients at Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province.

3. Materials and Method
3.1. Research format
This research utilizes quantitative research method. The research population includes 238 diabetic patients under care of Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province. These 238 patients are both male and female that are diagnosed by doctor to be any type of diabetes. The research is done to study about the mental health state of these patients. The concept and theory from Department of Mental Health is applied to this work along with a mental health enhancement program when studying these patients. The mental health enhancement program includes 3 parts:
- Emotionally stability required when adjusting to the new lifestyle and habit caused by being a diabetic including possessing a stable mind, unwavering to challenges and mental control to not get pulled in by the events.
- Keeping positive morale including staying motivated, self-encouragement, and receiving compliments from others.
- Managing problems from a crisis event including negotiation, finding multiple solutions to the problem, overcoming obstacles.

In addition to this, we also came up with our own mental health assessment and interviews to identify if the mental health enhancement program is required by the patients. This mental health assessment includes 3 mental health criteria: the ability to maintain emotional stability (resilience), the ability to maintain positive morale (optimism) and the ability to manage life problems (determination). The mental health assessment took
3.2. Research method
This research applied 3 types of interviews: general information interview, mental health assessment interview from Department of Mental Health and mental health promoting program interview. Details are as following:

1) General information interview is designed by researchers to gather general information from the interviewee. There’s a total of 8 general information questions regarding gender, age, religion, education, career, income, marital status and current address.

2) Mental health assessment interview from the Department of Mental Health in 2008 with a total of 20 questions: 10 questions on emotional stability, 5 questions on positive morale and 5 questions on managing life problems.

<table>
<thead>
<tr>
<th>Table 1 Mental Health Assessment Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components of Mental Health</td>
</tr>
<tr>
<td>Emotional Stability (Question 1-10)</td>
</tr>
<tr>
<td>Positive Morale (Question 11 – 15)</td>
</tr>
<tr>
<td>Managing Life Problems (Question 16 – 20)</td>
</tr>
<tr>
<td>Total Points</td>
</tr>
</tbody>
</table>

Two groups are divided and scored accordingly.

Group 1 received survey that included question 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 17, 18, 19 and 20.

Each question is scored as follow:
Not true 1 point
Sometimes true 2 points
Relatively true 3 points
Very true 4 points

Group 2 received survey that included question 1, 5, 14, 15, and 16.

Each question is scored as follow:
Not true 4 points
Sometimes true 3 points
Relatively true 2 points
Very true 1 point

3) Mental health promoting program interview is a questionnaire designed by the researchers. There’s a total of 20 questions: 9 questions on emotional durability, 5 questions on positive morale and 6 questions on managing life problems. These questions are designed to be answered by a 5-level rating scale as follow:
Strongly agree 5 points
Agree 4 points
Neutral 3 points

And the score range is registered as follow: 1.00 – 1.80 means interviewee’s opinion is at the lowest level. 1.81 – 2.60 means interviewee’s opinion is at a low level. 2.61 – 3.40 means interviewee’s opinion is at a neutral level. 3.41 – 4.20 means interviewee’s opinion is at an agreeable level. 4.21 – 5.00 means interviewee’s opinion is at the most agreeable level.

3.3. Data Analysis
Data gathered from the 3 types of interview (general information interview, mental health assessment interview from Department of Mental Health and mental health promoting program interview) is analyzed by utilizing quantitative methods such as percentage, mean and standard deviation from a ready-made software. This information is then presented in a grid along text explanation for the reader’s convenience.

4. Result
The research’s result is divided into 3 parts:
Part 1: General information analysis result of patients
Most diabetic patients are female with 170 patients (71.40%) while there’s only 68 male diabetic patients (28.60%). Most of them also falls under the age group of 61 years old and above with 136 patients or 57.10%. Second on the list belongs to the age group between 51-60 years old with a total of 56 patients or 23.50%. The main religion followed by our research population is Christianity with 231 people or 97.10%. Buddhist comes second with 7 patients or 2.90%. Most of our research population finished primary school with 204 people or 85.70%. the second most common education level is Thai’s Lower Secondary School with 14 people or 5.90%. Most of our research population finished primary school with 3,001 people or 120.60%. Second in the rank are 66 people as stay-at-home mom or dad or 27.70%. Most of the research population earn an income of lower than 3,000 Baht with 142 people or 59.70%. Second in line is 64 people or 26.90% with an income of 3,001 – 5,000 Baht. In terms of marital status, most of the population is married with 158 patients or 66.50%. The following status is widowed with 61 people or 25.60%. Most people are living in their own house with 237 or 99.60% and only 1 patient is living in their rental house or 0.40%.

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Part 2: Result of diabetic patient’s mental health undergoing treatment at Health Promoting Hospital in Tha Rae subdistrict, Muang district, Sakhon Nakhon province.

Table 2 Displays the amount and percentage of the classified samples according to the mental health level (N=238) as follows:

<table>
<thead>
<tr>
<th>Mental Health</th>
<th>Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low &lt;55</td>
<td>5</td>
<td>2.10</td>
</tr>
<tr>
<td>Normal 55-69</td>
<td>104</td>
<td>43.70</td>
</tr>
<tr>
<td>High &gt;69</td>
<td>129</td>
<td>54.20</td>
</tr>
<tr>
<td>Total Mental Health (μ = 69.36, σ = 6.59)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From Table 2, we can conclude that the majority of diabetic patients or 129 patients (54.20%) have a mental health that is classified as high level or being mentally healthy. Next, 104 patients (43.70%) are classified as normal mental health and the last 5 patients (2.10%) are classified as low mental health. (μ = 69.36, σ = 6.59)

Table 3 Displays amount, percentage, mean, standard deviation and mental health levels including both overall and classified levels as follow:

<table>
<thead>
<tr>
<th>Mental Health</th>
<th>Amount (N=238)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mental health criteria on emotional stability (μ = 32.04, σ = 4.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt;27</td>
<td>23</td>
<td>9.70</td>
</tr>
<tr>
<td>Medium 27-34</td>
<td>166</td>
<td>69.70</td>
</tr>
<tr>
<td>High &gt;34</td>
<td>49</td>
<td>20.60</td>
</tr>
<tr>
<td>Mental health criteria on positive morale (μ = 16.63, σ = 2.92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt;14</td>
<td>71</td>
<td>29.80</td>
</tr>
<tr>
<td>Medium 14-19</td>
<td>107</td>
<td>45.00</td>
</tr>
<tr>
<td>High &gt;19</td>
<td>50</td>
<td>25.20</td>
</tr>
<tr>
<td>Mental health criteria on managing life problems (μ = 20.68, σ = 2.62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt;13</td>
<td>8</td>
<td>3.40</td>
</tr>
<tr>
<td>Medium 13-18</td>
<td>26</td>
<td>10.90</td>
</tr>
<tr>
<td>High &gt;18</td>
<td>204</td>
<td>85.70</td>
</tr>
</tbody>
</table>

From Table 3, we can conclude that the majority of diabetic patients falls into the normal group for both mental health on emotionally stability and positive morale. For moral mental health on managing life problems falls into the high group. Details to this is shown as below.

For mental health on emotional stability, the majority of diabetic patients falls into the normal group at 69.70%. High group and low group follows as 20.60% and 9.70% accordingly. The average score is presented as follow (μ = 32.04, σ = 4.24).

For mental health on positive morale, the majority of diabetic patients falls into the normal group at 45%. Low group and high group follows as 29.80% and 25.20% accordingly. The average score is presented as follow (μ = 16.63, σ = 2.92).

For mental health on managing life problems, the majority of diabetic patients falls into the high group at 85.70%. Normal group and low group follows as 10.90% and 3.40% respectively. The average score is presented as follow (μ = 20.68, σ = 2.62).

Part 3: Result of diabetic patient’s opinion on mental health promoting activity classified into levels

The result of the mental health promoting activity analysis including 3 parts: emotional stability, positive morale and managing life problems of diabetic patients under treatment at Health Promoting Hospital in Tha Rae subdistrict, Mueang district, Sakhon Nakhon province. (N= 238)

Table 4 displays mean value, standard deviation and diabetic patient’s levels of opinion on the overview of the mental health promoting activity of each criteria as follow:

<table>
<thead>
<tr>
<th>Questions</th>
<th>μ</th>
<th>σ</th>
<th>Opinion Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotional Stability Activity</td>
<td>4.20</td>
<td>0.60</td>
<td>Strongly Agree</td>
</tr>
<tr>
<td>Positive Morale Activity</td>
<td>4.30</td>
<td>0.50</td>
<td>Strongly Agree</td>
</tr>
<tr>
<td>Managing Life Problems Activity</td>
<td>4.70</td>
<td>0.50</td>
<td>Strongly Agree</td>
</tr>
<tr>
<td>Total</td>
<td>4.40</td>
<td>0.40</td>
<td>Strongly Agree</td>
</tr>
</tbody>
</table>

Table 5 displays amount and percentage of mental health promoting activity including 3 criteria: emotional stability, positive morale and managing life problems as follow:

<table>
<thead>
<tr>
<th>Promoting Program</th>
<th>Strongly Agree</th>
<th>Agree</th>
<th>Neutral</th>
<th>Disagree</th>
<th>Strongly Disagree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotional Stability</td>
<td>159</td>
<td>57</td>
<td>14</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(66.90%)</td>
<td>(23.90%)</td>
<td>(5.90%)</td>
<td>(2.90%)</td>
<td>(0.00%)</td>
</tr>
<tr>
<td>Positive Morale</td>
<td>113</td>
<td>106</td>
<td>16</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(47.50%)</td>
<td>(44.50%)</td>
<td>(6.70%)</td>
<td>(1.30%)</td>
<td>(0.00%)</td>
</tr>
<tr>
<td>Managing Life Problems</td>
<td>206</td>
<td>21</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(86.60%)</td>
<td>(8.70%)</td>
<td>(3.40%)</td>
<td>(1.30%)</td>
<td>(0.00%)</td>
</tr>
</tbody>
</table>
From Table 4 and 5, we can conclude that in general, diabetic patients strongly agree with the mental health promoting activities ($\mu = 4.40$). When examining diabetic patient’s opinions on each individual part, we can conclude the part that diabetic patients agree the most with is managing problem program ($\mu = 4.70$). Positive Morale Program follows in second place with $\mu = 4.30$ and Emotional Stability Program ranks lowest at $\mu = 4.20$.

Additional Research Findings on Survey Sample

This analysis part is a fragment of the entire survey research which focuses on diabetic patients under treatment at Health Promoting Hospital in Tha Rae subdistrict. Gathering information from interviews with the survey sample and listening to their comments, we can analyze and conclude as follow:

1) For emotional stability activity, the interviewee gave feedback comments on many aspects. For example: “There’s no need to care too much about them. We don’t have to rely on them for food”, “If there’s anything I did wrong, then I would take fully accept my mistakes”, “When there’s anything problem that I have to face, I usually don’t share it with anyone else. I think I should keep it to myself because it’s my personal business”, “We don’t have to attach our feeling with things that happened in the past. We should just focus in the present”. After sharing these comments, many diabetic patients would burst out in laughter, smile and display a relaxing attitude towards the situation.

2) For positive morale activity, the survey sample gave feedback comments on many aspects. One patient said: “My family is my best motivation. Whenever there’s any problems, we would talk about it to them”. Another one commented: “I have no plans for the future. I am already old. Currently, I’m just spending time with my children and grandchildren”. Some other diabetic patients don’t have a family, remain single or widowed without the company of children and grandchildren. With this lack of company from children or grandchildren, these patients may not receive the motivation or care that family members can offer to them. Their only support that they can rely on are their cousins and relatives. For example, there is a case of 1 diabetic patient with minor mental health problem. The patient lives alone with any children and grandchildren. On the positive side, she is still living with her relatives in the same village. However, the relatives don’t really pay attention or take care of her anyway. In addition, she has no form of income except receiving social security fund for senior, disabled or unemployed citizen from the government. During the interview, she seemed really depressed and ready to burst out in tears at any point during the conversation. Her facial expression is extremely sad as she said that sometimes she felt that her life had no meaning or value.

3) For managing life problems, the diabetic also gave their feedback comments on many aspects. One said “Sometimes I want to escape from my life problems, but I don’t have any good reason to do so. Who will take care of my grandchildren? Their parents are working in Bangkok.” Another one commented “I do enjoy listening to other’s opinions, but I don’t like to input any opinion of my own. It is their own business, so I don’t like to get involved”. When managing and solving any life problems, diabetic patients would enjoy listening to other people’s opinion without showing their own opinion. They would also use past or present experience, and support from others to help them solve and manage their life problems successfully.

During the interviews, we also received many other unexpected comments as follow. One said: “When I am distressed, I would listen or sing to music to release stress and have fun”. Another patient commented: “When I feel sad, I would do house chores to forget about my unhappiness”. Another shared: “When I am mad or in distress, I would pray to the gods before sleep to attain peace of mind”. One shared: “When I am feeling furious about something, I would curse and swear anyone or anything. Then the anger will lessen.”

Since our survey sample background is mainly Christian, this would be a factor and support the patients to have a healthier mental health as follow:

1) Emotional Stability Part:

Many diabetic patients mentioned about the Christmas Star Parade during their interview. “Christmas Star Parade is a big provincial event. There will be Christmas star marching band on the street. People believe that the star is the symbol of Jesus Christ descending to our world. There will be decorations of big Christmas stars to the marching cars with colorful string lights and pictures that tell the stories of Jesus Christ. The villagers will decorate the star lamp in front of their houses. In addition, there will be other activities such as choir singing, Christmas singing contests, acting out religious plays, entertainment and night market all night”. And from my personal experience, Christians do
have a lot of preparation, excitement and happiness from this religious event.

a. Other patients also mentioned about candlelight memorial ceremony. “It is a religious ceremony or culture of Christians in the memory of the deceased. Generally, the event will be hosted once a year or on December the 31\textsuperscript{st} in a sacred forest or at the graveyard.

2) Positive Morale Part:

a. Giving merits and donating is a very common practice by the survey sample. “Donation of goods, money or volunteer work are ways to help others”. Many Catholics said that these were the teachings of Jesus Christ. Christ taught them the concept of sharing, kindness, forgiveness and helping others. Especially, these acts of kindness are not applied exclusively for Christians. Many commented: “Whenever there’s a volunteer activity for villagers on mountains, I would participate with others. I don’t have money and would contribute by doing volunteer work. The more work I volunteer to do, the better I feel. I don’t wish for anything in return by doing these deeds.”

3) Managing Life Problem Part:

a. Doing prayers are frequently mentioned in the interviews. One commented: “Praying is a form of concentration and making a wish to the gods”. Others commented: “I would pray to Jesus Christ before going to sleep. After praying, she would feel calm and peaceful” while showing us her holy bead bracelets that she always uses to pray with. One more patient shared: “I pray multiple times every night until I calm down and feel better”.

5. Discussions

From the findings of this research this project, we found out that in general, the mental health of diabetic patients from Health Promoting Hospital in Tha Rae subdistrict, Mueang district, Sakhon Nakhon province, is under the group of healthier (high) mental health than average. Specifically, mental health on emotional stability and positive morale both falls under the normal grade while mental health on managing life problems is classified into the healthier (high) grade. For emotional stability, patients can manage their emotion by thinking positively. For positive morale, patients receive emotional support from their family. For managing life problems, diabetic patients exchange advices between each other to find ways to relieve stress, especially through religious practices. In addition, we also find out that most of the diabetic patients is in the age group of 61-years-old and above with 136 patients (57.10%), which is consistent to Dr. Chaturudee Parayat’s work studying the predicting factors affecting mental health of older adults in 2015. From this study, Dr. Chaturudee Prayat also concluded that half of the survey sample achieved a healthier (high) mental health group. For age group from 60-69 years old, 51% of the patients are still capable of adapting to new changes or managing their own problems and helping others as well. Patients at this age group are still physically and mentally still very healthy. Many of them are very competent and still contribute their family, community or society more than other age groups, according to Wagnild and Young (1993). We also found out that their feeling and emotion can greatly affect the self-confidence they have. In addition, the reason for their mental health falls into a healthy (high) group stems the necessity to live alone, self-exist, perseverance and tolerance in their lives.

5.1) Department of mental health’s 3-part assessment

1) Emotional Stability Part:

For this part, we can conclude that diabetic patients fall into the normal group ($\mu = 32.04$) or 69.70%. This result is consistent with the study of Dr. Jansuda Janopakhun about mental health’s levels of new graduate nurses under job rotation at Chulalongkorn Hospital. Dr. Janopakhun’s study found out that most of the newly graduate nursing staff (74.00%) also falls into the normal group. During the interview, there were many signs that showed diabetic patients can control their emotions. For example, one patient mentioned: “There’s no need to care too much about them. We don’t have to rely on them for food”. Another commented similarly: “If there’s anything I did wrong, then I would take fully accept my mistakes”. Many adults of the age group 30-60 years old noted: “When there’s anything problem that I have to face, I usually don’t share it with anyone else. I think I should keep it to myself because it’s my personal business”. Meanwhile, older adults in the age group 60-70 years old would share: “We don’t have to attach our feeling with things that happened in the past. We should just focus in the present”. After answering with these comments, many diabetic patients would burst out in laughter, smile and display a relaxed attitude towards the situation. However, in some infrequent cases, interviewees showed a much more serious attitude towards the situation. In conclusion, from the patient’s comment, we can deduce that diabetic patients take full responsibility of being diagnosed
with this disease, understand their own feelings or emotion, and able to self-control their emotion by not paying attention to things that already happened in the past (Chanthaburi Pharapokklao Nursing College, 2015).

2) Positive Morale Part:
   In this part, we found out that diabetic patients fall under the normal group (μ = 16.64) or 66.40%. The finding in this research is consistent with a research team member’s previous work, Dr. Chaliya Wamalum. Dr. Chaliya Wamalum’s research work studied group-based psychology counseling for cancer patients. The program is designed to support the cancer patient to overcome the obstacles of being diagnosed with cancer. From the work, Dr. Chaliya Wamalum also concluded that the cancer patient’s emotional positive morale also falls under the normal group (μ = 16.64). We concluded that under the influence of the chronic diseases, patients must stay positive by themselves and look for external support from their family or close ones. This process of motivation and realization will help them identify their own strengths, weaknesses, and improve the relationship with other individuals in their lives. During the interview, we noted many comments from patients that further supports the research findings. Many diabetic patients usually comment: “I have no plan for the future. There’s no reason for me to do so. I’m already old. Nowadays, I only spend time with my children and grandchildren.” Elders under the age group of 60 years old and above regularly shared: “My family is my best motivation. Whenever there’s anything in my mind, I would talk and discuss with them”. There is a group of elderly patients that are living alone, without the care and motivation that children and grandchildren. They can only rely on their cousins and relatives. There is a case of an elder patient that lives alone. She has minor mental problems and her only caretaker is the relatives from the same village that pays very little attention to her. She has no income but social security funds from the government. During the interview, she was very depressed while answering: “Sometimes, I feel like I have no value or meaning to my life. There’s no one that will take care of me”. In conclusion, in general, diabetic patients possess good positive morale from the support of their family and close members.

3) Managing Life Part:
   From the research, we found out that diabetic patients fall under the normal group (μ = 16.64) or 61.30%. This finding is unique when comparing to other research work but still holds valid when comparing with the evidences retrieved from the interviews. Diabetic patients from all age groups shared: “Sometimes I want to escape from my life problems, but I don’t have any good reason to do so. Who will take care of my grandchildren? Their parents are working in Bangkok”. A group of patients shared their thoughts: “I enjoy listening to others, but I wouldn’t share my opinion because it’s their business and I wouldn’t want to get involved”. Many diabetic patients enjoy listening to other’s opinions that are different to their own. However, they wouldn’t share their own opinion. They would then use their personal experience and ask for advice from others to help them manage their life problems. In conclusion, we can confirm that diabetic patients are able to recognize their diabetes problems and are prepared for treatment, knowing it is a chronic disease and won’t heal. They would ask advice from professionals or friends in order to continue fighting diabetes for their family and love ones.

5.2) Promoting Mental Health Activities for Diabetic Patient
   1. Emotional Stability Part:
      After analyzing the results, the type of activity that received the most agreeable rate at (μ = 4.63) or 77.70% is relaxation activities. Examples of these relaxation activities are watching comedy, TV shows, TV series, and listening to music. This finding is consistent with the Department of Mental Health theory about Necessary Activities for a Healthy Mental Psychology (Department of Mental Health, Bureau of Mental Health, 2012). The theory mentioned that important activities that needs to be practiced are relaxation activities that brings happiness to the patient. In addition, according to the conversations between the interviewer and patients, we also found out 2 extra activities that are commonly mentioned are Christmas Star Parade and Candlelight Memorial Ceremony. During the Christmas Star Parade, people will decorate their houses with Christmas star lights, participate in Christmas singing contests, choir singing, perform in a Christmas play, and go for some shopping at a night market. From my own experience, during this festival, Christians are very excited, joyful while in preparation for the festival. For Candlelight Memorial Ceremony, Christians mentioned that it is a religious ceremony or culture of Christians in the memory of the deceased. During this festival, Christians will experience happiness, pride, enjoyment, and forget about sad memories. This event will be host once a year or on December the 31st in a sacred forest or at the graveyard. (Christianity in Thailand, 2013).
2. Positive Morale Part:
After analyzing the result, the type of activity that received the most agreeable rate at (\( \mu = 4.72 \)) or 87.00%, is self-talk. Examples of self-talks are self-talk to consolation yourself (believing everything will turn out well and problems will be solved somehow) or speaking subconsciously to yourself. This finding is consistent to Department of Mental Health’s theory on People Who Possess a Healthy Mental Psychology. The theory stated that self-support or self-talk such as “we must succeed” or “we must keep fighting”, will raise encouragement for people to continue to solve problems and overcome obstacles in life (Thiamsawet, 2014). In addition, we also found out extra information during the interview. Many Christian patients mentioned that giving merit, donation of goods, money or volunteer work are teachings from Jesus Christ. Christ taught them the concept of sharing, kindness, forgiveness and helping others and these acts of kindness are not applied exclusively for Christians. One patient commented: “Whenever there’s a volunteer activity for villagers on mountains, I would participate with others. I don’t have money and would contribute by doing volunteer work. The more work I volunteer to do, the better I feel. I don’t wish for anything in return by doing these deeds.”

3. Managing Life Problem Part:
After analyzing the result, the type of activity that received the most agreeable rate at (\( \mu = 4.70 \)) or 87.50% is meditation and praying. In addition, we also found out extra information during the interview. Many patients commented that praying is a form of concentration and making a wish to the gods. One commented: “Praying is a form of concentration and making a wish to the gods”. Others commented: “I would pray to Jesus Christ before going to sleep. After praying, she would feel calm and peaceful” while showing us her holy bead bracelets that she always uses to pray with. Another patient shared: “I pray multiple times every night until I calm down and feel better” (Christianity in Thailand, 2013).

6. Suggestion
6.1 Health organizations should host health promoting events for diabetics by provide training sessions or seminars.
6.2 Health organizations should set up public address system within the village to provide information about diabetes for diabetic patients and others.
6.3 Health organizations should set up a society/community for diabetic patients such as Part-time Career Club for Diabetics

7. Reference
http://www.kamsonbkk.com
http://www.thaihealth.or.th
https://www.doctor.or.th/article/detail/11212
Isolation and characterization of filter paper degrading bacteria from the guts of Coptotermes formosanus

Tochukwu Frank Egwuatu¹,², Osita Gabriel Appeh²
¹Department of Cell Biology and Genetics, Faculty of Science, University of Lagos, Akoaka 100213, Nigeria
²Michael Okpara University of Agriculture Umudike, P.M.B. 7267. Umuahia, Abia State, Nigeria

Corresponding author e-mail: tochukuwufrankegwuatu@gmail.com

Abstract
The filter paper utilizing capabilities of Pseudomonas mendocina, Burkholderia pseudomallei, Chryseobacterium luteola, Klebsiella oxytoca and Klebsiella terrigena isolated from the gut of a local termite Coptotermes formosanus were analysed. The aim of this study was to isolate and characterize cellulolytic microbes from the guts of Coptotermes formosanus. The isolates were inoculated into a buffered medium containing minerals and Whatman filter paper as sole source of carbon to observe the ability of these bacteria to digest solid substrate. The ability of the isolates to grow in this medium as well as to digest the filter paper was determined by visual observation after 30 days. Reducing sugar test and gravimetric analysis were also carried out at the end of 30 days. All bacteria cultures showed growth as the medium turned cloudy and the filter paper became macerated. The gravimetric analysis of the residual filter in the liquid medium at the end of 30 days incubation showed that Chryseobacterium luteola had the highest degradation rate of 95%, Pseudomonas mendocina had the degradation rate of 90%, whilst Burkholderia pseudomallei, Klebsiella oxytoca and Klebsiella terrigena had biodegradation rate of 75% each. Reducing sugar test and paper chromatography carried out for glucose production were positive showing their ability to convert cellulose to glucose. The bacterial isolates showed a potential to convert cellulose into reducing sugars which could be readily used in many applications like feed stock for production of valuable organic compounds; for example in simultaneous saccharification and fermentation of cellulose into ethanol.

Keywords: Biodegradation, Chromatography, Termite gut, Cloudy, Cellulose

1. Introduction
Termites are insects from the order Isoptera which in Greek ισος means equal and πετέρων means wing (Akpomie, Ubogun., & Ubogun, 2013). They are usually called white ants. They are small to medium size with a dull white to light brown body (Borji, Rahimi, Ghorbani, Vand Yoosefi, & Fazaeli, , 2003) and characterized by their colonial behaviour. Termites are among the most important lignocelluloses-ingesting insects and possess a variety of symbiotic microorganisms in their hindguts, including bacteria, Archaea and Eukarya (Borji, Rahimi, Ghorbani, Vand Yoosefi, & Fazaeli, , 2003). Termites can be classified into six families and fifteen subfamilies (Chakraborty et al., 2000). Higher termites make up about 85% of the known species of termites. Lower termites feed mainly on wood, utilizing the enzymes they make themselves, as well as those from bacteria, archaea, and protists in their guts to digest the wood (Dugas, Zurek, Paster, Keddie, 2001). The gut microbiota enables termites to efficiently hydrolyze cellulose. The cellulose activity of termite hindgut is attributed to cellulose-degrading bacteria. Termites are diverse in their feeding habits that lead to diverse microbiota. Many microorganisms have been reported with cellulolytic activities including many bacterial and fungal strains both aerobic and anaerobic. Chaetomium, Fusarium, Myrothecium, Trichoderma, Penicillium, Aspergillus and so forth are some of the reported fungal species responsible for cellulosic biomass hydrolysation. Cellulolytic bacterial species include Trichonympha, Clostridium, Actinomyces, Bacteroides, Succinogenes, Ruminococcus albus, Methanobrevibacter ruminantium (Gupta, Samant, & Sahu, 2012; Scheffrahn, & Su, 1994). The aim of this study was to isolate and characterize cellulolytic microbes from the guts of Coptotermes formosanus.
2. Materials and Methods

2.1 Sample collection

The termites used for the isolation and identification of cellulolytic microorganisms were collected from a termite hill at Oba, a town in Anambra State, Nigeria.

2.2 Isolation of filter paper degrading microorganisms

The termites collected were identified as *Coptotermes formosanus* (lower termites) as described by (Schwarz, 2001; Sindhu, & Dadarwal, 2001). The termites were taken out of their nests and placed in sterilized Petri dishes. Twenty worker termites were surface sterilized with 70% ethanol (Stanley, 1907) and then washed in sterile distilled water and allowed to air-dry for 1 minute. Under sterile conditions, each termite was separated into its head and body. After removing heads with forceps, the bodies were dissected with a sharp blade with the aid of a magnifying Hand lens. The guts of the dissected termites were picked out using a sterile syringe and mixed in 20 ml of 0.85% NaCl. Five milliliter of the suspension was inoculated into four different conical flasks (except the control), each containing seventy-five milliliter of Basal Salt Medium as described by Chakraborty, Sarkar, & Lahiri, 2000; Konig, 2006). The medium contained in g/l, 2.2 K2HPO4, 1.5 KH2PO4, 1.3 (NH4)2SO4, 0.1 MgCl, 0.02 CaCl, 0.001 FeSO4, 7H2O, five filter papers and the pH was adjusted to 7.2 (with 1M NaOH and HCl) and incubated at room temperature for 30 days. The medium was sterilized at 121°C for 15 minutes. The sterilized Whatman qualitative filter paper (diameter: 90 mm) served as the main carbon source (cellulose) for the microorganisms. Cloudiness of the medium indicated growth and maceration of the filter paper indicated cellulolytic activity. After 30 days, the culture was plated out on nutrient agar and pure colonies were obtained by several subsequent sub-culturing and plating.

2.3 Identification and characterization of bacterial isolates

Colonial examination of the isolates was carried out to determine the type of shape, elevation and pigmentation pattern they exhibited. Microscopic examination including Gram staining and cellular morphological appearances were also carried out. Analytical Profiling Index (API) 20E and 20 NE kits were then used to carry out further identification tests on the isolates. API 20E and 20 NE kits are used for the identification of enteric and non-enteric bacteria. The kit was prepared according to the manufacturer’s specifications.

2.4 API 20E and 20 NE identification

The API 20E (Biomérieux) strip contains 20 microtubules. The inoculums were prepared by culturing the organism on nutrient agar plate for 24 hours. The distinct colonies produced were then picked and transferred to 5 ml sterile normal saline to prepare a homogenous suspension. A sterile pipette was used to fill the tubules of CIT; VP and GEL positions were filled with the suspension. Mineral oil was used to overlay test ADH, LDC, ODC, H2S and URE to create anaerobiosis. The inoculated strip was placed in the incubation box into which sterile distilled water had been placed to create a humid condition during incubation. The strips were incubated at 30°C for 18-24 hours. Readings were taken after the 24 hours of incubation; other tests such as TDA, IND, VP and NIT were carried out by adding appropriate reagents into the tubules. Observations were recorded and subsequently analyzed using API kits software which presumptively identified and characterized the isolates to species level.

2.5 Filter paper degradation study

Each bacterial isolate was inoculated into a test tube containing nutrient broth, incubated overnight at room temperature and afterwards used as inoculum. The medium used for the cellulolytic activity study was as described by Chakraborty, Sarkar, & Lahiri (2000) (Konig, 2006). The medium was sterilized at 121°C for 15 minutes in an autoclave. Seventy-five milliliter of the medium was poured into twenty-two conical flasks containing 0.4g of sterilized filter paper as sole source of carbon (cellulose) for the microorganisms. Five milliliter of each isolate’s cell culture was washed and pipetted into each flask (except the control) and then incubated at room temperature for 30 days. Growth and cellulolytic activity was determined by observing the change in the medium as well as on the filter paper. Turbidity of the medium indicated growth and maceration of the filter paper indicated cellulolytic activity.

2.6 Gravimetric analysis

This analysis is by weight. All gravimetric analyses rely on final determination of weight as means of quantifying an analyte. Gravimetric analysis was used to determine the weight of residual filter paper present in the medium after 30 days of incubation and thus determined the degree

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of filter paper degradation. A standard profile was first obtained by determining the dry-weight of undegraded filter paper in the control experiment which was the same for all flasks before inoculation, incubation and degradation commenced. Media containing cellulolytic isolates and pieces of filter paper were filtered and washed. The residual filter paper pieces were dried to a constant weight at 60 °C. Filter paper degradation by these cellulolytic microorganisms was determined as the differences between filter paper present at the beginning and the end products of the culture period. The individual weight of residual filter paper pieces for each flask was determined. The weight of degraded filter paper for each flask was determined and converted to percentage.

### 2.7 Determination of reducing sugar with Fehling's solution

Reducing sugar determination using Fehling Solution was done as described by Stanley (Kodama, Kimura, & Komagata, 1985) to determine the presence of reducing sugars in the filter paper/ cellulose degradation culture medium. The culture medium was centrifuged and 2 ml of the supernatant was added into a test tube. One ml of Fehling Solution was added to it and heated for 15 minutes. Formation of yellow to brick red precipitate showed the presence of reducing sugars such as glucose and fructose.

### 2.8 Paper chromatography

Paper chromatography is a technique that involves placing a dot or line of sample solution onto a strip of cellulose paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. Paper chromatography was carried out using the supernatant from each of the bacterial isolate culture medium to determine the production of glucose from filter paper/ cellulose degradation by the bacterial isolates. Pure glucose was used as a control experiment. The spots on the Chromatograms were visualized by spraying them with ammonical silver nitrate and placing them in an oven for about 5-10 minutes. The reducing sugars appeared as brown spots and the Retardation factor of glucose on each Chromatogram was determined. Ammonia (1%) plus saturated phenol-water was used as the solvent for this procedure.

### 3. Results

Five bacterial species were isolated and identified using colonial morphology (as shown in Table 1), biochemical tests and the use of API test kit. The organisms obtained were identified using API test kit 20E and 20NE as Bac-1, *Pseudomonas mendocina*, Bac-2, *Burkholderia pseudomallei*, Bac-3, *Chryseobacterium luteola*, Bac-4, *Klebsiella oxytoca* and Bac-5, *Klebsiella terrigena* as shown in Tables 2 and 3.

The result of gravimetric analysis carried out on the residual filter paper from the filter paper degradation study culture medium by each isolate after 30 days of incubation is as follows: *Chryseobacterium luteola* gave the highest degradation value of 95% followed by *Pseudomonas mendocina* with the rate of 90%. *Burkholderia pseudomallei*, *Klebsiella oxytoca* and *Klebsiella terrigena* gave degradation rate of 75% each as shown in Table 4.

Reducing sugar determination test with Fehling Solution (Stanley, 1907) for each bacterial isolate culture medium was positive. Paper chromatography showed the production of glucose from the degradation of filter paper/ cellulose by the bacterial isolates. The Retardation factors were calculated and recorded as shown in Table 5.
Table 1. Characteristics of bacteria on nutrient agar

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Bac-1</th>
<th>Bac-2</th>
<th>Bac-3</th>
<th>Bac-4</th>
<th>Bac-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Size</td>
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<td>Small</td>
<td>Small</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
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<td>Cream</td>
<td>Cream</td>
<td>Cream</td>
<td>Cream mucoid</td>
<td>Cream</td>
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<td>Convex</td>
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<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
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<td>Cell morphology</td>
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<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
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<tr>
<td>Gram’s reaction</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

-, negative; +, positive

Table 2. Biochemical characteristics of isolates using API kit 20 NE

<table>
<thead>
<tr>
<th>Isolates</th>
<th>API</th>
<th>Probably Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac-1</td>
<td></td>
<td>Pseudomonas mendocina</td>
</tr>
<tr>
<td>Bac-2</td>
<td></td>
<td>Burkholderia pseudomallei</td>
</tr>
<tr>
<td>Bac-3</td>
<td></td>
<td>Chryseobacterium luteola</td>
</tr>
</tbody>
</table>

-, negative; +, positive

Table 3. Biochemical characteristics of isolates using API kit 20 E

<table>
<thead>
<tr>
<th>Isolates</th>
<th>API</th>
<th>Probably Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac-4</td>
<td></td>
<td>Klebsiella oxytoca</td>
</tr>
<tr>
<td>Bac-5</td>
<td></td>
<td>Klebsiella terrigena</td>
</tr>
</tbody>
</table>

-, negative; +, positive
Table 4. Residual filter paper content in filter paper liquid culture medium at the end of a 30-day incubation period

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual Filter paper (g/l)</th>
<th>Biodegradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40</td>
<td>0.00</td>
</tr>
<tr>
<td><em>P. mendocina</em></td>
<td>0.04</td>
<td>90.00</td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>0.10</td>
<td>75.00</td>
</tr>
<tr>
<td><em>C. luteola</em></td>
<td>0.02</td>
<td>95.00</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>0.10</td>
<td>75.00</td>
</tr>
<tr>
<td><em>K. terrigena</em></td>
<td>0.10</td>
<td>75.00</td>
</tr>
</tbody>
</table>

Table 5. Retention factors (Rf) of glucose produced from cellulose degradation by bacterial isolates determined by paper chromatography.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Retention factor (Rf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.640</td>
</tr>
<tr>
<td><em>P. mendocina</em></td>
<td>0.643</td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>0.642</td>
</tr>
<tr>
<td><em>C. luteola</em></td>
<td>0.643</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>0.639</td>
</tr>
<tr>
<td><em>K. terrigena</em></td>
<td>0.634</td>
</tr>
</tbody>
</table>

4. Discussion

In this study, microorganisms capable of degrading filter paper were isolated from *Coptotermes formosanus* (lower termites) (4). Termites are one of the planet’s most efficient bioreactors and may be capable of producing up to two litres of hydrogen from digesting a single piece of paper. Termites achieve this high degree of efficiency by exploiting the metabolic capabilities of about 200 different species of microorganisms that inhabit their hindguts (Lee & Wood, 1971). The complex lignocellulosic polymers within wood are broken down into simple sugars by fermenting bacteria in the termite’s gut, using enzymes that produce hydrogen as by-product. The potential for the production of biofuel from the fermentation of the simple sugars obtained from lignocellulosic decomposition in this way is tremendous (Matsui, Tokuda, & Shinzato, 2009). The world’s increasing need for an alternative source of energy has always urged scientists to turn to biofuel. Since many of the developed nations in the world have already started bioethanol production using cellulolytic bacteria obtained from many organisms including termites, it has become urgently necessary for our nation to exploit these organisms for biofuel production. In nature, cellulosic materials can be degraded by many bacteria. More than 50 species have been isolated. However, different strains possess different cellulose degradation capabilities (Millward-Sadler, Davidson, Hazlewood, & Black, 1995; Milala, Shugaba, Gidado, Ene, & Wafar, 2005). Konig (2006) had grouped bacteria from termites gut based on their lignocellulolytic activity into two, that is, hydrolytic and fermentative groups. In this study, all bacterial species were able to digest the filter paper as well as used the products for growth. Previous studies by Borji, Rahimi, Ghorbani, Vand Yousefi and Fazaeli, (2003) also reported that *Enterobacter* and *Acinetobacter* species showed cellulolytic activity. Dugas, Zurek, Paster and Keddie (2001) had isolated and identified a strain of *Chryseobacterium* from the gut of the American cockroach which was fed with a high fibre diet (Ohkuma, 2003; Ramin, Alimon, Sijam, & Abdulla, 2008) isolated and identified *Chryseobacterium kwangyangense* which belong to the family Flavobacteriaceae and (Akporie,


Optimal medium for watercress (Alternanthera sp.) micropropagation

Vachiraporn Pikulthong1*, Sirirat Phakpaknam1, Manussawee Dechkla1, Tharathorn Teerakathiti2

1Faculty of Science and Technology, Suan Sunandha Rajabhat University
U-thonthong Rd, Dusit, Bangkok 10300, Thailand
2National Center for Genetic Engineering and Biotechnology, Thailand Science Park Phahonyothin Road, Khlong Luang, Pathum Thani 12120, Thailand
Corresponding author e-mail: *vachiraporn.pi@ssru.ac.th

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Abstract

The objective of the present study was to find the optimal conditions for micropropagation of Alternanthera sp. The first experiment aimed to find the optimal sterilization of lateral bud meristems for in vitro culture. The results revealed that the best sterilization technique is to clean lateral bud meristem by bending shoot and dipping in 70% ethanol for 1 min. These shoots were then removed and immersed in 3% sodium hypochlorite solution for 10 min. This sterilization technique provided the highest survival percentage of 95% and the best microorganism removal. The second experiment aimed to determine the effects of the plant growth regulators benzyladenine (BA) on shoot proliferation. The shoot induction of sterile lateral bud meristems of Alternanthera sp. was then carried out in Murashige and Skoog (MS) media supplemented with benzyladenine (BA) at five different concentrations (0, 1, 2, 3 and 4 mg/l) under 25±2 °C and light condition for 4 weeks. It was found that MS media supplemented with 3 mg/l BA induced the optimal growth and development of shoots. This condition provided the highest root induction and the significant average shoot of 12.88±0.51 shoots/explant (p<0.05). The results from the present study could be the basic information for producing aquatic plants that have economic value and genetic conservation. In additions, it may be useful for biological activity tests or provide the possibility for callus induction for further somatic embryo culture technology.

Keywords: Alternanthera sp., Micropropagation, Sodium hypochlorite, Benzyladenine

1. Introduction

Watercress is a favorite vegetable as salad and other menu among people in Europe and some countries in Asia. There are two species of watercress grown in Thailand. The first one is Nasturtium officinale (family Brassicaceae), which is an imported species from Europe. It contains isothiocyanate which exhibits anti-cancer activity (Kopsell, Barickman, Sams, & McElroy, 2007).

Another watercress species that is widely grown in Thailand is Alternanthera sp. (family Amaranthaceae). Its commercial name is Asian watercress or Japanese watercress. It is a herbaceous plant, commonly found in freshwater pond and swamp. Watercress varieties might be imported, mutated or genetically modified to have different appearances. Phytochemical study revealed that watercress (Alternanthera sp.) comprises various bioactive compounds including phenolic compounds, alkaloids and flavonoids (Majumder, Rashid, Chowdhury, Gupta, & Mandal, 2016). The stem and leaf of watercress highly compose of ferric, vitamin A, proteins and fibers (Dutta, 2015). In folklore medicine, watercress is used as anti-pyretic and anti-ulcer agent (Rattanathongkom, Sripanidkulchai, & Kanchanapoom, 2008).

In additions, it is used for treatment of diarrhea, common cold and gastro-intestinal disorders (Kumar, Dheeba, Stalin, Maragatham, & Kannan, 2011; Nin, 1986). It is reported that watercress inhibits the growth of pathogenic virus in respiratory tracts, dengue virus (Jiang, Yang, Chen, Xiao, & Luo, 2007) and human immunodeficiency virus (HIV) (Zhang, He, Tabba, & Smith, 1988). Watercress has virus inhibitory activity. It may play a significant role on immune system stimulation (Rattanathongkom, Sripanidkulchai, & Kanchanapoom, 2008).

The present study was aimed to investigate the optimal conditions for micropropagation of watercress
(Alternanthera sp.) which is widely grown in Thailand. This is the basic information for producing aquatic plants that have economic value using tissue culture for consumption and genetic conservation. In addition, this may be useful for biological activity tests and for pharmaceutical purposes. Moreover, it could provide the possibility for callus induction for further gene transfer study by somatic embryo culture technology. Therefore, the objective of this study is to investigate the optimal sterilization technique for micropropagation and study the effect of BA on lateral bud meristems in Alternanthera sp.

2. Materials and Methods

2.1. The optimal sterilization technique for micropropagation

Mature watercress without diseases and pests were cleaned with tap water. There are two protocols for the investigation of the optimal sterilization technique for micropropagation of lateral bud meristems in Alternanthera sp., as follows:

1. The lateral bud meristems with length 1-1.5 cm were cleaned with tap water and liquid soap. These lateral buds were then compared in the sterilization technique by putting in sodium hypochlorite at the concentration of 10 and 15 % for 10 and 20 min. The specimens were then washed with sterile distilled water for 2 minutes, 3 times. The exceeded lateral buds were removed, then the optimal sterilization technique for micropropagation was determined by culture on MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2.5 g/l agar (Phytagar®) and pH 5.7 for 4 weeks. The experimental design was completely randomized design (CRD) with 24 replications. The results were recorded in terms of contamination and survival of the explants within 1 week (figure 1).

2. Watercress lateral bud meristems 1-1.5 cm in length were soaked in 70% ethanol for 1 min and left at room temperature for 15 min until dry. The shoots were cut for comparison of the sterilization technique with sodium hypochlorite at the concentration of 0, 1, 2, 3 and 4 mg/l, 3% sucrose, 2.5 g/l agar (Phytagar®) and pH 5.7 for 4 weeks. The experimental design was CRD with 24 replications. The results were recorded in terms of contamination and survival of the explants within 1 week (figure 1).

2.2. The effects of BA on shoot induction from lateral bud meristems

Watercress lateral bud meristems were sterilized from experiment 1. The exceeded parts were removed by cutting leaves. The shoots meristems were cut to -1.5 cm in length. They were then cultured on MS media supplemented with benzyladenine (BA) at different concentrations of 0, 1, 2, 3 and 4 mg/l, 3% sucrose, 2.5 g/l agar (Phytagar®) and pH 5.7 under temperature of 25±2°C for 4 weeks and sub cultured every two weeks. The growth parameters of the explants including shoot length, shoot counts and morphology of the explants were recorded. The experimental design was CRD with 15 replications. The statistical differences among means were compared by using Duncan’s new multiple range test (DMRT). The confidence interval was 95% (p<0.05).

3. Results

3.1. The optimal sterilization technique for micropropagation

Watercress lateral buds were compared for the sterilization technique with sodium hypochlorite at the concentration of 10 and 15 % for 10 and 20 min. The results revealed that lateral buds sterilized with sodium hypochlorite at the concentration of 10 and 15 % for 10 min had the percentage of bacterial and fungal contamination of 100 %. Lateral buds sterilized with sodium hypochlorite at the concentration of 10 % and shaken for 20 min had the percentage of bacterial and fungal contamination of 54.16 % and maximum survival rate of 45.83 %. Interestingly, lateral buds sterilized with sodium hypochlorite at the concentration of 15 % and shaken for 20 min had the percentage of bacterial and fungal contamination of 29.16 % and maximum survival rate of 70.83 % (Table 1).

Next, were the results for the method in which lateral bud meristems were soaked in 70% ethanol followed by sterilization with sodium hypochlorite at concentrations of 0, 0.5, 1, 2 and 3 % for 10 min. It was found that watercress shoots sterilized with 70% ethanol in combination with 3% sodium hypochlorite had the lowest microbial contamination percentage of 5 %, followed by shoots sterilized with 70% ethanol in combination with 2% sodium hypochlorite with a microbial contamination percentage of 21 %. For shoots sterilized with 70% ethanol in combination with 1% and 0.5% sodium hypochlorite, these had a contamination percentage of 100 % without any survival. The sterile explants were effectively induced as new shoots in the next experiments (figure 2).
Table 1. Contamination and survival rates of lateral bud meristems after sterilization with different treatments on MS media

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chemicals and sterilization methods</th>
<th>Contamination rate</th>
<th>Survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% NaOCl, 10 min</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10% NaOCl, 20 min</td>
<td>54.16</td>
<td>45.83</td>
</tr>
<tr>
<td>3</td>
<td>15% NaOCl, 10 min</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>15% NaOCl, 20 min</td>
<td>29.16</td>
<td>70.83</td>
</tr>
<tr>
<td>5</td>
<td>70% EtOH + 0.5% NaOCl</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>70% EtOH + 1.0% NaOCl</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>70% EtOH + 2.0% NaOCl</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>8</td>
<td>70% EtOH + 3.0% NaOCl</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

Figure 1. Characteristics of Alternanthera sp., A) before sterilization; B) after sterilization; C) lateral buds after exceeded part removal

Figure 2. Microbial contamination on MS media, A) bacterial contamination; B) fungal contamination; C) sterile and surviving lateral buds

3.2. Effects of BA on watercress lateral bud meristematic tissues for 4 weeks

Watercress lateral buds were cultured on MS media supplemented with benzyladenine (BA) at different concentrations of 0, 1, 2, 3 and 4 mg/l under temperature of 25±2 °C and light condition in order to induce shoots for 4 weeks. The results showed that lateral buds cultured on MS media supplemented with BA at different concentrations had significant different shoot counts and lengths (p<0.05). MS media supplemented with 4 mg/l BA induced the highest average shoot count of 13.66±1.86 shoots/explant, followed by MS media supplemented with 3, 2 and 1 mg/l BA which induced the shoot count of 12.88±0.51, 12.22±1.01 and 11.87±1.05 shoots/explant, respectively. The MS media without supplementation of BA induced the lowest shoot count of 4.44±0.84 shoots/explant (Table 2 and Figure 3).

The study on the effects of BA on the shoot lengths revealed that MS media without supplementation of BA induced the highest average shoot lengths of 3.37±0.32 cm, followed by MS media supplemented with 1, 2, 3 and 4 mg/l BA which induced the shoot lengths of 2.50±0.23, 2.42±0.23, 2.37±0.32 and 2.35±0.10 cm, respectively. MS media supplemented with 4 mg/l BA induced the lowest average shoot length of 2.35±0.10 cm. The MS media without BA had a significant number of induced shoot length. Whereas, explants cultured on MS media supplemented with BA at different concentrations did not have significant different shoot lengths when the means were compared by using DMRT at confidence interval of 95% (Table 2).

The observation of morphological changes of lateral bud meristematic tissues showed that MS media supplemented with 4 mg/l BA induced a lot of new shoots. When cultured for several weeks, leaves did not expand but were clustered. They were elongated with a yellowish green color and the root induction was very few. However, MS media supplemented with 3 mg/l BA induced the better explants. Leaves were completely green in color. The root induction was complete with more root counts (data not shown). Therefore, MS media supplemented with 3 mg/l BA is recommended for further micropropagation of Alternanthera sp.

Table 2. The effects of different concentrations of BA supplemented in MS media on shoot counts and shoot lengths in watercress lateral bud meristematic tissues cultured for 4 weeks

<table>
<thead>
<tr>
<th>Media formula</th>
<th>Average shoot counts (shoots/explant)</th>
<th>Average shoot lengths (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS only</td>
<td>4.44±0.84</td>
<td>3.37±0.32</td>
</tr>
<tr>
<td>MS supplemented with 1 mg/l BA</td>
<td>11.87±1.05</td>
<td>2.50±0.23</td>
</tr>
<tr>
<td>MS supplemented with 2 mg/l BA</td>
<td>12.22±1.01</td>
<td>2.42±0.23</td>
</tr>
<tr>
<td>MS supplemented with 3 mg/l BA</td>
<td>12.88±0.51</td>
<td>2.37±0.32</td>
</tr>
<tr>
<td>MS supplemented with 4 mg/l BA</td>
<td>13.66±1.86</td>
<td>2.35±0.10</td>
</tr>
</tbody>
</table>

Remarks: * is means from 3 replications
Different letters after numbers in the same column mean there is significant difference compared by DMRT at confidence interval of 95%
Data are expressed as mean±S.D. (standard deviation)
Figure 3. The growth of watercress lateral bud meristematic tissues cultured on MS media only and MS media supplemented with BA at four different concentrations for 4 weeks. A) MS only; B) MS + 1 mg/l BA; C) MS + 2 mg/l BA; D) MS + 3 mg/l BA; E) MS + 4 mg/l BA

4. Discussion

Disinfection is an important step in the tissue culture process. The most commonly used screening method is sodium hypochlorite solution, which has capacity in inhibiting microorganisms on the surface of tissues. The appropriate concentration of bleach depend on the type of plant and the parts to be sterilized. According to Rawdkhao (2000), plant tissues should be subjected to the removal of microorganisms on the surface using sodium hypochlorite solution in various concentration. The optimal concentration of sterilizing agents that provide the most sterile plant tissues should be further investigated. These sterilizing agents must be easily eradicated and not harmful to plant tissues.

In the present study, MS media (Murashige & Skoog, 1962) was selected in the investigation of optimal concentrations of BA in the micropropagation of watercress lateral bud meristematic tissues. MS media is a general media formula for plant tissue cultures. It comprises of various kinds of nutrients (Tanthai et al., 2015). MS media is usually supplemented with BA at different concentrations. It was found that all concentrations of BA affect the growth of watercress lateral bud meristematic tissues. BA belongs to cytokinin plant regulator. It stimulates and accelerates somatic cell division, cell expansion and differentiation of lateral buds into shoots in a very short time (Sakakibara, 2006; Zhang, Swarup, Bennett, Schaller, & Kieber, 2013). The previous studies showed that MS media supplemented with cytokinin plant regulators increases the shoot counts in micropropagation of the genus Alternanthera and other aquatic plants. Shekhawat, Manokari and Revathi (2017) reported that MS media supplemented with 2 mg/l BA induced a lot of shoot counts (23.8±1.9 shoots/explant) in micropropagation of Alternanthera philoxeroides. Flores, Flóres, Bempck, Maldaner and Marchioretto (2016) found that MS media supplemented with 1 µM Thidiazuron (TDZ) in combination with 30 or 40 g/l sucrose and 20 g/l glucose effectively increases the shoot counts in micropropagation of Alternanthera hirtula. Pongchawee, Pasugdee, Pradissan, Pipatcharoenchai and Kanthrong (2012) reported that MS media supplemented with 2 mg/l BA significantly increases induction of meristem tissues of Echinodorus osiris Rataj into shoots (P<0.05) with average shoot counts of 3.31±0.74 shoots/explant, average root counts of 4.81±1.17 roots/explant, average shoot height of 2.45±0.16 cm and average leaf counts of 7.75±0.82 leaves/explant. In addition, it was found that MS media supplemented with 1 mg/l BA significantly increases shoot counts (P<0.05) of water trumpet (Cryptocoryne tonkinensis) in comparison to the other concentrations with average shoot counts of 4.50±2.12 shoots/explant (Pongchawee, Chusang, & Tong-mee-aied, 1999).

In the present study, although MS media supplemented with 4 mg/l BA induced the higher average shoot counts than those of 3 mg/l BA, leaf growth in watercress is incomplete. This finding may be due to the increase of BA concentrations which will be toxic to meristematic tissues and therefore exhibiting plant growth inhibition. In additions, Montri et al. (2000) found that MS media supplemented with 10 mg/l BA decreases shoot counts in the micropropagation of lateral bud meristems of Phyllanthus emblica. Six weeks after culture of watercress lateral bud meristematic tissue, it was found that the shoots were very small in size. Leaf growth was incomplete. Root counts were quite low. These findings probably related to the high accumulation of BA in MS media in the early phase of the experiment. When the concentrations of BA were still high, there were changes in the balance of plant growth regulators in plant tissues resulting in the incomplete growth of plants (Pikulthong, Teerakathiti, Thamchaipenet, & Peyachoknagul, 2016). Likewise, Lee and Chan (2004) reported a stunted shoot of Orthosiphon stamineus developed in the medium with increasing concentrations of BA. However, MS media without supplementation of plant growth regulators induced the lowest average shoot counts. The shoots were elongated. Leaves were largely expanded. Root counts were very high. Cytokinin has an important role on transportation of nutrients including vitamins and minerals in plants. Therefore, meristematic tissues cultured on MS media without supplementation of BA had the decreased shoot counts.
5. Conclusion
The most suitable method for surface sterilization in Alternanthera sp. lateral bud meristematic tissues was the direct bending of shoot and exposure to 70% ethanol for 1 min and left at room temperature until dry. The shoots were then cut and soaked in 3% sodium hypochlorite for 10 minutes. This technique provided the contamination rate of 5% with the highest survival rate of 95%. It should be recommended for further micropropagation of lateral bud meristematic tissues of Alternanthera sp.

The optimal conditions for the culture of the aquatic plant Alternanthera sp. using tissue culture were through the use of MS medium containing 3 mg/L BA. The plant grown for a further one week could develop new shoots. The results from the present study could be applied for micropropagation of lateral bud meristematic tissues for other aquatic plants and biological activity tests as well as secondary metabolite production for pharmaceutical purposes. In addition, it could provide the possibility for callus induction for further gene transfer study by somatic embryo culture technology.

6. Acknowledgement
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7. References


