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Improvement of microbiological quality, antioxidant content and shelf life of jujube (*Ziziphus mauritiana* cv. BAU Kul) fruit by gamma irradiation

Farzana Mridha\(^a\), Roksana Huque\(^b\), Mst. Afifa Khatun\(^b\), Mahfuza Islam\(^b\), Arzina Hossain\(^b\), Afzal Hossain\(^b\), Md. Shahinur Kabir\(^a\)*

\(^a\)Department of Botany, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh
\(^b\)Food Technology Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka, Bangladesh

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**Abstract.** Postharvest loss of inherently perishable fruits is a matter of serious concern for the farmers and traders. Reduction of postharvest loss is one of the key components for ensuring food security. A study was carried out to reduce the postharvest loss of BAU Kul, an improved variety of jujube fruit (*Ziziphus mauritiana*), by using gamma irradiation. Different doses of gamma irradiation (0.5, 1.0 and 1.5 kGy) was applied to the jujube fruit samples and the microbiological quality, antioxidant content and shelf life of those fruits were evaluated. Gamma irradiation initially caused significant reduction of the total heterotrophic bacteria, coliform as well as yeast and mold counts. However, the counts increased in both irradiated and non-irradiated fruit samples with the passage of storage period but the increment was significantly less in the 1.5 kGy irradiated samples. Irradiation played active role in the enhancement of total phenolics and flavonoids contents. The concentration of these antioxidants remained higher in irradiated samples in comparison to non-irradiated control samples throughout the storage period. However, the ascorbic acid content decreased gradually with the increase of radiation dose and storage period. The overall acceptability of the fruit samples was determined by the taste-taking panelist. The irradiated (1.0 and 1.5 kGy) fruits were acceptable up to 8 days whereas control and 0.5 kGy irradiated fruits lost their acceptability during storage. The study revealed that 1.5 kGy irradiation can improve microbiological quality and extend the shelf life of jujube fruits (cv. BAU Kul) without significant loss of overall antioxidant content and sensory attributes.

**Keywords:** antioxidant markers, gamma irradiation, jujube, microbes, shelf life

**INTRODUCTION**

Jujube (*Ziziphus mauritiana* Lamk.), a member of the family Rhamnaceae, is a fast growing tropical tree and its fruit is a good source of flavonoids, organic acids, vitamins, polysaccharides and microelements (Li *et al.*, 2007). This fruit is generally consumed directly as a fresh fruit. Moreover, this fruit is also consumed as dried, candied, pickled and other products like squash or jujube butter. This fruit is used in Chinese system of medicine for spleen diseases and nourishment of blood (Shena *et al.*, 2009). In Bangladesh, jujube (locally called Boroi or Kul) is popular among people of all age groups and usually consumed directly without any processing. BAU Kul, a new variety developed at the germplasm center of Bangladesh Agricultural University (BAU), has...
gained huge popularity among the available varieties. The demand of this variety is very high all over the country mainly because of its taste, high yield, suitability for cultivation, nutritional value and reasonable price. BAU Kul contains a complex microbial flora including potential plant pathogens and saprophytes. Prevalence of various fungi on jujube fruits is high (46.86%) and BAU Kul was found as the most disease vulnerable variety than other jujube varieties available in Bangladesh (Hoque et al., 2016). Like other jujube fruits, BAU Kul has a short shelf-life of only 4-5 days at ambient temperature. Such a short postharvest shelf-life results huge financial loss for the farmers and traders.

Ionizing radiation of food products is now recognized as a safe and effective technology for improving quality of fruits, vegetables and juices (Kume et al., 2009; Song et al., 2006). Furthermore, no toxic radioactive effect remains on treated foods (Farkas and Mohácsi, 2011). Most of the organization related to food and health safety reported it as an effective technology for the elimination of food spoilage organisms, extension of shelf life of fresh food and reduction of post-harvest loss (IAEA, 1977; ICGFI, 1998; WHO, 1981). Gamma irradiation is mostly used than other ionizing radiations (e.g., x-rays, electron beam) in food sector. It is widely used as an alternative to chemical fumigants and preservatives which have adverse effects on human health and environment.

Despite of high popularity and productivity of BAU Kul, few studies have been conducted on this variety (Hoque et al., 2016; Talukdar et al., 2014; Tanvir et al., 2015; Uddin and Hussain, 2012) and no published data available to date on the effect of gamma irradiation on the microbiological and biochemical attributes of BAU Kul. Thus, this study was performed to evaluate the effect of gamma irradiation on microbial quality, antioxidant content and shelf life of this variety of jujube during storage at ambient temperature.

MATERIALS AND METHODS

Sample collection, irradiation and storage. Jujube fruits (cv. BAU Kul) were procured from local retailers and transported to the laboratory immediately after collection in a clean sterilized container. The samples used in this study were uniform in size, apparently free from any visible insect infestation, mechanical injury or deterioration. Collected fruit samples were put into sterilized perforated low-density polyethylene zipper bags. The packed samples were labeled properly with three selected doses of gamma irradiation (0.5 kGy, 1.0 kGy and 1.5 kGy) and a non-irradiated packed sample was kept as control. There were 3 replicates for each treatment and control (non-irradiated). Samples labeled as treated were irradiated in a 50 kCi Co60 gamma irradiator (dose rate 6.4 kGy/hr) located at Institute of Food and Radiation Biology, Bangladesh Atomic Energy Research Establishment, Savar, Dhaka, Bangladesh. Both irradiated and control samples were stored at ambient temperature in air. Microbiological, biochemical and sensory attributes of all samples were assessed before gamma irradiation, immediately after irradiation (0 day of storage) and also after 4 and 8 days of storage.

Determination of pH and moisture content. An automatic electric pH meter (JENWAY 3510, UK) was used to determine the pH of the samples. For this purpose, fruit of each sample was separately homogenized in a homogenizer. After adjusting and stabilizing the pH meter, the pH was recorded. The moisture content of jujube samples was determined following the method described previously (AOAC, 1975).

Microbiological analysis. For microbiological analysis, standard techniques (Cappuccino and Sherman, 1996; ICMSF, 1978) were used to enumerate bacterial load. Fruit sample (10 g) from each sample was separately homogenized in 90 ml 0.9% saline solution. Subsequent ten-fold serial dilutions were made and 100 µl diluted sample was spread onto the plate containing appropriate medium (HiMedia). Nutrient agar (NA), MacConkey agar and potato dextrose agar (PDA) were used to enumerate total heterotrophic bacterial count (THBC), total coliform count (TCC) and total yeast mold count (TYMC), respectively. The NA and MacConkey plates were incubated at 37°C for 24 hours. The PDA plates were incubated at room temperature for 5 days.
After incubation, result was recorded by counting the characteristic colonies formed on the plates. The counts, after appropriate calculation, were expressed as colony forming units per gram (cfu/g) of fresh fruit (Mridha et al., 2017).

**Total phenolics content and total flavonoids content.** BAU Kul fruit samples of both control (non-irradiated) and irradiated were separately homogenized with 80% methanol in a homogenizer (Wisetriss 20). The homogenate was then centrifuged at 5000 rpm for 10 minutes and the supernatant was filtered through filter paper (Whatman No.1). All the sample extracts (filtrate) were used for subsequent phenolics or flavonoid content determination.

The Folin-Ciocalteu (FC) method was used to determine the total phenolics content (Singleton and Rossi, 1965). Briefly, an aliquot of extract was added to FC reagent (1:10). After few minutes, 7.5% sodium carbonate solution was added and the volume of the mixture was adjusted to 10 ml with distilled water. The tubes were kept in dark and the absorbance of the solution was measured by a spectrophotometer at 765 nm (Spectro UV-VIS, Labomed). Calculation was done using gallic acid as a standard and results were reported as mg gallic acid equivalents (mg GAE) per 100 g of fresh weight.

Total flavonoids (TF) content was determined according to colorimetric method (Chang et al., 2002; Shibata et al., 1975). Briefly, an aliquot of the extract was taken in test tube. One hundred microlitre of 10% aluminum chloride and 100 µl 1 M potassium acetate were added sequentially to the test tube. The volume of the mixture was adjusted to 5 ml by distilled water. The tubes were kept at room temperature for 30 minutes and absorbance of the solution was measured by a spectrophotometer at 415 nm (Spectro UV-VIS, Labomed). Standard curve was prepared using Quercetin hydrate solution and results were expressed as milligram of quercetin equivalent (mg QE) per 100 g of fresh fruit.

**Ascorbic acid content.** Titrimetric method was used to determine the ascorbic acid content (Ranganna, 1986). Two grams of fruit from each sample was homogenized with 25 ml 3% metaphosphoric acid and filtered through a filter paper. Then titration was done by 2, 6-

**Sensory attributes.** Samples were evaluated by a taste testing panelist of ten judges who observed the sensory attributes (color, flavor and texture) from randomly assigned irradiated and non-irradiated samples. They marked the samples on a 9-point Hedonic scale (Krum, 1955) where 0-2 represents extremely dislike, 3-5 dislike, 6-8 acceptable or good and 9 excellent for color, flavor and texture. An overall acceptability was calculated taking average of the three attributes.

**Statistical analysis.** The data were analyzed and expressed as an average. Significant differences were analyzed by the Student's t-test and considered significant at the level of \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**The pH and moisture content.** The initial average pH value of jujube sample was 4.81±0.06. This pH value is slightly higher than that of the findings of Uddin and Hussain (2012) who observed pH 4.50 for fresh BAU Kul variety. In another study, pH value of 4.39 to 4.56 for Tohafy and 4.72 to 4.57 for Balahy varieties of jujube was recorded in 2007 and 2008, respectively (Ezz et al., 2011). No significant changes in pH values were observed between irradiated and control samples for all the storage treatments (Table 1). Previous studies also revealed that low dose gamma irradiation (0.1 to 1.5 kGy) does not affect the pH value in strawberry, raspberry and passion fruits (Golding et al., 2015; Majeed et al., 2014; Verde et al., 2013). In the present study, the moisture content of non-irradiated sample was 87.58%. Almost similar level of moisture content was also reported in BAU Kul in two separate studies (Uddin and Hussain, 2012; Talukdar et al., 2014). Moisture content in all irradiated samples was increased immediately (at 0 day) after treatment, but not for storage at 4 day and 8 day (Table 1). This increase may be due to tissue softening by partial degradation of polysaccharides in response to irradiation (D’Amour et al., 1993). During storage period, moisture content reduced in all
Improvement of quality and shelf life of jujube fruit experimental samples which might be due to the transpiration.

Microbiological quality. Data on the microbiological quality of gamma-irradiated and non-irradiated jujube fruits is shown in Figures 1-3. In fresh non-irradiated jujube fruit samples, the average THBC, TYMC and TCC were 5.04±0.63 log cfu/g, 4.39±0.51 log cfu/g and 3.20±0.35 log cfu/g, respectively. These microorganisms represent both the indigenous microbial flora and contamination during post-harvest handling of the fruits. The total counts were higher than the maximum criterion value set by GCC Standardization Organization (GSO, 2014).

Gamma radiation applied in this study caused the reduction of the THBC, TYMC and TCC in a dose dependent manner but the applied doses did not completely eliminate the microbes present in the fruit samples. The average THBC decreased 1.9 log at 1.5 kGy dose immediately after irradiation (Figure 1). Application of gamma radiation at 0.5 and 1.0 kGy doses initially caused 0.24 and 0.81 log reduction of THBC. Highest reduction of TYMC (1.35 log) and TCC (2.09 log) was achieved by the application of 1.5 kGy gamma irradiation while the other two doses (0.5 and 1.0 kGy) caused relatively less reduction of those counts. According to the IGCFI (1998), irradiation with low doses does not kill all the microbes but decrease the total contamination. With the passage of the storage time, THBC, TYMC and TCC increased significantly in the control (non-irradiated) samples. These counts also increased in the irradiated fruits samples but numbers were far below that of the non-irradiated fruit sample. An increase of the number of microbial loads in 1.0 kGy irradiated fruits of blackberries were also described by Oliveira et al. (2013). However, it is noteworthy to mention that microbial counts of irradiated samples increased in a slow rate than that of non-irradiated sample during storage. The THBC, TCC and TYMC increased significantly in the non-irradiated sample after 8 days of storage at ambient temperature compared to the initial counts obtained immediately after the collection of fruits. In case of THBC, around 1.9 log increase was recorded in the non-irradiated control sample after 8 days of storage while 54.74% less increase of the heterotrophic bacteria was recorded in 1.5 kGy gamma irradiated samples. The TYMC and TCC of the samples were also decreased significantly immediately after gamma irradiation. Iqtedar et al. (2016) also observed the lowest increase of microbial count in storage after the application of 2 kGy gamma irradiation in apples.

Antioxidant content. Ascorbic acid, phenolics and flavonoids present in fruits and vegetables have received attention of the health-conscious persons because of having antioxidant activity. Phenolics are important free radical scavengers and plays significant role to minimize molecular

Improvement of quality and shelf life of jujube fruit

The total phenolics content of BAU Kul was found 44.17±4.46 mg GAE/100 g for the control sample. Nearly similar phenolics content (41.40±0.2 mg GAE/100g) was also reported in *Ziziphus rugosa* (Karuppusamy *et al.*, 2011). Application of gamma irradiation increased the phenolics content in treated samples (Figure 4).

Table 1. Effect of gamma irradiation on pH and moisture content of jujube samples during storage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Moisture Content (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day</td>
<td>4 Day</td>
<td>8 Day</td>
<td>0 Day</td>
<td>4 Day</td>
<td>8 Day</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.81±0.06a</td>
<td>4.84±0.12a</td>
<td>4.85±0.06a</td>
<td>87.58±0.54a</td>
<td>85.60±0.57a</td>
<td>82.22±0.04a</td>
<td></td>
</tr>
<tr>
<td>0.5 kGy</td>
<td>4.83±0.08a</td>
<td>4.84±0.09a</td>
<td>4.85±0.01a</td>
<td>90.50±0.15b</td>
<td>85.41±0.48b</td>
<td>81.00±0.05b</td>
<td></td>
</tr>
<tr>
<td>1.0 kGy</td>
<td>4.80±0.01a</td>
<td>4.80±0.07a</td>
<td>4.82±0.04a</td>
<td>89.27±0.24c</td>
<td>84.70±0.31b</td>
<td>81.61±1.08b</td>
<td></td>
</tr>
<tr>
<td>1.5 kGy</td>
<td>4.83±0.03a</td>
<td>4.81±0.05a</td>
<td>4.82±0.08a</td>
<td>90.71±0.20c</td>
<td>86.19±1.02b</td>
<td>82.80±0.02a</td>
<td></td>
</tr>
</tbody>
</table>

Values are the average of 3 replicates. Values with different lowercase letters within a column differ significantly ($p <0.05$).

![Graph showing effect of gamma irradiation on total phenolics content of BAU Kul during storage. Bars with different lowercase letters differ significantly ($p <0.05$).](image)

In this study, significant increase of phenolics content (27.40%) was observed in the samples treated with 1.0 kGy gamma irradiation. The observed increase of total phenolics after gamma irradiation may be due to the better extractability of these compounds and decomposition of some insoluble phenolic compounds (Villavicencio *et al.*, 2000). Increased concentration of total phenolics treated with gamma irradiation (0.25 and 0.5 kGy) was also observed in bitter gourd (Khatun *et al.*, 2012). Accumulation of phenolics at high doses of gamma irradiation was also reported in other study (Beltagi *et al.*, 2011). With the passage of storage time, gradual decrease of total phenolics content observed in the non-irradiated sample. After 8 days of storage, 29.08 % reduction of phenolics was observed in the control sample. However, the phenolics content in the gamma irradiated samples remained above the initial concentration found in non-irradiated jujube fruits.

Flavonoids are ubiquitous secondary products among which epicatechin, quercetin, epicatechin gallate are well-known for their antioxidant activity. Antioxidant activities of flavonoids are stronger than those of vitamins C and phenolic compound because of multiple hydroxyl groups present in their chemicals (Geldof and Engeseth, 2002). The fresh non-irradiated BAU Kul had 15.87±1.97 mg QE/100 g of total flavonoids
which is higher than the value (10.02±3.53 mg CE/100 g) reported by Tanvir et al. (2015). The Total flavonoids content of studied fruits was increased upon irradiation compared to non-irradiated control sample (Figure 5). The sample which received 1.0 kGy gamma radiation also showed significant increase of flavonoid content compared to other treated samples. The increase pattern can be attributed to the phenylalanine ammonia lyase (PAL) activity, which is one of the key enzymes in the synthesis of flavonoids compounds in plant (Frohnmeyer and Staiger, 2003; Gitz et al., 2004). Cheng et al. (2005) also reported that the PAL directly induces flavonoid formation. In another study, Moghaddam et al. (2011) also reported the activity of PAL affects the flavonoid synthesis in Centella asiatica in response to gamma and UV-B irradiation. The flavonoid content in the 1.0 kGy and 1.5 kGy treated samples were significantly higher even after 8 days of storage than that of initial concentration of the untreated control samples.

The ascorbic acid content of studied jujube fruits is shown in the Figure 6. The initial value of ascorbic acid in fresh non-irradiated BAU Kul fruit was 10.77±1.43 mg/100 g. In this study, ascorbic acid concentration decreased with the increase of radiation dose. In storage, reduced amount of ascorbic acid was observed in both radiation-treated and non-treated samples but radiation-treated samples showed less reduction. About 38% reduction of ascorbic acid content was recorded in control sample whereas only 9.73% reduction was observed in case of 1.5 kGy irradiated samples after 8 days of storage. Our findings corroborate with the findings of Owureku-Asare et al. (2014) who observed the decrease of vitamin C content in both irradiated and non-irradiated samples over the storage period and found that irradiated samples retained higher vitamin C than control samples in pineapples (Ananas comosus). In another study, presence of similar level of vitamin C was reported in citrus fruits which decreased with the increase of radiation doses and storage time (Ahmad et al., 2012). Dose-dependent decreases in total vitamin C content compared to control, both immediately after irradiation and after 5 and 10 days of storage was also observed in strawberries (Graham and Stevenson, 1997). Ascorbic acid is a heat sensitive bioactive compound and degraded by oxidative processes. Therefore, oxidation of ascorbic acid might be one of the causes of the observed decrease of the ascorbic acid content (Mohammad et al., 2009).

**Sensory evaluation.** Sensory evaluation of jujube fruits revealed that treated samples had more acceptability in terms of color, flavor, and texture up to 8 days of storage at ambient temperature compared to control (Table 2). Both irradiated and non-irradiated samples were good enough up to 4 days of observation. After 4 days, non-irradiated samples gradually lost its color, flavor and texture which ultimately lead to complete loss of acceptability within 8 days. This also corroborates with our findings that microbial counts increased significantly compared to that of initial counts in the non-irradiated jujube fruit samples. The overall acceptability of sample treated with 1.5 kGy radiation was found...
significant ($p<0.05$) in terms of all sensory attributes up to 8 days compared to the control and the other irradiated samples. These results indicate that irradiation at 1.5 kGy can improve the shelf life of jujube fruits without adverse effects on sensory attributes. Shelf life extension of strawberries, cherries, apricots and apple without affecting the sensory quality of the fruits was also achieved by the use of electron accelerators and gamma irradiation (Minea et al., 1996; Mridha et al., 2017).

Table 2. Sensory evaluation of jujube fruit treated with different doses of gamma irradiation during storage.

<table>
<thead>
<tr>
<th>Sensory attributes</th>
<th>Treatments</th>
<th>0 Day</th>
<th>4 Day</th>
<th>8 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Control</td>
<td>8.8a</td>
<td>5.3a</td>
<td>1.1a</td>
</tr>
<tr>
<td>0.5 kGy</td>
<td></td>
<td>8.9a</td>
<td>5.5a</td>
<td>3.3b</td>
</tr>
<tr>
<td>1.0 kGy</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>7.4b</td>
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<tr>
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</table>

Values are the average of 5 replicates. Values with different lowercase letters in column of each attributes differ significantly ($p<0.05$).

CONCLUSION

This is the first report on the effects of gamma irradiation on the physico-chemical and microbiological attributes of BAU Kul variety of jujube. Briefly, findings of the present study demonstrate that gamma irradiation can be used as an effective technology to improve microbial quality, increase phenolics and flavonoids contents and extend shelf life of jujube fruit (cv. BAU Kul). Gamma irradiation might be useful to extend shelf life and reduce post harvest losses of other perishable fruits.

ACKNOWLEDGEMENT

The authors are grateful to Gamma Source Division for providing irradiation facilities and to the technical personnel for their support.

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A rhizosphere isolate from *Oryza sativa*, *Enterobacter cloacae* VITTPN2, as a potential plant growth promoting rhizobacteria; an *in vitro* study

Thahiya Naushad, Neethu Kamarudheen, Poorna Chandrika Gopal, Kokati Venkata Bhaskara Rao

Molecular and Microbiology Research Laboratory, Division of Environmental Biotechnology, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu 632 014, India

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**Abstract.** The increasing need for Plant Growth Promoting Rhizobacteria (PGPR) for biofertilizer development is warranted owing to the environmental hazards caused by chemical fertilizers. Our investigation was to isolate, screen and characterize PGPR from rhizospheric soil with potential PGPR properties. *Oryza sativa* and *Saccharum officinarum* rhizosphere were collected from the agricultural research station, Virinjipuram, Vellore (12.9202°N, 79.1333°E), Tamil Nadu, India for PGPR isolation. Eleven distinct isolates of bacteria were grown on Jensen’s (seven) and Pikovskaya’s media (four). Among these, four isolates (TPN1 to TPN4) showed phosphate solubilisation activity. And one isolate TPN2 particularly showed both nitrogen fixation and phosphate solubilization with other PGPR properties. Furthermore, the isolate TPN2 demonstrated promising results in Indole 3-Acetic Acid production (99.29±0.945µg ml⁻¹). Since the isolate TPN2 displayed all PGPR characteristics under study, it was selected for pot culture studies. The seeds treated with TPN2 revealed an increase of 63.6% in shoot length and 14.63% in root length of the okra plant. There was a 74.6% increase in shoot length and a 16% increase in the root length of the tomato plant. Additionally, there was extensive developmental of lateral roots in okra plant. Henceforth TPN2 was identified as *Enterobacter cloacae* VITTPN2 (ku951582). This report produced remarkable results which promise the bacterial strain *Enterobacter cloacae* strain VITTPN2 can be further studied as a prospective biofertilizer.

**Keywords:** *Enterobacter cloacae*, IAA, nitrogen fixing, phosphate solubilisation, pot culture, shoot elongation

**INTRODUCTION**

The soil is one of the most exploited scientific frontiers and rhizosphere is the active part of that frontier as it contains many microorganisms. The rhizosphere is a fine sheet of soil around the roots of the plants which hold a large number of active bacteria including rapidly colonizing Plant Growth Promoting Rhizobacteria (PGPR) (Villacieros et al., 2003; Suslow et al., 1979).

Lately, a great deal of consciousness has been given to PGPR as a valuable substitute for agrochemicals. Also, there has been much interest shown towards these bacteria to substitute chemicals, which promote the growth of plants in various number of ways, including production of plant growth promoting phytohormones, root growth enhancement, biocontrol of pathogens, degradation of organic pollutants, solubilization of unavailable forms of nutrients etc (Gupta et al.,...
The rhizospheric soil contains root exudates secreted by plants and rhizodeposits which act as chemical attractants for PGPR (Lowe et al., 2012). The exudates include amino acids, sugars, vitamins, nucleosides, enzymes, inorganic ions and gaseous molecules. Activities of rhizobacteria are observed to be high in this region. Many bacteria like Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Rhizobium, and Serratia are accounted as inhabitants of rhizosphere which enhances plant growth. (Bhattacharya and Jha 2012) The PGPR bacteria are characterised by (i) they must be capable of colonizing on the root surface (ii) during their expression of plant growth promoting activities, they should thrive, reproduce and be able to win other microbes present around (iii) they must enhance the plant growth (Ahmad and Khan 2011).

The PGPR increases plant growth by two different mechanisms (i) direct (ii) indirect. Direct means are, fixing the unavailable atmospheric nitrogen, phytohormone production like indole acetic acid, gibberellic acid, siderophores, reduction in ethylene and phosphate solubilisation (Glick et al., 1998) whereas production of antibiotics, iron in the rhizosphere, antifungal metabolites and enzyme production and induced systemic resistance, are included in indirect mechanisms of growth enhancement by PGPR (Gupta et al., 2015; Kloepper et al., 1989).

Nitrogen (N\textsubscript{2}) is a majorly important macronutrient essential for the growth of plants. Despite the presence of 78% nitrogen in the air, it is not available for the use of plants. PGPR helps convert N\textsubscript{2} to NH\textsubscript{3} by nitrogen fixation, thus making nitrogen available for plants. Phosphorus is the next major growth-limiting nutrient, that exists in both organically and inorganically in soil (Glick, 1995). A major amount of phosphorus is encountered in forms that are not soluble like apatite, inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters which lowers its uptake by plants (Khan et al., 2009). Plants take up phosphorous in monobasic (H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−} and dibasic (HPO\textsubscript{4}\textsuperscript{2−}) ion forms which are soluble (Glick, 2012). PGPR solubilises the soil phosphates by producing organic acids and phosphatase enzymes. This results in uptake of soluble phosphorous by plants.

Indole 3-Acetic Acid (IAA) is regarded as the major indigenous auxins (Bhattacharya and Jha 2012). IAA acts as signal molecules in regulating the growth of plants like organ formation, tropic and cellular response and gene regulation. PGPR strains are well documented for the biosynthesis of the IAA which subsequently increases plant root system and thereby enhances the nutrient uptake by plants. L- Tryptophan functions as a predecessor for IAA production, thus its presence in culture medium enhances the IAA production in vitro (Kloepper and Schroth 1978).

PGPR strains are protective agents against plant pathogens disseminated through soil by hydrogen cyanide (HCN) production (Glick, 2012). HCN inhibits the growth of plant pathogenic fungi. Biofertilizers have been increasingly recognized as substitutes to chemical fertilizers as these microbes help enhance the plant growth, yield and augment the nutrients in the soil they inhabit (Cappucino and Sherman 1992). A substantial rise in the productivity of crops is accounted after applying PGPR microbial inoculants (Lwin et al., 2012).

In this study, we have screened and identified PGPR strains. Rhizospheric soil of Oryza sativa and Saccharum officinarum was used as a source to isolate PGPR. PGPR isolates obtained on various media were screened for PGPR properties such as nitrogen fixation, phosphate solubilisation, synthesis of IAA, HCN, ammonia synthesis and catalase production. The most active isolate was identified genomically and subjected to root and shoot elongation study for the primary conclusion of PGPR aiding plant growth.

**MATERIALS AND METHODS**

**Chemicals and reagents.** All the bacteriological media used in this study were purchased from Hi-media (Mumbai, India). Every analytical grade chemicals and reagents such as indole acetic acid, L-tryptophan were purchased from Merck (Bangalore, India).

**Sample collection and isolation of PGPR strains.** Rhizosphere sample was obtained from the rhizosphere region of Oryza Sativa and Saccharum officinarum from the agricultural research
station, Virinjipuram of Vellore (12.9202°N, 79.1333°E) Tamil Nadu, India. The isolation of rhizospheric bacteria was carried out through traditional microbiological procedures (Cappuccino and Sherman 1992). Jensen’s agar medium and Pikovskaya’s agar medium were employed for the selective isolation of nitrogen fixers and phosphate solubilizers respectively. The plates were incubated at an optimal temperature of 30±2°C for a period of 2-8 days with continuous monitoring for the presence of bacterial growth. The colonies with morphological differences were selected and preserved at 4°C on the corresponding medium.

**Determination of dual nature of the isolates.**

To evaluate the isolates in terms of both nitrogen fixation as well as phosphate solubilisation the isolates from Jensen’s agar were grown on Pikovskaya’s agar and vice-verse. The isolates which virtually exhibited both the properties were selected and preserved for further studies (Deolankar et al., 2015).

**Phytohormone studies - Indole Acetic Acid (IAA) assay.** One ml of overnight bacterial culture was inoculated into Luria Bertani broth with 5 µg ml⁻¹ of L-tryptophan and placed in an orbital shaker (120 rpm) at room temperature for 48 hours. Upon incubation period, 2 ml was withdrawn and centrifuged at 4307 g for 30 mins. IAA production was estimated by UV-Vis Spectrophotometer by Salkowski’s reagent at 530 nm as mentioned by Patten and Glick, 2000. IAA produced by isolated strain was calculated in comparison to indole acetic acid curve and the highest IAA producer was established (Ashrafuzzman et al., 2009).

**Phosphate solubilisation activity.** For determination of solubilisation capacity of the isolates, they were incubated on modified Pikovskaya’s medium at 37±2°C for 5 days. The plates were observed continuously until 5 days for the appearance of the zone of clearance indicative of the potency of the isolate (Vessey, 2003; Salamone 2000).

**Hydrogen cyanide and catalase production.** Isolates were screened for the ability to produce hydrogen cyanide (HCN) according to the method of Castric (Castric et al., 2010). For the purpose of detection of HCN production, the selected isolates were inoculated on nutrient agar plates with glycine concentration of 4.4 g ml⁻¹. A strip of Whatman filter paper no. 1 soaked in sodium carbonate in 0.5% picric acid solution and was placed inside sticking on to the surface of the lid of the agar plate. The plate was sealed with paraffin film and incubated at 36±2°C for 72 hours. The filter paper turning orange indicates the production of HCN.

For assessing catalase activity, 250 µl of 48 hr old bacterial isolates was placed on the surface of a glass slide and mixed with an equal volume of 3% H₂O₂ with a sterile applicator stick. The effervescence indicates the catalase activity (Schaad, 1992).

**Ammonia production.** To investigate the production of ammonia, peptone water was used to grow the isolate. 5-10 ml of peptone water was inoculated with 100 µl of overnight culture and incubated at 37±2°C for 2-3 days. Production of ammonia was detected by addition of Nessler’s reagent (Colins and Lyne 1980). Production of ammonia was indicated by the brown to yellow colouration.

**Characterization and identification of the potential isolate.** The isolate showing all the activities viz., nitrogen-fixing ability, phosphate solubilisation, maximum IAA production and catalase activity were identified by following microscopic, biochemical and molecular analyses. The potential isolate TPN2 was identified through 16S rRNA sequence analysis. InstaGene Matrix Genomic DNA isolation kit (Bio-Rad) was used to isolate bacterial genomic DNA. The segments were amplified using the universal primers 27F 5’-AGAGTTTGATCMTGCTGAG-3’ and 1492 R 5’-TACGGYTACCTTGTTACGACTT-3’. The PCR product was purified with the help of montage PCR clean-up kit (Millipore). Sequencing was performed on ABI 3730xl sequencer. The resultant 16S rRNA sequence was observed for similarity using NCBI BLAST similarity sequence tool. The sequence was further analysed for phylgeny with the highest similar sequences. Multiple alignments of the sequences were performed by MUSCLE 3.7. Mega 5 was used for constructing the phylogenetic tree by
neighbour joining method with interior branch test.

**Pot culture study for determining plant growth promotion.** The most potential isolate exhibiting all the above plant growth promoting properties were selected for the in vitro studies in pot cultures. Seeds of two important vegetable crops, tomato and Okra plant were obtained from the agricultural research station, Virinjipuram, Vellore, Tamil Nadu, India. Surface sterilization of the seeds was performed using sodium hypochlorite for 10-20 mins and they were washed thoroughly with distilled water. The seeds were immersed in contact with the suspension of bacteria prepared prior to the experiment. The suspension was prepared by inoculating a loopful of bacterial suspension in 100 ml of Luria Bertani broth on an orbital shaker at 180 rpm for 48 hours. The treated seeds were planted in pots as deep as 4 to 5 cm of 300 g sterile soil for a period of 15 days. A control pot was also maintained along with the experimental pots.

The plants were maintained and observed daily for a period of 3 weeks through seed sowing to harvesting. The elongation (cm plant⁻¹) of shoot and root of each plant (n=8) was studied after the period of growth. The comparison was made to the control set of plants (Yadav et al., 2010).

**Statistical analysis.** Experiments involved were performed in triplicates. Pot culture experiment result was examined statistically by Two-way Analysis of Variance (ANOVA) using GraphPad Prism 6.0 (GraphPad Software Inc. San Diego, USA). The two independent variables root and shoot length were found to have an interaction with the dependent variable total height and the study was found to be statistically significant.

**RESULTS AND DISCUSSION**

**Isolation and screening for dual nature.** The process of isolation yielded four bacterial colonies showing nitrogen fixing ability and seven phosphate solubilising colonies on Jensen’s medium and Pikovskaya’s medium respectively. Upon the cross-media inoculation studies, four bacterial isolates which showed the dual nature of phosphate solubilisation and nitrogen fixation were selected for further studies. These isolates were designated as TPN1 to TPN4. These isolates were found potent to fix atmospheric nitrogen and also to make available the phosphate present in the soil to plants by exhibiting phosphate solubilisation.

**Phytohormone studies- IAA Production.** IAA is obtained at the stationary phase of the bacterial culture as IAA as the secondary metabolite and varies with species, strains and their culture conditions (Yadav et al., 2010; Gupta et al., 1994). The isolates TPN2 and TPN4 showed higher IAA production at tryptophan concentrations 5µg/ml. The synthesis of auxin in plants and microbes are regulated by tryptophan as it is the main precursor (Vincent, 1970). This variation in concentration determines the PGPR bacterial ability to produce IAA. Previous reports of some rhizosphere isolate studied by Lwin et al. shows 121 µg ml⁻¹ of IAA at a concentration of 0.5 µg ml⁻¹ whereas TPN2 exhibited 97.24 µg ml⁻¹ of IAA production at a concentration of 5 µg ml⁻¹ of tryptophan (Lwin et al., 2012) (Figure 1). The production was indicated by dark pink. IAA synthesis by Pseudomonas putida has been reported as 25.65 µg ml⁻¹ and 48.46 µg ml⁻¹ at a tryptophan concentration of 100 µg ml⁻¹ and 200 µg ml⁻¹ respectively while Bacillus subtilis 16.23 µg ml⁻¹ at 100 µg ml⁻¹ and 36.38 µg ml⁻¹ at 200 µg ml⁻¹ (Yadav et al., 2010).

**Figure 1.** Quantification of IAA produced by the isolates

**Phosphate solubilization.** Phosphate solubilization is another vital characteristic of PGPR bacteria, a mechanism of making available the insoluble phosphates by the release of any
dissolving compounds, extracellular enzymes or release of phosphate on degradation (Rodriguez and Reynaldo 1999). It is a characteristic of members of *Arthrobacter*, *Bacillus*, *Rhizobium*, *Flavobacterium*, *Enterobacter* and few other genera (Khalid et al., 2004). Determination of phosphate solubilization was performed on Pikovskaya’s media and the zone of clearance was noted. Isolates exhibited different degree of clearance with TPN2 been the best of the isolates showing an inhibition of 11.2 mm followed by TPN4 with 9.4 mm. The other two isolates exhibited low inhibition of 3.2 mm and 1.7 mm by TPN1 and TPN3 respectively. Thus, TPN2 and TPN4 were selected for further studies.

**Hydrogen cyanide, catalase and ammonia production.** HCN is one of the metabolites that inhibit the growth of microbes and may also deter plant growth (Gupta et al., 1994; Vincent, 1970). HCN activity was reported negative for TPN4 whereas the isolate TPN2 was positive.

Catalase activity is critical for the regulation of oxidative stress. The isolate TPN2 demonstrated catalase activity but it was absent in TPN4. This adds on to the importance of TPN2 as a biofertilizer.

For the above reasons TPN2 was tested for another PGPR property namely, ammonia production. If produced ammonia helps in inducing plant growth as it is the most common assimilatory form of nitrogen. The ammonia released by a rhizobacterial strain plays a signalling role in interaction between PGPR and plants and also increase the glutamine synthetase activity (Chitra et al., 2002). The isolate was potentially showing ammonia production after 72 hours of incubation. In a similar fashion, Ahmed and Khan (2010) reported the production of ammonia by *Enterobacter asburiae*.

**Phenotypical and genotypical identification of the isolate.** Similar to many reported PGPR bacteria, isolate TPN2 was also identified as a Gram-negative motile rod. All the basic biochemical characteristics of the isolate were determined (as shown in Table 1). The ability of motility becomes beneficial being a PGPR bacterium. The 16S rRNA sequencing is a technique for determining bacterial phylogeny and defining the taxonomy. Therefore, the potential isolate was subjected to 16S rRNA sequencing and the sequence was checked for similarity using BLAST search which displayed 99% similarity with *Enterobacter cloacae*. Based on morphological, biochemical and phylogenetic character similarities, the isolate was thus identified as *Enterobacter cloacae* VITTPN2 and was deposited in GenBank under the accession no: ku951582 (see Figure 2).

### Table 1. Biochemical characterization of TPN2.

<table>
<thead>
<tr>
<th>Test</th>
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</tr>
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<tbody>
<tr>
<td>Morphology</td>
<td>Rod</td>
</tr>
<tr>
<td>Gram stain</td>
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</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red</td>
<td>-</td>
</tr>
<tr>
<td>Voges–Proskauer</td>
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</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>H2S</td>
<td>-</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Glucose utilization</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
</tbody>
</table>

(+ ) presence; (–) absence

**Hydrogen cyanide, catalase and ammonia production.** Our study demonstrates that seeds of the plants appear to have been affected by the PGPR isolate TPN2 as the growth was observed 4 days prior to the untreated control seeds. The PGPR isolate also significantly affected the height of both tomato and Okra plant (as shown in Table 2 and Figure 3). Results indicate that the height of the plant has increased in the PGPR treated plants over un-inoculated control.

The treatment with PGPR isolates considerably augmented the root and shoot length of both tomato and Okra. Root length of tomato and okra was recorded to be 3.50±0.721 cm plant⁻¹ and 5.60±0.361 cm plant⁻¹. The shoot length of tomato was observed to be 7.7±1.908 cm plant⁻¹ whereas in Okra it was 16.00±2.784 cm plant⁻¹ (Figures 4 and 5). In a previous study, the root and shoot elongation was found to be 8.4±1.1 cm plant⁻¹ and 12.2±1.6 cm plant⁻¹ and 6.7±1.2 cm plant⁻¹ for *B.subtilis* (Yadav et al., 2010). The differences can
Isolation and characterization of Rhizobacteria

vary among in vitro set up. Also, TPN2 would require optimization in terms of production of higher amounts of IAA and ammonia for being a better PGPR. On contrary to the control plants, plants treated with TPN2 isolate showed commendable root and shoot elongation. The growth and advancement of the lateral root system were also observed in the system of the okra plant. The increase of shoot and length can be attributed to the overexpression of H-NS genes as reported by English et al. (2010).

Nitrogen fixation is yet another important element for the enhancement of soil fertility. Both free-living, as well as symbionts, are capable of the above due to the presence of nif genes. Members of genera Enterobacter, Acetobacter, Azotobacter and a few more are able to fix nitrogen (Siddiqui, 2006). TPN1, TPN2, TPN3 and TPN4 showed the dual nature of being good nitrogen fixers and phosphate solubilizers. In 2011, Shankar et al. reported the efficacy of Enterobacter cloaca strain GS1 as PGPR by direct mechanisms exhibited by the bacterium whereas in our study we explain both direct and indirect mechanisms involving catalase production (Shankar et al., 2011). Therefore the two isolates although of the same species exhibited different capabilities as PGPR. Our isolate is concluded to have more diverse PGPR characteristics.

Table 2. The root and shoot elongation of tomato plant.

<table>
<thead>
<tr>
<th></th>
<th>Shoot length plant$^1$ (cm)</th>
<th>Root length plant$^1$ (cm)</th>
<th>Total height plant$^1$ (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>5.40±0.100</td>
<td>3.50±0.721</td>
<td>8.43±0.066</td>
</tr>
<tr>
<td>Treated seeds</td>
<td>7.7±1.908</td>
<td>2.80±0.721</td>
<td>10.5±1.24</td>
</tr>
</tbody>
</table>

The values are represented as mean ± SD

Table 3. The root and shoot elongation of okra plant.

<table>
<thead>
<tr>
<th></th>
<th>Shoot length plant$^1$ (cm)</th>
<th>Root length plant$^1$ (cm)</th>
<th>Total height plant$^1$ (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>11.44±0.087</td>
<td>5.10±0.100</td>
<td>16.50±0.015</td>
</tr>
<tr>
<td>Treated seeds</td>
<td>16.00±2.784</td>
<td>5.60±0.361</td>
<td>22.60±1.682</td>
</tr>
</tbody>
</table>

The values are represented as mean ± SD

Figure 2. Molecular phylogenetic tree of TPN2 constructed by Neighbour joining tree with interior branch analysis.
Plant growth promoting rhizobacteria colonize in plant roots have many beneficial roles in plant growth. These may vary with types and nature of the soil and depends on the bacterial species. The isolate TPN2 (Enterobacter cloacae VITTPN2) showed good IAA production, phosphate solubilisation, ammonia production and catalase activity. It also showed good activity in the root and shoot elongation. This shows that TPN2 can be a prospective biofertilizer.

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**In vitro** antimicrobial assessment on lactic acid bacteria isolated from common freshwater fishes

Wai-Wei Chong\(^a\), Crystale Siew-Ying Lim\(^a\), Kok-Song Lai\(^b\), Jiun-Yan Loh\(^*\)

\(^a\)Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, Cheras, 56000 Kuala Lumpur, Malaysia
\(^b\)Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

**Abstract.** Probiotic is well-known as an effective agent to control and manage diseases in aquaculture. Unlike antibiotics and chemotherapeutic agents, probiotic does not trigger the emergence of antibiotic-chemo-resistant bacteria. This study was aimed to isolate, identify and evaluate lactic acid bacteria from intestines of three common food fish, i.e. tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*) and rohu (*Labeo rohita*). Thirty-four lactic acid isolates were isolated and screened for inhibitory effect against fish pathogens e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella enterica*. Positive antagonists were subsequently tested in haemolytic, salt tolerance and bacteriocin-like inhibitory substances (BLIS) assays. Our results showed only three isolates displayed positive inhibitory effect against all four pathogens. These three isolates were classified as \(\gamma\)-haemolytic bacteria. Our results revealed that bacterial isolates (T2.1.2 - *Pediococcus acidilactici* and T2.2.2 - *Lactobacillus fermentum*) isolated from *O. niloticus* (tilapia) showed a better adaptation in the range of 0 - 20 ppt; while, the bacteria isolated from *L. rohita* (R1.1.1 - *P. acidilactici*) could survive up to 35 ppt. These isolates were then identified based on 16S rRNA gene sequences. BLIS data revealed that both *P. acidilactici* and *L. fermentum* isolated from *O. niloticus* and *L. rohita* could suppress the growth of pathogens with cell density as low as \(10^4\) cfu/ml. Our study shows that *P. acidilactici* and *L. fermentum* have the potential to be further explored as biocontrol/probiotic agents in aquaculture.

**Keywords:** aquaculture, lactic acid bacteria, pathogen, probiotic

**INTRODUCTION**

Intensive aquaculture often associated with high frequency of diseases outbreak. Fish pathogens such as *Aeromonas* sp., *Edwardsiella* sp., *Pseudomonas* sp. and *Vibrio* sp. are commonly found to be highly infectious in both freshwater and marine environments (Banerjee and Ray, 2017). The early onset of these symptoms could be associated with fin rot, exopthalmia, and skin ulcers. Infected fish showed a high rate of mortality or stunted growth in the severe stage (Dong et al., 2017; Fečkaninová et al., 2017).

Antibiotics are the common practices used in many animal productions to manage and control diseases. In aquaculture, antibiotics are administered through the feeds or directly into the water. Although antibiotics are effective in treating fish diseases, the rampant use of an antibiotic can cause negative impacts on the host and the environment (Lazado et al., 2015). Antibiotics increase the emergence of antibiotic-
resistant bacteria that can tolerate the use of antibiotics, which will in turn, detrimental to the host’s health. Therefore, higher concentration or different antibiotics are required to control the infection during the onset of the same disease in the future. Furthermore, antibiotic residues indirectly discharge through excretion, can lead to inevitable stress effects on the environmental microbial community.

Probiotics are live and beneficial microorganisms that modulate intestinal microbial flora in the host. The term probiotic in Greek language “pro bios” means “for life” (Gismondo et al., 1998). Probiotics can be originated from a wide range of bacteria from Gram-negative to Gram-positive species. The important criteria of a group of microbes to be considered as probiotic; it should not be causing any pathogenic effect to the host, and it should be able to enhance the bowel/intestinal health (Banerjee and Ray, 2017). Study by Fečkaninová et al. (2017) showed the application of lactic acid probiotics could reduce Aeromonas infection in salmonids, the host-associated symbiotic relationship not only contribute to the diversity of microbial flora in the gut, but it also improves digestion, growth, stress tolerance and regulate nutritional factors of the hosts (Wang et al., 2008; Lazado et al., 2015).

The present study aimed to discover the potential of intestinal lactic acid bacteria in probiotic development for aquaculture species. Intestinal microflora was isolated from three common food fish included tilapia (Oreochromis niloticus), catfish (Clarias gariepinus) and rohu (Labeo rohita). The bacteria isolates were screened through agar well diffusion assay against Salmonella enterica, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa. Hemolytic activities and salt tolerance test was used to evaluate the pathogenicity of the probiotics. After multi-screening tests, the potential probiotic candidates were identified based on molecular approach. Bacteriocin-like inhibitory substances (BLIS) assay was performed to determine the minimum inhibitory effect against the pathogens.

MATERIALS AND METHODS

Sampling of common food fish. Three common food fish (n=6) (Table 1) were purchased from a local fish market at Kuchai Entrepreneurs Park, Kuala Lumpur, Malaysia. Prior to the dissection, the surface body of common food fish was disinfected with 70% (v/v) ethanol. The fish were then dissected using a sterile dissecting kit, incision was made from the anus to post-stomach of the fish to remove the intestinal samples for subsequent processes.

Isolation of intestinal microflora from common food fish. Intestinal samples (1 g) from each fish were homogenized using an electric homogenizer (LabGEN®125, Cole-Parmer, USA) in deMan, Rogosa, and Sharpe (MRS) broth and incubated for 24 h at 37±2°C. Serial dilution was performed (10⁻¹ to 10⁻⁶), and 1 mL from each intestinal sample was spread plated onto MRS agar and incubated for 24 h at 37±2°C. The MRS plates were then incubated at room temperature (26±2°C) for 24 h. Pure cultures were collected from single colonies and established on corresponding fresh agar plates. Individual colonies were taken based on morphological differences and subsequently streaked on MRS agar. Pure bacteria were obtained after three times of plate streaking.

In vitro screening for antagonistic bacteria. Bacterial isolates were screened for their antagonistic activity against E. coli, K. pneumoniae, P. aeruginosa and S. enterica (UCSI laboratory’s collection, Microbiology Lab in South Wing campus, KL) using agar well diffusion method. Supernatants of overnight bacterial culture were collected by centrifugation at 7,500 rpm for 15 min at room temperature using a mini-spin centrifuge (MiniSpin® plus, Eppendorf, China). The supernatant of bacteria was then filtered through 0.45 μm pore size filter (Sortunos, USA) to obtain cell-free supernatant (CFS). An overnight culture of pathogens was added into 20 ml of soft nutrient agar (NA) at 37°C, and mixed before pouring into the Petri dishes. To each agar plate, five wells were prepared using a cork borer with a diameter of 2 cm. Each well was filled with 100 μL of CFS including a blank control of sterile
MRS broth. The plates were then incubated at 37±2°C for 24 h. The experiment was performed in triplicates. Antimicrobial activity was expressed as the diameter of the inhibition zones formed around the wells. Inhibitory strength was defined as: zone of inhibition >20 mm = strong inhibition; 10-20 mm = intermediate/moderate inhibition; and <10 mm = low inhibition (Shokryazdan et al., 2014).

**Haemolytic activity assay.** Bacteria exhibited positive antimicrobial activity were then streaked onto horse blood agar (Thermo Scientific, Malaysia) and incubated at 37±2°C for 24 h. Haemolysis was classified as α-haemolysis, β-haemolysis and γ-haemolysis based on red blood cell lysis patterns. Bacteria isolates with only γ-haemolysis were selected for subsequent study (Loh et al., 2014).

**Salt tolerance assay.** Salt tolerance was determined by incubating γ-haemolytic bacterial isolates in MRS broth containing a various concentration of sodium chloride (NaCl, Merck, Germany). Sodium chloride was supplemented to MRS broth at a different concentration of 0, 5, 10, 15, 20, 25, 30 and 35 ppt. Ten mL of MRS broth was used to culture the bacterial isolates in 15 mL tubes. The experiment was performed in triplicates. Bacterial culture was incubated for 24 h at 37±2°C. Optical density was determined at 600 nm using a UV-spectrophotometer (Eppendorf, Malaysia).

**Table 1.** Specifications of common food fish specimens (n=6).

<table>
<thead>
<tr>
<th>Common Food Fish</th>
<th>Species Name</th>
<th>Weight (g)</th>
<th>Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish</td>
<td>Clarias gariepinus</td>
<td>334±10.0</td>
<td>45.2±3.0</td>
</tr>
<tr>
<td>Rohu</td>
<td>Labeo robita</td>
<td>716±20.2</td>
<td>63.5±4.5</td>
</tr>
<tr>
<td>Tilapia</td>
<td>Oreochromis niloticus</td>
<td>480±12.1</td>
<td>47.8±2.8</td>
</tr>
</tbody>
</table>

**16S rRNA characterization of selected bacterial isolates.** Selected bacterial isolates were cultured in MRS broth at 37±2°C for 24 h prior to DNA extraction. Genomic DNA was extracted using a GF-1 Nucleic Acid extraction kit (Vivantis Tech, Malaysia). The 16S rRNA gene of the extracted DNA was amplified using primers 27f (5’–AGA GTT TGA TCC TGG CTC AG–3’) forward primer and 1492r (5’–GGT TAC CTT GTT ACG ACT T–3’) reverse primer to recover nearly full length of the 16S rRNA (Loh et al., 2017). The polymerase chain reaction (PCR) process was performed on 25 μL reaction mixtures containing 2.5 μL of extracted DNA, 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 2.5 μL of 10X reaction buffer, 0.5 μL of 10 mM dNTPs, 0.5 μL of 5 U polymerase enzyme, 0.5 μL of 25 mM magnesium chloride and 17.5 μL of Milli-Q water. PCR reaction was carried out using the thermal cycler (Eppendorf, Malaysia) with the following parameters: pre-denaturation at 94°C for 5.00 min, followed by 30 cycles of denaturation at 94°C for 0.30 min, annealing at 57°C for 0.45 min, and extension at 72°C for 1.30 min, with a final extension at 72°C for 7.00 min. DNA purification was performed in 50 μL of PCR reaction mixture prepared for each bacteria isolate using MEGAquick-spin™ Total Fragment DNA purification kit (YMS, Korea) prior to bi-directional sequencing using primers 27f and 1492r. After purification, PCR products were sent for sequencing (MyTACG, Malaysia). The results of gene sequences were analyzed using BioEdit 7.0.5.3 and matched with bacterial sequences in the BLAST (NCBI) database.

**BLIS assay.** The bacteriocin-like inhibitory substances (BLIS) assay was performed to determine the minimum inhibitory effect of bacteria isolates on the pathogens. The identified bacterial isolates and pathogens were cultured separately in 30 mL MRS broth and nutrient broth (NB) and subsequently incubated for 24 h at 37±2°C. The intestinal bacterial culture was then adjusted to the densities of 10⁶, 10⁵, 10⁴ and 10³ cfu/ml using a microplate reader (Infinite 200 PRO NanoQuant, Switzerland) at OD₅₆₂. Subsequently, bacterial isolates with various cell densities were streaked onto NA in a single line using a sterile cotton swab. Pathogens at a cell density of 10⁢³ cfu/ml were streaked perpendicularly to the line of the testing bacterial
isolates of different cell densities (Loh et al., 2014). The inoculated plates were then incubated for 24 h at 37±2°C. The experiment was performed in triplicates. Inhibitory lengths were measured and compared with the control (sterile MRS broth).

**Data analysis.** Data were analyzed using statistical analyzing software SPSS version 23. One-way analysis variance (ANOVA) was used to compare the means and Tukey’s (p<0.05) to determine the significance of different bacterial activities in agar well diffusion, salt tolerance, and BLIS assay. Statistical significance was accepted at p<0.05.

**RESULTS**

A total of 34 bacterial isolates were isolated from the intestinal tract of fish specimens. There were 16 isolates from *Clarias gariepinus* (catfish), 10 isolates from *Labeo rohita* (rohu) and eight isolates from *Oreochromis niloticus* (tilapia). Among these isolates, five isolates showed positive antagonistic activity towards the pathogens tested, with one potential antagonist isolated from *C. gariepinus*; one potential antagonist from *L. rohita* and three potential antagonists from *O. niloticus* (Table 2). Out of the five potential antagonists tested for haemolysis. Only three isolates showed γ-haemolytic activity. The other two bacterial isolates showed either α- or β-haemolytic activity. Only γ-haemolytic activity was selected for salt tolerance test. Out of three γ-haemolytic bacterial isolates, two bacterial isolates were originated from *O. niloticus* and one bacterial isolate was isolated from *L. rohita*. None of the bacterial isolates from *C. gariepinus* showed a positive result (γ-haemolysis) (Figure 1). Salt tolerance test revealed that all three bacterial isolates were able to grow in a wide range of salt-rich environment, i.e. 15, 30 and 35 ppt (Table 3). Bacterial isolates with γ-haemolytic activity were characterized using a molecular approach to determine their species. Bacteria isolated from *O. niloticus* (T2.1.2) and *L. rohita* (R1.1.1) showed 98-100% similarity to *Lactobacillus fermentum* (Table 4). *P. acidilactici* (T2.1.2) and *L. fermentum* (T2.2.2) were subsequently selected for BLIS assay in this study. The growth of *E. coli* (10^8 cfu/ml) was significantly inhibited by *P. acidilactici* at 10^4 cfu/ml and 10^7 cfu/ml. For *K. pneumonia*, the inhibitory effect exerted by *P. acidilactici* at 10^6 cfu/ml was recorded the lowest at 5±0.82 mm and the highest at 10^8 cfu/ml (9.67±0.47 mm). While for *P. aeruginosa*, lowest inhibition was found when treated with 10^4 and 10^5 cfu/ml *P. acidilactici*. There is no significant different (p>0.05) of the inhibitory effect showed by different bacterial cell density against the pathogen. On the other hand, different trend was observed in *S. enterica*, moderate inhibition was recorded when pathogen was treated with 10^7 cfu/ml *P. acidilactici* (Table 5). In the case of *L. fermentum*, low inhibition was recorded in *E. coli* treatment, whereby the pathogen showed rather mild suppression in their growth at all concentration. Moderate inhibition was found when *K. pneumoniae* treated with *L. fermentum* at 10^5 cfu/ml (10.67±0.94 mm), and mild inhibitory effect at 10^4 and 10^5 cfu/ml, respectively. Likewise, *P. aeruginosa* and *S. enterica* shared the similar inhibitory trend as in *K. pneumonia*, where moderate inhibition was found when these pathogens were treated with 10^7 cfu/ml *L. fermentum*, both recorded at 12.33±2.10 and 12.67±1.25 mm, respectively. Nonetheless, mild inhibition of the pathogens was still observable when treated with <10^6 cfu/ml *L. fermentum* (Table 6).

![Figure 1](image-url) Haemolytic activities of intestinal bacteria isolated from *C. gariepinus*, *L. rohita* and *O. niloticus*. 

**Figure 1.** Haemolytic activities of intestinal bacteria isolated from *C. gariepinus*, *L. rohita* and *O. niloticus*.
Table 2. Inhibitory effect of potential antagonists against pathogens.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>T2.1.2</th>
<th>T2.2.1</th>
<th>T2.2.2</th>
<th>R1.1.1</th>
<th>C1.2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>1.63±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>1.83±0.047&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>1.63±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>1.57±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13±0.047&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93±0.047&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1.77±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07±0.094&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6±0.082&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. enterica</td>
<td>1.67±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>1.63±0.047&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p<0.05) among the pathogens; “-” indicates no inhibition. “T” in front of the numbering denotes as Tilapia, “R” denotes as rohu, “C” denotes as catfish.

Table 3. Growth performance of bacterial isolates exposed under various NaCl concentrations.

<table>
<thead>
<tr>
<th>Salinity (ppt)</th>
<th>Absorbance (nm) of bacterial isolates</th>
<th>T2.1.2</th>
<th>T2.2.2</th>
<th>R1.1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.592 ± 0.189&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.392 ± 0.037&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.295 ± 0.032&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.653 ± 0.029&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.495 ± 0.038&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.231 ± 0.013&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.503 ± 0.017&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.417 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.216 ± 0.094&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.536 ± 0.006&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.519 ± 0.027&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.312 ± 0.070&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.587 ± 0.031&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.512 ± 0.045&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.247 ± 0.044&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2.415 ± 0.024&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.488 ± 0.077&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.190 ± 0.013&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.339 ± 0.017&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.416 ± 0.022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.151 ± 0.013&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>2.372 ± 0.004&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.419 ± 0.020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.232 ± 0.009&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among different salinities. “T” if front of the numbering denotes as Tilapia, “R” denotes as rohu, “C” denotes as catfish.

Table 4. Bacterial identification through 16S rRNA partial sequences genes.

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate</th>
<th>Host</th>
<th>Bacterial species</th>
<th>Strain</th>
<th>Max. identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>T2.1.2</td>
<td>O. niloticus</td>
<td>Pedicoccus acidilactici</td>
<td>ML96</td>
<td>100%</td>
</tr>
<tr>
<td>2.</td>
<td>T2.2.2</td>
<td>O. niloticus</td>
<td>Lactobacillus fermentum</td>
<td>NWAFU1463</td>
<td>99%</td>
</tr>
<tr>
<td>3.</td>
<td>R1.1.1</td>
<td>L. rohita</td>
<td>Pedicoccus acidilactici</td>
<td>JFP1</td>
<td>98%</td>
</tr>
</tbody>
</table>

Table 5. Inhibitory effect of *Pediococcus acidilactici*.

<table>
<thead>
<tr>
<th>Cell density of bacteria (cfu/ml)</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>S. enterica</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.67±0.471&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5±0.816&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7±1.414&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33±0.471&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.67±1.247&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8±1.633&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7±0.816&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7±0.816&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.67±1.886&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.67±0.471&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.33±2.055&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.67±1.247&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9.67±0.943&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.33±1.700&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.33±0.943&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10±0.816&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among the bacterial cell densities. All pathogenic concentration was set at 10<sup>6</sup> cfu/ml. Zone of inhibition >20 mm = strong inhibition; 10-20 mm = intermediate/moderate inhibition; and <10 mm = low inhibition.

Table 6. Inhibitory effect of *Lactobacillus fermentum*.

<table>
<thead>
<tr>
<th>Cell density of bacteria (CFU/ml)</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>S. enterica</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5±0.816&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.67±2.357&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.67±0.943&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33±0.471&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.67±0.943&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.67±0.943&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33±0.471&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33±0.943&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.33±1.247&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.33±0.471&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.67±0.471&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.67±1.247&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9.67±1.247&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.67±0.943&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.33±2.055&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.67±1.247&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among the bacterial cell densities. All pathogenic concentration was set at 10<sup>6</sup> CFU/ml. Zone of inhibition >20 mm = strong inhibition; 10-20 mm = intermediate/moderate inhibition; and <10 mm = low inhibition.
DISCUSSION

Among 34 bacteria isolates, only five isolates showed weak to intermediate inhibitory activity towards pathogens i.e. E. coli, K. pneumoniae, P. aeruginosa and S. enterica. Although catfish had the highest number of bacteria isolates, however, only one bacteria isolate was found to have weak inhibitory activity towards the pathogens. On the other hand, tilapia possessed the highest number of bacteria isolates that showed an intermediate inhibitory effect against the pathogens.

Haemolytic assay further tested the suitability of the bacterial isolates and their haemolytic properties. Haemolysis is a resultant of red blood cells breakdown caused by pathogenic bacteria (Sowemimo-Coker, 2002). In the present study, of the five bacterial isolates, only three isolates showed γ-haemolysis. Two isolates showed α-haemolysis and none of the bacterial isolate showed β-haemolysis. The haemolytic assay is a safety measure that can reduce/eliminate suspected pathogens during the screening process in probiotic development. Any bacteria displayed α- / β-haemolysis activity would not be considered as they may potentially cause immunological diseases or malfunction in the defence system of fish (Loh et al., 2014).

The salt tolerance level of three bacterial isolates was further tested at 0 - 35 ppt. The assay aimed to understand survivorship of the bacteria in the presence of salt content. The outcome of the assay could further elucidate the feasibility of these probiotic’s application in the marine environment setting. The tolerance level of selected potential probiotics was determined through salt tolerance assay in the study. An increment of turbidity signifying a growing number of bacteria in this case (Walter and Deborah, 1952). According to Wong and Rawls (2012), the intestinal environment of a host is largely dependent on its ambient living condition; which implies that intestinal microbiota composition of fish could be significantly influenced by their living habitat. However, our result showed that microflora isolated from freshwater fish’s intestines could also survive in a broad range of salinity. Two bacterial isolates were identified as P. acidilactici (Strain ML96: O. niloticus; Strain JFP1: L. robota), and one bacterial isolate was L. fermentum (Strain NWAFU1463: O. niloticus) through 16S rRNA sequence identification. In our case, Pediococcus acidilactici (T2.1.2) isolated from O. niloticus (tilapia) showed a better adaptation in the range of 0-20 ppt; however, Lactobacillus fermentum (T2.2.2) isolated from the same species, survived the best at the range of 5 - 20 ppt. On the other hand, P. acidilactici (R1.1.1) isolated from Labeo rohita (rohu) could adapt from 0 - 35 ppt (Table 3). Our result showed that P. acidilactici originated from different host’s intestines could strive at a different level of salinity. It is interesting to note that, these bacterial isolates have no doubt to survive in the environment of higher salinity. Our findings somehow were in the agreement with the findings of Wong and Rawls (2012), where intestinal bacteria could tolerate different salinities like their hosts. For instances, O. niloticus survive in a wide range of salinity e.g. 0 - 25 ppt (Watanabe et al., 1985) while L. robota survive <12 ppt (Islam et al., 2014). Some literates also indicated that certain lactic acid bacteria (LAB) able to tolerate different temperatures, salt contained environment and a wide range of pH (Papadimitriou et al., 2016). According to Justé et al. (2014), Tetragenococcus spp. could survive in the extreme high salt environment (25% (w/v) or 250 ppt). However, high salt concentration could induce osmotic stress in certain LABs e.g. L. delbrueckii subsp. lactis, which can kill the bacteria through autolytic activity (Koch et al., 2007). Thus, it is important to understand the salt tolerance level of proposed probiotic candidates to ensure the effectiveness of its application in various fish species, especially to species inhabiting at intertidal zones or estuaries (Cahill, 1990; Balcázar et al., 2008; Das et al., 2008).

BLIS assay results showed that P. acidilactici and L. fermentum were able to suppress the growth of E. coli, K. pneumoniae, P. aeruginosa and S. enterica, treated with LAB’s cell density as low as 10⁴ cfu/ml within 24 hours of application. Pathogenic concentration was set at 10⁵ cfu/ml in this study. The infectious concentration of pathogen to its host is varying among host’s species, likewise, it also depends on pathogenic species. In general, pathogens that causing fish disease could be as low as 10⁵ cfu/ml (Schmid-Hempel and Franke, 2007). Other BLIS producing bacteria e.g. Bacillus subtilis, B. methylotrophicus, Enterococcus faecium, L. fermentum and L. plantarum are abundantly found in
freshwater fish’s intestines (e.g. common carp, catla, snakehead and tilapia). They are able to suppress the growth of many pathogenic bacteria in aquaculture such as *A. hydrophila*, *P. aeruginosa* and *S. putrefaciens* (Cai et al., 1999; Del’Duca et al., 2013; Mukherjee and Ghosh, 2014). A recent study by Loh et al. (2018), showed that incorporation of LAB e.g. *Lactococcus lactis* subsp. *lactis* in live feed (e.g. Artemia) could significantly reduce the occurrence of edwardsiellosis in fish farming. This evidence further emphasizes the need for probiotic to improve the immune system of fish larvae, in order to reduce the mortality rate during the early stages.

For the past few decades, probiotic has been used for disease prophylactic control and management in aquaculture. Among the probiotic candidates, LABs are the most common probiotics used in fish farming to improve overall health status and to reduce the outbreak of diseases in fish. Two probiotic candidates proposed in the present study i.e. *P. acidilactici* and *L. fermentum* can be considered as a potential probiotic in aquaculture. It is also interesting to note that, *P. acidilactici* was recently suggested as a potential probiotic in the food industry (Barbosa et al., 2015). While, solid shreds of evidence showed that *L. fermentum* isolated from snakehead fish displayed positive inhibitory activity against several fish pathogens such as *A. hydrophila*, *P. aeruginosa* and *S. putrefaciens* (Allameh et al., 2013).

Our preliminary results support the fact that the inclusion of *P. acidilactici* and *L. fermentum* as functional foods in a dietary formulation is viable for the aquaculture industry.

**CONCLUSION**

In conclusion, *P. acidilactici* and *L. fermentum* isolated from food fish able to control against human–fish pathogens with the cell density as low as $10^4$ cfu/ml. Overall, our results indicate that *P. acidilactici* and *L. fermentum* could be potentially used in fish diet formulation. However, in *vivo* tests are still required to validate the efficacy of its application on living hosts.

**ACKNOWLEDGEMENT**

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**REFERENCES**


Extraction and preliminary study of antibacterial compounds of three species of Aspergillus genus

Amina Bramki\textsuperscript{a}, Meriem Frahtia\textsuperscript{b}, Atef Jaouani\textsuperscript{c}, Laid Dahimat\textsuperscript{a}, Noreddine Kacem Chaouche\textsuperscript{a}

\textsuperscript{a}Laboratory of Mycology, Biotechnology and Microbial Activity, University of Mentouri Brothers-Constantine, Algeria
\textsuperscript{b}Laboratory of Medical Analysis, Public Establishment of Local Health, Mila, Algeria
\textsuperscript{c}Laboratory of Microorganisms and Active Biomolecules, University of Tunis El Manar, Tunisia

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Abstract. In the interest of discovering new antibiotic molecules, the antibacterial activity of three fungal strains namely: Aspergillus quadrilineatus, Aspergillus niveus, and Aspergillus wentii isolated from particular ecosystems was sought against six bacterial strains including three with Gram-positive staining (Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis) and three with Gram-negative staining (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae). The results of the agar cylinder technique highlighted that the three fungal strains showed a considerable antibacterial activity. In order to optimize the extraction conditions of the bioactive molecules, five solvents in different polarities were tested, of which chloroform turned out to be the best one. After the selection of this solvent, four culture media of different compositions were used in order to determine the most adequate medium for the production of antibacterial substances. The results revealed that Czapek-dox medium supplemented with yeast extract turned out to be the most favorable one for the production of bioactive molecules from both strains: A. quadrilineatus and A. niveus, while the most suitable medium for the A. wentii strain was Sabouraud. In addition, a study of the antibacterial effect of organic extracts by the Biolog micro-culture system was performed using a range of concentrations. The obtained results revealed that the extracts of the three fungal strains presented a remarkable activity with different concentrations and this, against all the tested bacterial strains. It was recorded only for the three used fungal species, the antibacterial activity was studied for the first time by the Biolog system.

Keywords: antibacterial activity, Aspergillus, bioactive molecules, Biolog

INTRODUCTION
The bacterial resistance to antibiotics is one of the most serious threats to global health today. Every day, new mechanisms of resistance emerge and spread on a global scale, compromising our ability to treat the most common infectious diseases (Azoun, 2016).

In front of this alarming situation, the appropriate use of antibiotics is absolutely necessary to extend the clinical validity of these molecules. However, the long-term solution to counteract microbial resistance is to develop or search for new molecules (Madigan and Martinko, 2007; Saisivam \textit{et al}., 2008; Berger Savin, 2014). In the 1990s, the discovery of bioactive metabolites of microbial origin grew exponentially thanks to technological advances. Unfortunately, among the discovered metabolites, many are analogues of already known molecules, compounds with no antibiotic activity or even minor compounds. The invention of new innovative chemical families becomes scarce (Berdy, 2005). Faced with these emerging needs and multiresistant germs, the
search of new molecules represents a need (Sharma et al., 2005) which cannot be filled either by: the extraction of new derivatives in mutants of listed strains, the realization of new semi-synthetic molecules from known structure, the synthesis of new derivatives or the obtaining of new substances produced by new bacterial or fungal species isolated from explored or less explored ecosystems (Kitouni, 2007). This last path that was adopted during our study.

In addition, fungi are among the best candidates for the production of biologically active secondary metabolites. This kind of microorganisms is responsible for the production of about 22% active molecules with antibacterial effect on an industrial scale (Atoui, 2006). In fact, the genera Aspergillus and Penicillium as well as the species of the order Monilliales constitute the most important reservoirs (Botton et al., 1990).

For this reason, we sought to study the antibacterial activity of Aspergillus genus fungal strains isolated from different ecosystems as producers of active metabolites against resistant bacteria for the purpose of using their bioactive molecules.

MATERIALS AND METHODS

Producing strains. Three strains of Aspergillus genus were used to evaluate their antibacterial activity: Aspergillus quadrilineatus (MH109538) isolated from the soil taken from an arid zone; Laghouat (located at 400 km south of Algiers), Aspergillus niveus (MH109544), and Aspergillus wentii (MH109545) isolated from a thermal soil in the region of Teleghma (located in north-eastern Algeria). It should be noted that isolation and identification steps of fungal strains have been detailed in another article (Bramki et al., 2017).

Demonstration of the antibacterial activity of fungal strains by the agar cylinder technique.
The used microbial support was composed of six bacterial strains, four ATCC strains (American Type Culture Collection), which were: Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Escherichia coli (ATCC, 25922), Pseudomonas aeruginosa (ATCC, 27853), and two clinical strains namely: Enterococcus faecalis and Klebsiella pneumoniae.

Antibacterial tests must be performed from young cultures (18 to 24 h), in the exponential growth phase. The opacity of the bacterial suspensions in sterile physiological water was equivalent to 0.5 McFarland (a bacterial concentration estimated at 10^6 CFU/ml) (Cavalla and Eberlin, 1994).

The fungal strains were seeded on PDA medium (Potato Dextrose Agar). After 14 days of incubation at 28°C, agar cylinders of 6 mm in diameter were removed and deposited on the surface of Mueller-Hinton medium previously seeded following the NCCLS technique (National Committee for Clinical Laboratory Standard) by tested bacteria. The Petri dishes carrying the agar cylinders were placed at 4°C for 4 h, to allow the diffusion of the bioactive substances elaborated by the fungal strains, then incubated at 37°C for 18 to 24 h (Tortorano et al., 1979; Gungi et al., 1983).

Study of the antibacterial activity

Choice of the optimum solvent for the extraction of bioactive molecules

Preparation of extracts. Five solvents of different polarities were tested separately; chloroform, hexane, methanol, butanol and ethyl acetate to extract bioactive substances (Loucif, 2011; Boughachiche, 2012). 500 ml flasks, containing 100 ml of PDB (Potato Dextrose Broth) culture medium, were inoculated with eight disks of a seven days aged culture for each strain. After 14 days of incubation at 28°C (static fermentation), an identical volume of the tested solvent (100 ml) was added to each flask containing the fungal culture. The mixture was crushed using ultra turrax, then filtered on Whatman paper No. 1. The filtrate was transferred to a separatory funnel, then, the organic phase was concentrated by vacuum evaporation using a Rotavapor. (Gengan et al., 1999; Ghorri, 2015). Each fungal extract was dissolved in dimethylsulfoxide (DMSO) in order to obtain a concentration of 100 mg/ml.

Discs technique. To evaluate the antibacterial activity of organic extracts, sterile discs of 6 mm
Antibacterial compounds from Aspergillus genus

in diameter prepared from Wattman paper No. 1, containing 10 μl of organic extracts. After drying, the discs were carefully placed on Petri dishes containing Mueller-Hinton medium previously seeded by the tested bacteria. Before incubation at 37°C, the dishes were left for 2 h at 4°C to allow the diffusion of the bioactive substances (Yamaç and Bilgili, 2006; Hazalin et al., 2009). Results’ reading was done after 18 to 24 h. Any growth of inhibition zone around discs, even of a small diameter, was considered a positive result.

Choice of the optimum medium for the production of bioactive substances. Four liquid culture media of different composition: PDB, Sabouraud, Czapek dox supplemented with 2.5 g/l of yeast extract and MEB (Malt Extract Broth) recommended for the production of secondary metabolites were tested in order to determine the most adequate medium for the production of bioactive molecules. After a static fermentation of 14 days, an extraction of secondary metabolites with chloroform was performed. The preparation and the antibacterial activity test of the obtained extracts have been done as described previously (choice of the solvent) (Mathan et al., 2013). It should be noted that three repetitions were performed for each test, and the diameters of the inhibition zones were measured in millimeter.

Study of the antibacterial activity of organic extracts by the micro-culture system (Biolog). From a concentration of 100 mg/ml (100 mg of extract in 1 ml of DMSO solvent), a successive one-half dilutions have allowed to prepare a range of concentrations ranging from 100 to 1.56 mg/ml (Harrar, 2012). The bacterial suspensions were prepared from cultures aged from 18 to 24 h, and were calibrated using a Biolog turbidimeter to reach a final turbidity of 85% T, then, a volume of 220 μl of Mueller Hinton Broth (supplemented with 1% of « Biolog Redox Dye Mix F » for Gram-positive bacteria and « Biolog Redox Dye Mix A » for Gram-negative bacteria), 15 μl of the diluting extract and the same volume of the bacterial suspension were placed into each well of the microplate.

Both DMSO solvent and bacteria only were performed as negative and positive controls respectively were performed. The microplates were placed in an Omnilog incubator-reader for 24 h at 37°C, and the phenotypic behavior was recorded by a CCD camera (Al-Bayati, 2008; Benslama, 2014; Ghorri, 2015).

Indeed, this test was confirmed by the disc technique on Mueller Hinton Agar, and the discs were impregnated with 15 μl of each extract.

RESULTS AND DISCUSSION

The demonstration of the antibacterial activity of fungal strains by the agar cylinder technique. The antagonism test by the agar cylinder technique showed the ability of the three fungal strains to produce molecules with antibacterial effect. In fact, the strongest inhibition zones were observed against S. aureus with diameters of 33.67 mm, 33 mm, and 31.33 mm for the three fungal strains A. quadrilineatus, A. niveus, and A. wentii respectively. In contrast, E. faecalis showed a sensitivity only with A. quadrilineatus with a diameter of 20 mm. On the other hand, no inhibition zone was observed with the four tested strains B. subtilis, E. coli, P. aeruginosa, and K. pneumoniae (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter of inhibition zones (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. quadrilineatus</td>
</tr>
<tr>
<td>S. aureus</td>
<td>33.67±6.03</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>20.00±2.00</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>-</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>-</td>
</tr>
</tbody>
</table>

* (-): Diameter of the inhibition zone <6 mm.
Our results agreed with those of Irobi et al. in 2000, whereby the *A. quadrilineatus* fungal strain has a considerable antibacterial effect against Gram-positive bacteria. Abdelaziz in 2006, also showed that *Aspergillus* fungal strains had a considerable antibacterial effect against the tested bacteria. However, *Aspergillus* species are known by their production of antibacterial substances (Maria et al., 2005, Madki et al., 2010). These biologically active secondary metabolites are synthesized at the end of growth (Attalah and Kacem-chaouche, 1992).

**Study of antibacterial activity**

**Choice of the optimum solvent for the extraction of bioactive molecules.** The obtained results from antibacterial test showed that all the organic extracts have an antibacterial activity on at least one of the tested bacteria, with the exception of hexane extract of *A. quadrilineatus* strain, and that the most significant effect was especially obtained from the chloroform extracts, followed by ethyl acetate and butanol (Figures 1, 2, and 3).

Figure 1. Antibacterial activity of organic extracts of the fungal strain *A. quadrilineatus*.

Figure 2. Antibacterial activity of organic extracts of the fungal strain *A. niveus*.

Figure 3. Antibacterial activity of organic extracts of the fungal strain *A. wentii*.

Regarding the chloroform extracts, considerable inhibition zones were observed by *A. niveus* strain extract against both *B. subtilis* and *S. aureus* with diameters of 25 mm and 18.33 mm, in that order. As well as, *A. quadrilineatus* strain extract exhibited a remarkable effect on *S. aureus* bacterium with an inhibition zone of 16.67 mm.

The same results were detected by ethyl acetate and butanol extracts of *A. niveus* fungal strain, where the average of inhibition zones’ diameters ranged from 15 mm to 20 mm against the same bacterial strains: *B. subtilis* and *S. aureus*.

These results revealed that the chloroform was the best solvent for the extraction of fungal metabolites, especially that have an antibacterial effect, is in agreement with the results of Kitouni’s and Loucif’s results in 2007 and 2011, successively, who found that chloroform is among the best solvents used for the extraction of bioactive molecules. However, this solvent is often used for the extraction of secondary metabolites (Kosalec et al., 2004; Abdelaziz, 2006, Praveena and Padmini, 2011).

The appearance of the antibacterial activity by polar solvents (methanol and butanol), solvents of intermediate polarity (chloroform and ethyl acetate) and another non-polar (hexane), assumes that it is either several molecules of different polarity (each soluble in a different solvent) or one or more molecules soluble in various solvents. This last hypothesis is more probable since, according to Guernet and Hamon (1981), antibiotics’ structure is generally complex, containing polar and non-polar characters, resulting in an affinity for very diverse solvents.
Choice of the optimum medium for the production of bioactive substances. The antibacterial effect test of each fungal strain showed that the diameters of the inhibition zones vary from one culture medium to another and from one tested bacterium to another.

For the *A. quadrilineatus* fungal strain, the represented results in Figure 4 showed that the most important zones of inhibition were obtained by the disks impregnated with molecules from Czapek-dox medium, with diameters of 17.67 mm against *S. aureus* bacterium and 15 mm against *E. faecalis* bacterium, followed by the PDB medium with diameters of 16.67 mm and 13.67 mm against the bacteria *S. aureus* and *E. faecalis*, respectively.

Regarding the *A. niveus* fungal strain, the results in Figure 5 proved Czapek-dox medium as the most suitable medium for the production of antibacterial metabolites, followed by the PDB medium, in which the highest diameters against the two bacterial strains *B. subtilis* and *S. aureus* were shown.

In effect, Czapek-dox liquid medium supplemented with yeast extract was chosen as a specific medium for optimal production of secondary metabolites (Pamel *et al.*, 2010). In addition, this medium contains yeast extract and sodium nitrate, which are good sources of nitrogen, adequate for the production of antibiotics; it also contains sucrose as a carbon source as well as mineral salts (Pandey *et al.*, 2005).

However, the extract of the *A. wentii* fungal strain, coming from Sabouraud medium, reacted differently compared to other media, giving more distinct results. Moreover, considerable zones of inhibition: 24.33 mm, 20.33 mm, and 18.67 mm in diameter were observed against the three bacterial strains: *S. aureus*, *B. subtilis*, and *E. faecalis*, respectively (Figure 6).

The best performance of Sabouraud's medium compared to other media can be explained by the presence of peptone, which is a source difficultly metabolized and therefore favors the production of antibiotics (Voelker and Altaba, 2001).

Several studies have shown that the nature of carbon, nitrogen, and the mineral sources of culture media influences enormously the production capacity of antibiotics in fungi. Furthermore, Boussaber *et al*. in 2012, showed that the antibacterial activity of the actinomycete strains depends mainly on the tested bacteria and the composition of the culture media.
strains presented a remarkable activity with different concentrations, against all the tested bacteria.

The results in Figures 7, 9, and 11 showed that the chloroformic solubilized extracts in DMSO with the concentrations 100, 50, and 25 mg/ml could almost inhibit the total development of the tested bacterial cells, unlike positive control (bacteria only) and negative control (DMSO + bacteria) that demonstrated a good development in wells.

Otherwise, the results mentioned in Figures 8, 10, and 12 corresponding to the concentrations of the extracts ranged from 12.5 to 1.56 mg/ml, indicated a diminution in the antibacterial effect during the decrease of the extracts’ concentrations with all the tested bacteria.

However, the antagonism test performed by the discs imbibed with the fungal extracts confirmed the Biolog test (Tables 2, 3, and 4).

In regards to the tested germs, Gram-positive bacteria appear to be more sensitive to the bioactive molecules of the three fungal strains in comparison with Gram-negative bacteria. This is related to the results obtained by Hasavada et al. (2006), Atta et al. (2009), and Prabavathy and Nachiyar (2012). These results can be explained by the fact that these two groups of microorganisms differ morphologically (Kumara et al., 2010).

Figure 7. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of A. quadrilineatus strain with different concentrations. 1-2: test bacteria (2 repetitions); 3: the bacterium with DMSO; 4-12: the different concentrations (from 100 to 25 mg/ml); A-F: the different bacterial strains.

Figure 8. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of A. quadrilineatus strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Figure 9. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of A. niveus strain with different concentrations. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Figure 10. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of A. niveus strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.
Figure 11. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. wentii* strain with different concentrations. 1-2: test bacteria (2 repetitions); 3: the bacterium with DMSO; 4-12: the different concentrations (from 100 to 25 mg/ml); A-F: the different bacterial strains.

Figure 12. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. wentii* strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Table 2. Effect of different concentrations of chloroformic extract of *A. quadrilineatus* fungal strain on the test bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>100 mg/ml</th>
<th>50 mg/ml</th>
<th>25 mg/ml</th>
<th>12.5 mg/ml</th>
<th>6.25 mg/ml</th>
<th>3.125 mg/ml</th>
<th>1.56 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>17.00±1.41</td>
<td>14.00±1.00</td>
<td>12.50±0.71</td>
<td>10.50±0.71</td>
<td>9.00±0.00</td>
<td>8.00±0.00</td>
<td>7.00±0.00</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>14.00±1.41</td>
<td>12.50±0.71</td>
<td>11.33±0.58</td>
<td>11.00±0.00</td>
<td>10.00±0.00</td>
<td>9.50±0.71</td>
<td>8.00±0.00</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>14.50±2.12</td>
<td>12.50±0.71</td>
<td>12.00±0.00</td>
<td>11.50±0.71</td>
<td>11.00±0.00</td>
<td>10.00±0.00</td>
<td>9.33±0.58</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>15.50±0.71</td>
<td>15.00±0.00</td>
<td>13.50±0.71</td>
<td>13.00±1.41</td>
<td>10.00±0.00</td>
<td>9.33±0.58</td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>9.50±0.71</td>
<td>8.33±0.58</td>
<td>7.00±0.00</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>K. pneumoniae</em></td>
<td>10.00±1.41</td>
<td>9.50±0.71</td>
<td>9.33±0.58</td>
<td>8.00±0.00</td>
<td>8.00±0.00</td>
<td>7.00±0.00</td>
<td>-</td>
</tr>
</tbody>
</table>

* (·): Diameter of inhibition zone ≤ 6 mm

Table 3. Effect of different concentrations of chloroformic extract of *A. niveus* fungal strain on the test bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>100 mg/ml</th>
<th>50 mg/ml</th>
<th>25 mg/ml</th>
<th>12.5 mg/ml</th>
<th>6.25 mg/ml</th>
<th>3.125 mg/ml</th>
<th>1.56 mg/ml</th>
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<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>19.00±1.41</td>
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<td>12.50±0.71</td>
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</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>26.50±2.12</td>
<td>17.50±0.71</td>
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<td>10.50±0.71</td>
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<td>7.50±0.71</td>
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<tr>
<td><em>E. faecalis</em></td>
<td>16.50±0.71</td>
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<tr>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>10.00±0.00</td>
<td>9.50±0.71</td>
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<td>7.00±0.00</td>
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<tr>
<td><em>K. pneumoniae</em></td>
<td>11.33±0.58</td>
<td>10.00±0.00</td>
<td>9.50±0.71</td>
<td>9.00±0.00</td>
<td>7.50±0.71</td>
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</tr>
</tbody>
</table>

* (·): Diameter of inhibition zone ≤ 6 mm

Table 4. Effect of different concentrations of chloroformic extract of *A. wentii* fungal strain on the test bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>100 mg/ml</th>
<th>50 mg/ml</th>
<th>25 mg/ml</th>
<th>12.5 mg/ml</th>
<th>6.25 mg/ml</th>
<th>3.125 mg/ml</th>
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</tr>
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<td>12.33±0.58</td>
<td>11.00±0.00</td>
<td>11.00±0.00</td>
<td>9.50±0.71</td>
<td>7.50±0.71</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>14.50±2.12</td>
<td>13.00±1.41</td>
<td>11.50±0.71</td>
<td>11.00±1.41</td>
<td>10.02±0.00</td>
<td>8.50±0.71</td>
<td>7.00±0.00</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>16.50±0.71</td>
<td>14.00±0.00</td>
<td>13.33±0.58</td>
<td>11.50±0.71</td>
<td>8.00±0.00</td>
<td>7.00±0.00</td>
<td>7.00±0.00</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>13.00±1.41</td>
<td>11.50±0.71</td>
<td>11.00±0.00</td>
<td>11.00±0.00</td>
<td>10.00±0.00</td>
<td>8.33±0.58</td>
<td>8.00±0.00</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>10.00±0.00</td>
<td>9.33±0.58</td>
<td>8.00±1.41</td>
<td>8.00±0.00</td>
<td>7.00±0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>13.00±1.41</td>
<td>10.00±0.00</td>
<td>9.33±0.58</td>
<td>9.00±0.00</td>
<td>9.00±0.00</td>
<td>8.00±0.00</td>
<td>7.50±0.71</td>
</tr>
</tbody>
</table>

* (·): Diameter of inhibition zone ≤ 6 mm
CONCLUSION

In the context of research for new antibiotic molecules, three fungal strains of Aspergillus genus were tested against six bacterial strains. The highlighting test revealed that the three fungal strains have the ability to produce antibacterial molecules. In order to optimize the extraction conditions of these bioactive molecules, five solvents with different polarities were tested, in which chloroform turned out to be the best solvent. Fermentation on different media has shown that the nature of carbon, nitrogen, and mineral sources of the culture media influences enormously the antibiotics' production capacity in fungi. Therefore, the research perspectives would consist in completing the characterization, the purification and the identification of these molecules, as well as the optimization of their biosynthesis for medical purposes.

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Characterization of phenol-degrading fungi isolated from industrial waste water in Malaysia

Nadila Hanafeea, Nor ‘Azzah Mohd Sallehb, Siti Aqlima Ahmadb, Wan Zuhainis Saada, Mohd Termizi Yusofb*

aDepartment of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
bDepartment of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Abstract. Microorganisms have the ability to degrade phenol. However, in Malaysia, there are lack of study on indigenous microorganisms (fungi) that have the ability to degrade phenol. A total of 141 phenol-degrading fungi isolates were isolated from soil and water samples collected from various industrial areas located in Malaysia. The fungi isolate N12 P6C3 was chosen based on its high efficiency in degrading phenol. The fungi isolate N12 P6C3 isolated from a heavy metal factory, Dungun, Terengganu was able to degrade 700 mg/L of phenol within 6 days and the mycelium growth had increased to 0.25 g. The phylogenetic tree based on the ITS sequence analysis confirmed that the fungal identity was closely related to Penicillium janthinellum strain ATCC 4845. The optimum conditions of this fungus to degrade phenol was attained at temperature of 35°C, ammonium sulphate at 3 g/L, 0.05 g/L of sodium chloride, and pH 6. The ability of P. janthinellum strain N12 P6C3 in the degradation of phenol may provide additional knowledge on locally isolated phenol-degrading fungi which could contribute towards phenol waste management in Malaysia.

Keywords: biodegradation, fungi, phenol

INTRODUCTION

Environmental pollution is one of the critical problems the world is facing today. Rapid industrial growth, urbanization, as well as increasing of hazardous and toxic waste discharged, eventually leads to the increasing of xenobiotic levels in the environment (Gami et al., 2014; Javed and Usmani, 2015). Based on reports by Agency for Toxic Substances and Disease Registry, phenol is one of the major industrial chemicals found in waste effluent which can be present in the environment through natural or chemical processes (ATSDR, 2008).

Phenol is an aromatic compound that is widely distributed as environmental pollutants due to their existence in the waste effluents of many industrial processes including petroleum refineries, pharmaceuticals, paper manufacture, textiles, plastics, dyes, and phenolic resin industries (Santos and Linardi, 2004; Leitão, 2009; Wang et al., 2010). Phenols can also naturally present in decomposition of organic materials or in coal (Basha et al., 2010). Excessive level of phenolic compounds in the environment leads to phenol’s accumulation, affecting groundwater and
soil quality (Michalowicz and Duda, 2007; Panagos et al., 2013).

Distributions of phenol provide a great impact to the environment and human beings. Even at low concentrations, phenol can be toxic and lethal to aquatic organisms (Rittmann and McCarty, 2001). Phenol is rapidly absorbed by human body through inhalation, ingestion, and dermal contact (HPA, 2007). Exposure to phenol can cause irritation to the eye, severe skin damage, serious gastrointestinal damage, cardiovascular disease, and even death (ATSDR, 2008). Therefore, priority and great concern have been placed in these issues, toward the exposure of phenol and the removal of phenol effluent from the ecosystems (Barlow et al., 2007).

Various treatment techniques have been developed for phenol removal including chemical, biological, and physical methods. Solvent extraction, chemical oxidation, adsorption, and incineration have been widely applied, but all these methods are very costly and most probably will produce hazardous by products (Basha et al., 2010; Bui et al., 2012; Thappu et al., 2012). This is why biological approach, biodegradation is preferred to degrade phenol since it is environmentally friendly and cost effective (Wang et al., 2010).

A number of microorganisms including bacteria and fungi have the ability to utilize phenol as their carbon source for growth (Chen et al., 2006). Most of the phenol-degrading microorganisms reported were bacteria including Acinetobacter spp., Pseudomonas spp., Bacillus spp., and Rhodococcus spp. (Ahmad et al., 2011; Sridevi et al., 2011; Mahiuddin et al., 2012; Aravindhan et al., 2014; Hasan and Jabeen, 2015). Besides bacteria, fungi also have been reported as phenol degrader. Some of examples are Aspergillus fumigatus, Fusarium flociferum, Aspergillus awamori, Penicillium chrysogenum, Trichosporum cutaneum, and Candida tropicalis (Mendonça et al., 2004; Stoilova et al., 2006; Leitão et al., 2007; Tuah et al., 2009; Ravikumar et al., 2011).

Phenol-degrading microorganisms may utilize phenol in two pathways either through aerobic or anaerobic pathway (Haiyin et al., 2007; Tuah et al., 2009; Sarwade and Gawai, 2014). The absence or presence of oxygen is very important in determining the fate of phenol biodegradation pathway (Sridevi et al., 2012). In aerobic condition, oxygen is used by the enzyme phenol hydroxylase to produce catechol. Depending on the microorganisms involved, the resulting catechol can be degraded into two pathways either ortho or meta pathway. In the ortho pathway, the enzyme catechol 1, 2-dioxygenase transforms catechol to cis, cis muconate whereas in the meta pathway, the enzyme catechol 2, 3-dioxygenase convert catechol to 2-hydroxymuconic semialdehyde (van Schie and Young, 2000; Tuah et al., 2009; Supriya and Neehar, 2014). The products for both pathways are further incorporated into the Krebs cycle (Soudi and Kolahchi, 2011).

In contrast to bacteria, fungi are able to adapt easily to the ecosystem and able to grow at extreme condition (Atagana, 2004; Stoilova et al., 2008). However, in Malaysia, the investigation on phenol-degrading fungi is poorly reported. Therefore, this study was conducted to provide preliminary information on phenol-degrading fungi in Malaysia.

MATERIALS AND METHODS

Isolation and screening of phenol-degrading fungi. Water and soil samples were collected from various industrial areas located in Selangor, Kedah and Terengganu, Malaysia. The water and soil samples were collected from 10-20 cm from the soil and water surface. Aseptically the samples were transferred into sterile polypropylene tubes and stored at 4°C. The samples were diluted in 10 mL of sterile distilled water and were grown in Liquid Mineral Salt medium (LMS); made up of 0.5 g MgSO₄.7H₂O, 11.8 g KH₂PO₄, 2.3 g K₂HPO₄, 0.05 g CuSO₄, 0.05 g NaCl, 0.25 g NH₄Cl, 0.01 g MnSO₄, 0.1 g FeSO₄ and 0.01 g ZnSO₄ (Cai et al., 2007). All the chemicals were dissolved in distilled water, pH to 6 and brought up to 1 L. Appropriate amount of phenol and 100 μg/mL of penicillin-streptomycin antibiotic were added to the autoclave’s medium prior to inoculation. The cultures were incubated at room temperature for 3 days.

Sensitivity of phenol-degrading fungi. The ability of each isolate to utilize phenol was tested at different phenol concentrations. Concentration
of phenol was gradually increased from (mg/L) 100, 300, 500, and up to 700. Mycelium of the isolate was homogenized and incubated into Potato Dextrose Broth (PDB) for 24 h at 125 rpm under room temperature. After 24 h, 0.1 g of fungal mycelium was harvested and incubated into 50 mL LMS solution supplemented with various concentrations of phenol at 125 rpm under room temperature. All samples were periodically taken for dry weight measurement and phenol degradation rate analysis. Mycelia from LMS media were filtered and washed with sterile distilled water for several times. Mycelia obtained were then kept in a small pack of aluminium foil and dried at 60°C. The dry weight of the mycelia was measured by subtracting the initial weight of mycelia and aluminium foil. 4-amino antipyrine colorimetric method was used for phenol degradation rate analysis (APHA, 1998). All experiments were carried out in three replicates.

**Identification of phenol-degrading fungi.** DNA extraction was done as described by Raeder and Broda with some modification (Raeder and Broda, 1985). The extracted genomic DNA was amplified using Internal Transcribed Spacer (ITS) primers, ITS1 (5'TCCGTAGGGAACCTGCGG-3') and ITS4 (5'TCCTCCGGCTTATGATATGC-3') (White et al., 1990; Pryce et al., 2006). The PCR reaction mixture consisted of 25 µL of 2X Taq master mix (Vivantis Technologies), 19 µL of free nuclease water, 2 µL of 0.5 µM of each primer, and 2 µL of DNA template. The reaction was performed in a thermal cycler under the following temperature: denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 30 s, and primer extension at 72°C for 25 s, with the penultimate step of 72°C for 10 s and lastly cooling at 4°C. DNA sequence was analyzed using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.gov). The sequence was aligned using CLUSTAL W in Molecular Evolutionary Genetics Analysis version 6 (MEGA6). Phylogenetic tree was constructed using Neighbour-Joining method with bootstrap value replication of 1000.

**Fungal growth and phenol degradation activity of the selected isolates.** To study the physiological factors affecting the growth and phenol degradation of the selected isolate, different temperature (10, 15, 20, 25, 30, 35, 40, 45, 50, and 55°C), nitrogen source (ammonium nitrate, ammonium sulphate, ammonium chloride, sodium nitrate, serine, isoleucine, valine, and phenylalanine), concentration of nitrogen source (0, 0.25, 0.5, 1, 2, 3, 4, and 5 g/L), sodium chloride (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1 g/L) and pH range of 4.0 – 9.0 were investigated. Fungal isolate (0.1 g) was inoculated in 50 mL LMS and incubated on an orbital shaker at 125 rpm for 2 days. The degradation of phenol was detected using 4-AAP colorimetric method and the fungal growth was measured by measuring the dry weight of the mycelium.

**Statistical analysis.** All data obtained were subjected to statistical analysis. The SPSS 17.0 software was used for the analysis. Comparison among the groups was performed using one-way analysis of variance (ANOVA) with post hoc analysis by Tukey’s test (Haynes, 2013).

**RESULTS AND DISCUSSION**

**Isolation and screening for phenol-degrading fungi.** A total of 141 phenol-degrading fungi were isolated from soil and water samples collected from various industrial areas located in Malaysia. Most samples were obtained from charcoal factories, heavy metal waste sites, drains, sludges, agricultural sites, and oil contaminated areas. Sixteen fungal isolates were selected for further investigation based on their ability to degrade phenol (Table 1). All isolates were grown on LMS agar plate supplemented with phenol as the sole carbon source. All 16 isolates of phenol-degrading fungi were assessed daily for phenol degradation (100 mg/L phenol) in LMS. Among the 16 isolates, 6 isolates (N7 P1C1, N5 P2C2, N3 P2C2, DD P1C2, N12 P6C3 and N12 P4C1) were able to degrade 100% phenol within 2 days (Figure 1a). Isolate N4 P2C1 consumes phenol completely in 4 days while isolates 3 LP2, I LP1, N15 P2C1 and N2 P9C1 required 6 days to completely degrade 100 mg/L of phenol. Isolates N4 P2C1 and N3 P2C1 only able to degrade less than 30% of phenol at the end of incubation period (day 8). Growth rate of each fungal isolates was recorded.
(Figure 1b). The mycelia cell increased as the phenol concentration started to decrease. This proved phenol was being utilized as carbon source for cellular growth (Wang et al., 2010).

Six fungal isolates were further analyzed for phenol tolerance at different phenol concentrations (300, 500, and 700 mg/L). At 300 mg/L of phenol, all isolates required 3 days to completely degrade the phenol except for isolate N3 P2C2 which needed 4 days (Figure 2a). While at 500 mg/L phenol, only two isolates (N12 P6C3 and DD P1C2) were able to degrade 100% phenol within 4 days of incubation time (Figure 2b). As the phenol concentration increased to 700 mg/L, only isolate N12 P6C3 was able to completely degrade 700 mg/L of phenol within 6 days (p<0.05) compared to other isolates (Figure 2c). While isolates DD P1C2 and N7 P1C1 required 7 days to completely degrade 700 mg/L of phenol. The least potent fungi were isolates N5 P2C2, N12 P4C1, and N3 P2C2 which degraded 2 - 20% of phenol at the end of incubation time. The mycelia dry weight had increased to 0.25 g for 700 mg/L of phenol concentration (Figure 3). Isolate N12 P6C3 was further analysed due to its efficiency in degrading phenol compared to other isolates. The results show that the higher phenol concentration, the more time required to completely degrade phenol and also a longer lag phase was observed (Figure 2) (Stoilova et al., 2006; Wang et al., 2010; Supriya and Neehar, 2014).

Figure 1. (a) Phenol degradation of 16 fungal isolates grown in LMS solution supplemented with 100 mg/L of phenol for 8 days at 125 rpm under room temperature and (b) dry weight of 16 fungal isolates within 8 days. Each point represents the mean of triplicate ± SD.

Figure 2. Phenol degradation of 6 fungal isolates: N12 P6C3, DD P1C2, N7 P1C1, N5 P2C2, N12 P4C1, and N3 P2C2 incubated in LMS supplemented with various phenol concentration: (a) 300 mg/L, (b) 500 mg/L and (c) 700 mg/L. Values shown are mean ± SD, n=3.
Characterization of phenol-degrading fungi

Figure 3. Dry weight of 6 fungal isolates: (—) N12 P6C3, (—) DD P1C2, (—) N7 P1C1, (—) N5 P2C2, (—) N12 P4C1, and (—) N3 P2C2 incubated in LMS supplemented with various phenol concentration: (a) 300 mg/L, (b) 500 mg/L and (c) 700 mg/L. Values shown are the mean ± SD, n=3.

Optimization of conditions for growth and phenol degradation of Penicillium janthinellum strain N12 P6C3. The effect of temperature on the growth of Penicillium janthinellum strain N12 P6C3 in the presence of 300 mg/L phenol was studied at different temperatures ranging from 10 to 60°C. The mycelial growth and the phenol degradation gradually increased as the temperature increased until it reached an optimum temperature, 35°C (p<0.05) (Figure 6). The mycelial growth and phenol degradation ability of the fungus decrease at 40°C and ceased at 60°C. Supriya and Neehar (2014) reported similar observation in Aspergillus niger where the optimum temperature for the degradation of phenol was recorded at 35°C and the growth decreases drastically at low (25°C) and high (60°C) temperature. The growth and phenol degradation rate decrease at low temperature due to the delayed activity in cold conditions. While at high temperature, the enzyme responsible for phenol degradation has been denatured, thus exhibit lower growth rate (El-Naas et al., 2009).

Ammonium sulphate appeared to be the best nitrogen source for P. janthinellum strain N12 P6C3 with 73.4% phenol degradation (Figure 7a). Ammonium sulphate gave the highest phenol degradation activity and fungal growth compared to other nitrogen sources (p<0.05), followed by ammonium chloride and ammonium nitrate. The contribution of nitrogen sources leads to faster phenol degradation rate and increases in cell biomass. It is probable that the simultaneous metabolization of these nutrients with phenol allows the cells to overcome the inhibition effect of fungal growth caused by phenol (Neumann et al., 2004; Khleifat, 2006). Figure 7b shows the effects of different concentration of ammonium sulphate ranges from 0-5 g/L on phenol degradation and fungal growth. The optimum concentration of ammonium sulphate for the highest phenol degradation activity was recorded at 3 g/L with 89.9% degradation (p<0.05).
Table 1. Growth of fungal isolates on LMS supplemented with 100 mg/L of phenol for 3 days at room temperature.

<table>
<thead>
<tr>
<th>No</th>
<th>Location</th>
<th>Isolates</th>
<th>Diameter of mycelia (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Charcoal factory, Dungun, Terengganu</td>
<td>N7 P1C1</td>
<td>2.633±0.115e</td>
</tr>
<tr>
<td>2</td>
<td>Charcoal factory, Dungun, Terengganu</td>
<td>N5 P2C2</td>
<td>2.193±0.210de</td>
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<tr>
<td>3</td>
<td>Dungun, Terengganu</td>
<td>N3 P2C2</td>
<td>2.200±0.100de</td>
</tr>
<tr>
<td>4</td>
<td>Bangi, Selangor</td>
<td>DD P1C2</td>
<td>2.733±0.058e</td>
</tr>
<tr>
<td>5</td>
<td>Awie Metal, Dungun, Terengganu</td>
<td>N12 P6C3</td>
<td>2.667±0.208e</td>
</tr>
<tr>
<td>6</td>
<td>Awie Metal, Dungun, Terengganu</td>
<td>N12 P4C1</td>
<td>2.167±0.321d</td>
</tr>
<tr>
<td>7</td>
<td>Charcoal factory, Dungun, Terengganu</td>
<td>N7 P3C1</td>
<td>1.567±0.208bc</td>
</tr>
<tr>
<td>8</td>
<td>Serdang, Selangor</td>
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</tr>
<tr>
<td>9</td>
<td>Klang River</td>
<td>3 LP2</td>
<td>1.533±0.153b</td>
</tr>
<tr>
<td>10</td>
<td>Penang, Kedah</td>
<td>U3 P1C3</td>
<td>1.167±0.208ab</td>
</tr>
<tr>
<td>11</td>
<td>Shah Alam, Selangor</td>
<td>1 LP1</td>
<td>1.200±0.200ab</td>
</tr>
<tr>
<td>12</td>
<td>Charcoal factory, Dungun, Terengganu</td>
<td>N4 P2C1</td>
<td>1.667±0.153bcd</td>
</tr>
<tr>
<td>13</td>
<td>Awie Metal, Dungun, Terengganu</td>
<td>N15 P2C1</td>
<td>0.933±0.231a</td>
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<td>14</td>
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<td>N3 P2C1</td>
<td>1.833±0.153cd</td>
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<tr>
<td>15</td>
<td>Dungun, Terengganu</td>
<td>N2 P9C1</td>
<td>1.700±0.200bcd</td>
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<td>16</td>
<td>Awie Metal, Dungun Terengganu</td>
<td>N12 P7C1</td>
<td>1.333±0.115abc</td>
</tr>
</tbody>
</table>

Note: Values are mean of triplicate ± standard deviation.
*Mean values with different superscripts are significantly different (p<0.05).

Figure 5. A phylogenetic tree (neighbor-joining method) showing genetic relationship between fungal isolate N12 P6C3 and other related reference fungi based on the ITS sequence analysis. Species name are followed by the accession numbers of their ITS region sequences. *Fusarium oxysporum* strain ATCC 52434 is the outgroup.
Characterization of phenol-degrading fungi

Figure 6. The effect of temperatures on the growth and phenol degradation of *Penicillium janthinellum* strain N12 P6C3. The isolate was grown in LMS supplemented with 300 mg/L phenol incubated in orbital shaker (125 rpm) for 2 days. Phenol degradation rate was determined using 4-AAP assay. Data represents mean ± SD, n=3.

Figure 7. Phenol degradation activity and growth of *Penicillium janthinellum* strain N12 P6C3. (a) Phenol degradation activity and growth in various nitrogen sources; (b) phenol degradation activity and growth in various ammonium sulphate concentrations. Data represents mean ± SD, n=3.

The effect of various concentration of NaCl on growth of strain N12 P6C3 was observed at 0 to 1 g/L (Figure 8). The results show *P. janthinellum* strain N12 P6C3 grew best at 0.05 g/L NaCl and was able to degrade 88% of phenol after 2 days of incubation compared to other concentrations (p<0.05). The reduction of growth and phenol degradation were recorded at high NaCl concentrations. This indicates *P. janthinellum* strain N12 P6C3 requires low amount of NaCl for their optimum growth. High NaCl concentration could affect the degradation of phenol due to osmotic stress to the fungal growth which eventually decreases the phenol degradation rates (Annadurai et al., 2008).

The effect of pH on the growth and degradation of phenol by *P. janthinellum* strain N12 P6C3 was studied using an overlapping buffer system consisting of acetate, phosphate, and Tris-HCl buffer at the pH ranging from 4.0-9.0 (Ahmad et al., 2011). Overlapping buffer system was employed to cancel out the different effect of buffers on fungal growth and phenol degradation (Ahmad, 2012). The results (Figure 9) show the fungus able to grow at pH ranging from 5.5-6.5 (p<0.05) in phosphate buffer. The fungal growth increased with the increased of pH and peaked at pH 6. The highest degradation rate was achieved at pH 6 with 88.7% phenol degradation (p<0.05), indicating *P. janthinellum* strain N12 P6C3 prefer slightly acidic condition. The growth of this fungus and phenol degradation rate was dramatically decreased at pH using acetate and Tris-HCl buffers. Extremely low or high pH usually leads to loss of activity for most of the microbial enzyme. Enzymes produced by microorganisms are significantly affected by the change in pH as it is a crucial factor in the stability of the enzyme and can affect the solubility of
enzymatic compounds (Banerjee and Ghoshal, 2010).

![Graph](image)

**Figure 9.** The effect of pH on phenol degradation activity and growth of *Penicillium janthinellum* strain N12 P6C3 using three overlapping buffers (---) acetate, (---) phosphate and (---) tris-HCl buffers. (a) Phenol degradation activity; (b) fungal growth. Data represents mean ± SD, n=3.

**CONCLUSION**

A total of 141 phenol-degrading fungi were isolated from phenol-contaminated sites and tested for their ability to degrade phenol. Based on the results, strains N12 P6C3 was selected as the best fungal isolate for further investigations. Strain N12 P6C3 was identified as *Penicillium janthinellum*. Various parameters including temperature, nitrogen source, salinity, pH, and phenol concentration affecting phenol degradation activity of *P. janthinellum* strain N12 P6C3 were studied. The optimum conditions for phenol degradation were achieved at temperature of 35°C, ammonium sulphate at 3 g/L, 0.05 g/L of sodium chloride, and pH 6 using phosphate buffer system.

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Molecular cloning and characterization of NAC genes from four foxtail millet genotypes

Sintho Wahyuning Ardie*, Nurul Khumaida*, Tetsuo Takano*, Nike Karjunita*, Muhammad Habib Widyawan*

*Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University (IPB), Jl. Meranti, IPB Darmaga Campus, Bogor, 16680, West Java, Indonesia

*Laboratory of Environmental Stress Tolerance Mechanisms, The University of Tokyo, 1-1-1 Midori-cho, Nishitokyo-shi, 188-0002, Tokyo, Japan

*Graduate School of Plant Breeding and Biotechnology, Bogor Agricultural University (IPB), Jl. Meranti, IPB Darmaga Campus, Bogor, 16680, West Java, Indonesia

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Abstract. Transcription factor gene family of NAC (NAM, ATAF, CUC) is tightly involved in plant development and in the response to stresses. In this study, we reported the isolation and the characterization of NAC gene homolog from four foxtail millet genotypes. Band with approximately 1300 bp size was successfully amplified from the genomic DNA of four foxtail millet genotypes (ICERI-4, ICERI-5, ICERI-6 and ICERI-10) using gene specific primer. The fragment was designated as SiNAC065 after showing high similarity with NAC gene homologs in the GenBank. Sequence analysis results showed that the SiNAC065 genes isolated from the four genotypes were 1265 bp in length with one intron and two exons. The two exons encode 325 amino acids with the conserved domain located between amino acid 19-325. The SiNAC065 protein identified in this study have 8 conserved motives in the conserved region which categorized them as SNAC (stress responsive NACs) orthologs that are involved in the abiotic stress responses. Different features of SiNAC065 isolated from the tolerant- and the sensitive-genotypes should provide information of the gene’s role in salinity tolerance mechanism of foxtail millet.

Keywords: abiotic stress, gene cloning, Setaria italica, stress-responsive NAC, transcription factor

INTRODUCTION

Foxtail millet (Setaria italica L. Beauv) is carbohydrate producing crop with high nutritional value (Amadou et al., 2013). This crop ranks second in total millet production globally (Yang et al., 2012) and became an essential food crop particularly in areas with drought and saline conditions due to its tolerance to those conditions (Kafi et al., 2009; Karyudi and Fletcher, 2003; Panaud 2006). Several studies have been conducted to reveal the tolerance mechanism of foxtail millet to abiotic stresses, particularly to drought (Lata et al., 2010) and salinity (Islam et al., 2011) stresses.

Extensive studies in plant abiotic response showed that the genes involved in abiotic stress tolerance mechanisms were evolutionary conserved among plant species (Akpinar et al., 2012). Generally, stress related genes are classified into down-stream genes (functional genes) and up-stream genes (regulatory genes). Functional genes encode enzymes and metabolic proteins which directly function to protect cells from...
stress, such as detoxification enzymes, ion transporters, late embryogenesis abundant (LEA) proteins, and heat shock proteins (HSP). Regulatory genes encode various regulatory proteins which regulate the signal transduction and gene expression in response to the stress, such as protein kinases and transcription factors (TFs) (Hirayama and Shinozaki, 2010; Akpinar et al., 2012). The NAM, ATAF1,2 and CUC2 (NAC) proteins constitute a major plant specific transcription factor (TF) family which have been shown to play wide-range of roles in various developmental programs as well as in abiotic and biotic stress responses (Olsen et al., 2005; Nakashima et al., 2012; Puranik et al., 2012). Several studies reported that the expressions NAC TF members were upregulated by salinity stress in many plants, including cereals such as foxtail millet (Puranik et al., 2013), wheat (Huang et al., 2015), rice (Hong et al., 2016), and pearl millet (Shinde et al., 2019). These studies indicated the pivotal role of NAC TF in salinity stress responses. In this study, we reported the isolation of NAC-containing domain genes from four foxtail millet genotypes. Ardie et al. (2015) has reported the salinity tolerance variation between the four foxtail millet genotypes, hence characterization of NAC TF from these genotypes would provide useful information in the breeding strategy of foxtail millet.

MATERIALS AND METHODS

NAC gene homolog isolation from genomic DNA. Four foxtail millet genotypes from Indonesian Cereal Research Institute (ICERI), namely ICERI-4, ICERI-5, ICERI-6 and ICERI-10, were used in this study due to their contrasting tolerance to salinity. ICERI-5 and ICERI-6 genotypes were reported to have better tolerance to salinity compared to ICERI-4 and ICERI-10 (Ardie et al., 2015; Lapuimakuni et al., 2018). Genomic DNA was isolated from leaves of 10 days-old foxtail millet seedlings using CTAB method (Murray and Thompson 1980). Each PCR reaction mixture of 30 µL consisted of 5 µL (12 ng/µL) genomic DNA, 15 µL GoTaq®Green Master Mix (Promega, USA), 50 pmol of each forward (5'-ATGGGAGAACGAGCAAGCAGTC AGC-3') and reverse primer (5'-CTAGAACATT TCCTCGGCCCTCGG-3'), and 5 µL ddH2O. The PCR cycle consisted of initial denaturing at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 58°C annealing for 60 s, and extension at 72°C for 30 s. The final extension step was done at 72°C for 7 min, then the PCR products were stored at 4°C. PCR product was gel-purified using FastGene™ Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Germany) following the manufacturer instruction and directly sequenced to Eurofin (https://eurofinsgenomics.jp/). BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/) analysis was performed to ensure the homology of the fragment with the NAC genes stored in the GenBank. Sequenced fragments were then inserted into T-vector pMD20 (TaKaRa, Japan) with the ligation reaction of ±80 ng/µL PCR product, 1 µL plasmid pMD20 (50 ng/µL), and 1x volume of DNA Ligation Kit Mighty Mix (TaKaRa, Japan) at 16°C for 45 mins. Ligation products were transformed into E. coli strain DH5α, purified using FastGene™ Plasmid MiniKit (Nippon Genetics Europe GmbH, Germany), and sequenced to Macrogen (http://www.macrogen-japan.co.jp/).

Sequence analysis of NAC gene homolog from foxtail millet. The NAC nucleotide sequences obtained were aligned and translated into amino acid sequence using Geneious software version 8.1.6 (Biomatters Ltd). Amino acid sequences were aligned using web-based program ClustalW2 (Larkin et al., 2007). Phylogenetic tree was developed by MEGA7 software (Kumar et al., 2016) using Neighbor-Joining method at 1000 replication bootstrap. Conserved domain analysis was conducted using BLAST on Plant Transcription Factor Database v. 4.0 (http://planttfdb.cbi.pku.edu.cn/blast. php). Visualization on conserved domain was done using WebLogo program (Crooks et al., 2004).
RESULTS AND DISCUSSION

Fragment with the targeted size (1265 bp) was amplified by using gene specific primer (Figure 1). The gene specific primer was deduced from SiNAC065 sequence deposited in the plant genome database Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Sequence alignment analysis between the four amplified fragments with the reference sequence in Phytozome database (Phytozome identifier: Si012319m) showed that the fragments shared 99% identity with the reference sequence, thus the fragments isolated in this study were designated as SiNAC065 followed by the foxtail millet genotype name. The fragments were deposited in the GenBank as SiNAC065_ICERI4 (Accession no. KY404102), SiNAC065_ICERI5 (Accession no. KY404103), SiNAC065_ICERI6 (Accession no. KY404104), and SiNAC065_ICERI10 (Accession no. KY404105). The SiNAC065 genes isolated have one intron and two exons. The first exon spans between base 1-196, the second exon spans between base 487-1265, while the intron spans between base 197-486. The two exons encode 325 amino acids that showed high homology to similar sequences from other species in GenBank, such as Oryza brachyantha, Zea mays, Sorghum bicolor, Panicum virgatum, Brachypodium distachyon, Selaginella moellendorffii, Bambusa emeiensis, Hordeum vulgare, Aegilops tauschii, and Triticum aestivum (Figure 2).

Multiple sequence alignment analysis of the deduced amino acid sequences of SiNAC065 fragments isolated from the four foxtail millet genotypes and the deduced amino acid sequence of SiNAC065 from S. italica inbred ‘Yugu1’ reported by Bennetzen et al. (2012) deposited in NCBI GenBank database and PHYTOZOME v8.0 database (www.phytozome.net/) revealed the presence of conserved domains located in the N-terminal region (Figure 3). Domain identification analyses conducted by Puranik et al. (2013) showed that the N-terminal region of SiNAC065 share the general structure of NAC proteins. In general, the N-terminal NAC-domain (~150 aa) can be divided into five sub-domains, A-E, which each possess particular function (Nakashima et al., 2012; Puranik et al., 2012). The DNA binding ability is reported to be possessed by the highly conserved positively charged C and D sub-domains, whereas the A sub-domain may be involved in the functional dimer formation,
and the B and E sub-domains may be responsible for the functional diversity of NAC genes (Jensen et al., 2010; Chen et al., 2011; Puranik et al., 2012). The SiNAC065 proteins isolated in this study also have the well conserved N-terminal NAC-domain. Based on the conserved motif determination of NAC TF family member in foxtail millet by Puranik et al. (2013), the N-terminal NAC-domain of SiNAC065 can be divided into 8 conserved motifs (Table 1). Two putative Nuclear Localization Signal (NLS) domains were found within the NAC domain in SiNAC065 protein, which is previously reported by Puranik et al. 2011 (Figure 2). The presence of NLS domains suggest that the SiNAC065 protein was directed into the nucleus. NLS is a short peptide motif that able to mediate the import of proteins into nuclear by binding to their receptors (Kosugi et al., 2009; Sakuma et al. 2006). It is no surprise if NLS domains are present in SiNAC065 proteins since this protein has a DNA binding ability as a transcription factor. Meanwhile, the C-terminal region of NAC TF is greatly varied among the family member and it serves as a potential transcriptional activation regions (TARs) which has either activator or repressor function and some of them possess protein binding activity (Olsen et al., 2005; Puranik et al., 2012).

Figure 3. Identification of conserved domain found in SiNAC065 proteins. The NAC domain located in the N-terminal was boxed and the shaded amino acid sequences was predicted as Nuclear Localization Signal. Phyllogenetic analyses and gene functional studies have clearly classified the stress-responsive NAC proteins into one group, the stress-responsive NAC (SNAC) group (Fang et al., 2008; You et al., 2015). Experimental evidences are available for some members of the SNAC group that demonstrated their functions in abiotic stress responses. For example, over-expression of SNAC1 from rice improved drought and salt tolerance in rice (Hu et al., 2006), cotton (Liu et al., 2014), and ramie (An et al., 2015), while the over-expression of SNAC2 increased salt and cold tolerance in transgenic rice (Hu et al., 2008) and the over-expression of ATAF1 from Arabidopsis also enhanced drought tolerance in transgenic Arabidopsis (Wu et al., 2009). Puranik et al. (2013) reported the identification of 147 putative NAC domain-encoding genes from foxtail millet and distributed them into 11 distinct sub-families. Phylogeny-based function prediction in that study indicated that the member of sub-family VII probably involved in similar regulatory roles as the SNAC orthologous in other species. SiNAC065 is one member of the 18 members of sub-family VII which was up-regulated in the early stress of drought and salinity, indicating the role of this gene in drought and salinity stress responses.

A study utilized foxtail millet cultivars with different tolerance to salinity, cv. Prasad (salt-tolerant) and cv. Lepakshi (salt-sensitive), showed that the salt-tolerant cultivar had better salt-induced-oxidative tolerance due to increased total superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity, whereas both enzyme activities decreased in the salt-sensitive cultivar (Sreenivasulu et al., 2001). It is long-known that the increased of SOD and CAT activities correlates with lower oxidative stress induced by various abiotic stress (Dhindsa et al., 1981). The overexpression of JUB1, one of NAC TFs, dampened intracellular H₂O₂ levels and enhances tolerance to various abiotic stresses (Wu et al., 2012). Those studies indicate the possible role of NAC TFs in improving tolerance to salt stress by escalating antioxidant components in the tolerant genotypes. Whether or not SiNAC065 is involved in antioxidant component modulation still need to be further elaborated.
Characterization of genes associated with abiotic stress would be useful to develop efficient breeding strategy for tolerant variety, such as through genetic engineering (Turan et al., 2012) and molecular marker assisted breeding (Ashraf and Foolad, 2013). Previous studies reported transgenic plants overexpressing NAC gene exhibited enhanced tolerance to salinity stress (Huang et al., 2015; Hong et al., 2016; Shinde et al., 2019). Study conducted by Widyawan et al. (2018) utilized single nucleotide polymorphism (SNP) at the 558th base of SiDREB2 gene, a TF gene which tightly associated with abiotic stress responses, to develop SNAP marker related to drought stress in foxtail millet. Similar approaches can be performed utilizing SiNAC065 gene. As a conclusion, this study has successfully isolated SiNAC065 genes from foxtail millet genotypes which differ in their salinity tolerance. Different features of SiNAC065 isolated from the tolerant and the sensitive-genotypes should provide information of the gene’s role in salinity tolerance mechanism of foxtail millet.

ACKNOWLEDGEMENT

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Table 1. Conserved motifs identified in SiNAC065 protein.

<table>
<thead>
<tr>
<th>Motif No.</th>
<th>Amino acid sequence composition of motif</th>
<th>Amino acid span</th>
<th>NAC sub-domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W[YF][FF][SC][PR][DK][KYP][TN][GSR][TP][NRAT]</td>
<td>76-96</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>LPPGFRFHPDEEL[VI]x[HYF]</td>
<td>19-32</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>[I][ED][VD][LI][N][S][KR][E][PW][DE][EP][E][KAKIG]</td>
<td>50-68</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>xx[DF][DE][LV][C][R][V][FY][K][KS][PR]</td>
<td>160-171</td>
<td>E</td>
</tr>
<tr>
<td>7</td>
<td>x[SA][YF][K][AT][K][K][D][K]-[KR]</td>
<td>98-107</td>
<td>C</td>
</tr>
<tr>
<td>8</td>
<td>YL[KK]RR[VA]Agx-[PR][LI][PL][DI][VI]</td>
<td>36-41</td>
<td>-</td>
</tr>
</tbody>
</table>

The amino acid sequences were aligned with the reference sequence (Phytozome identifier: Si012319m) and conserved motifs were identified according to Puranik et al. (2013).

REFERENCES


Factors contributing to the enhanced production of protease and lipase in *Bacillus pumilus* SG2 mutant

R. Sangeetha*, A. Geetha*, I. Arul Pandi*

*Department of Biochemistry, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies, Chennai, India
*Department of Biochemistry, Bharathi Women’s College, Chennai, India
*PG and Research Department of Microbiology, Asan Memorial College of Arts and Science, Chennai, India

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Abstract. A random mutagenesis on a wild strain *Bacillus pumilus* SG2 using physical and chemical agents was performed previously and a mutant *Bacillus pumilus* SGMM8 which produced comparatively higher levels of protease and lipase was isolated. This study was attempted to analyse the causes underlying the enhanced enzyme production by the mutant. Substrate uptake, repression/de-repression studies and determination of Km and Vmax were done to understand if de-repression and enhanced affinity to substrate are the plausible reasons for enhanced enzyme activity. It was observed that the protease production was not repressed at high glucose concentrations in the mutant. The Km and Vmax of SG2 lipase were 2.265 mg/ml and 158 U/ml respectively and those of SGMM8 lipase were 1.619 mg/ml and 159 U/ml respectively and thus enhanced affinity may be the underlying cause for enhanced lipase activity. The genome sequence of the mutant enzymes were analysed and transversion mutations were identified in the coding regions. The mutations in the signal peptide of the protease and near the active site of the lipase may have caused increased enzyme production/activity.

Keywords: *Bacillus pumilus*, Km/Vmax, lipase, protease, repression studies, strain improvement

INTRODUCTION

Economical industrial production of microbial enzymes can be achieved by the following approaches: developing a suitable medium for fermentation, optimizing the parameters for cultivation and refining the fermentation process and improving the productivity of the strains. However, the strain improvement approach offers more enzyme yield. The techniques used to modify the strains genetically in order to increase the production of a desired product are collectively referred to as strain improvement. Strain improvement is achieved by either mutant selection or recombinant DNA technology (Mahadik et al., 2004; Sangeetha et al., 2011).

We had earlier reported the isolation of an improved mutant of *Bacillus pumilus* SG2. The parent strain was subjected to physical mutagenesis with UV irradiation and chemical mutagenesis using ethyl methane sulphonate and the most potent mutant that produced remarkably higher quantities of protease and lipase was isolated. The enzymes of the mutant were partially purified and characterized (Sangeetha et al., 2011). However, the factors which contributed to improved enzyme production were not reported earlier. Alterations involving either enhancement or decrement in enzyme production/activity can be attributed to many factors like repression/
derepression and cell membrane permeability (Stulke and Hillen, 1999). Strain improvement processes result in alterations in the base pairs as well.

This study was carried out to understand the factors contributing to the enhanced enzyme production by the mutant SGMM8. Hence uptake and repression studies and enzyme kinetic studies were performed. The study also aimed at sequencing the genes encoding protease and lipase in the parent strain and the mutant for comparison and identification of base alterations that had contributed to the enhanced enzyme activity in the non-genetically modified mutant.

**MATERIALS AND METHODS**

**Bacterial strains, growth medium and conditions.** The parent strain Bacillus pumilus SG2 was isolated from an industrial effluent and was subjected to strain improvement by physical mutagenesis using UV light followed by chemical mutagenesis using ethyl methane sulphonate. An improved strain which exhibited enhanced production of protease and lipase was isolated and designated as SGMM8. The production medium consisted of (w/v) 0.04% CaCl₂, 0.02% MgCl₂, 1% glucose, 0.5% NaCl, 0.3% yeast extract and 1% tributyrin (in sodium phosphate buffer, pH 9.0). Overnight culture (5 ml) (O.D₆₀₀=1.0) of parent strain Bacillus pumilus SG2 and its hyper-producing mutant SGMM8 were inoculated into separate 100 ml medium and incubated on a rotary shaker (180 rpm) for 48 h at 37°C.

**Enzyme kinetics.** The proteolytic activity at different concentrations (2-10 mg/ml) of casein and lipolytic activities at different concentrations (2-10 mg/ml) of tributyrin were studied using methods described by Rahman et al. (2005) and Kordel et al. (1991) respectively. The Km and Vmax values were determined from the Lineweaver-Burk plot.

**Uptake and repression studies with glucose and inorganic phosphate.** Glucose and phosphate uptake by the parent and mutant cells were studied by determining the residual concentrations of glucose and phosphate in the culture medium at regular intervals. Glucose was measured using the method described by Miller (1959) and phosphate was measured using the method described by Fiske and Subbarow (1925).

The catabolite repression of the synthesis and secretion of SGMM8 protease and lipase by glucose, inorganic phosphate (KH₂PO₄) at different concentrations (0-2% w/v) was also studied. For the investigation on repression by glucose, the production medium with varying concentrations of glucose were inoculated with 1.0% (v/v) of overnight culture (O.D₆₀₀=0.6) and incubated at 37°C in shaking incubator at 120 rpm for 28 hours. The production medium containing varying concentrations of inorganic phosphate was used for study on repression by phosphate. At the end of the incubation period, the liquid cultures were centrifuged at 10000 g for 15 min and the supernatants were subjected to protease and lipase assay. The proteolytic activity was measured by the photometric method described by Rahman et al. (2005). One unit (U) of protease activity is equivalent to 0.5 µg of tyrosine liberated by 1.0 ml of enzyme solution under the assay conditions. The lipase activity was assayed by the photometric method described by Kordel et al. (1991). One unit (U) of lipase activity is equal to 1µmol of p-nitrophenol liberated per min under the assay conditions.

**Amplification and sequencing of alp and lip genes of SG2 and SGMM8.** DNA isolation from SG2 and SGMM8 was performed based on the method of Tripathi and Rawal (1998). The PCR amplification was carried out in Ericomp Delta cycler I system (Ericomp, Inc, CA) and PTC-100 (MJ Research Inc. USA) thermocycler machines. The alp and lip gene amplification was performed using the primers described by Rahman et al. (2007) and Sangeetha et al. (2014) respectively. The primers used for alp gene amplification were forward: 5’- ATG TGC GTG AAA AAG AAA AAT GTG -3’ and reverse: 5’-TTA GTT AGA AGC TGC TTG AAC GTT -3’. The reaction conditions were as follows: one denaturation step at 94°C for 4 min, 30 cycles of annealing of primers at 60°C for 45 sec, extension at 72°C for 2 min except for the final cycle for which extension proceeded for 5 min. The primers and the reaction conditions used for lip
Production of protease and lipase in B. pumilus SG2 mutant
gene amplification were reported earlier (Sangeetha et al., 2014).

**Gene sequencing.** The direct gene sequencing was carried out by the method of Sanger et al. (1997) using DTCS quick start Dye terminator kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The original sequences of the parent and mutant strains were submitted to GenBank, NCBI.

### RESULTS AND DISCUSSION

**Uptake and repression studies.** To investigate if the enhanced enzyme production by the parent SG2 and mutant SGMM8 was due to enhanced synthesis and/or secretion, uptake and repression studies with glucose and phosphate were performed. Glucose uptake by the parent SG2 and the hyper-producing mutant SGMM8 was not similar. Strong catabolite repression was observed with glucose concentrations higher than 1% (w/v). Protease production by the parent was repressed from 126 to 10 U/ml while the lipase production was repressed from 94 to 12 U/ml with increase in concentrations of glucose from 1% (w/v) to 2% (w/v) (Figure 1). The mutant exhibited seven fold higher activities of protease and lipase than the parent. The production of both protease and lipase by the parent and the mutant gradually increased with increase in glucose concentrations from 0 to 1% (w/v) and the production was optimum when the concentration of glucose was 1% (w/v). However, uptake of phosphate by both the parent and the mutant was similar. The decreased uptake or consumption rate of glucose could be attributed to a cis/trans acting mutation (Fickers et al., 2003). Repression studies show that enzyme production by the mutant was not repressed at 1.5% (w/v) of glucose although a decline in enzyme activity is observed (Figure 1).

Catabolic genes and operons in Gram-positive bacteria are subjected to carbon catabolite repression (CCR) by glucose and other metabolized carbon sources. The mechanisms underlying glucose repression include catabolite repression which involves global regulators, inducer exclusion and induction prevention. Negative regulation of the transcription of catabolite-repressive genes involves a trans-acting repressor protein called catabolite control protein, Ccp A and cis-acting elements referred to as cre, catabolite responsive elements (Deutscher et al., 2002; Inacio et al., 2003).

![Figure 1. Glucose repression studies. The values expressed are mean ± S.D of three independent experiments.](image)

The enhanced production of SGMM8 protease and lipase is therefore most likely due to decreased levels of glycolytic intermediates when the glucose uptake by the hyper-producing mutant was decreased. The glucose uptake by the bacteria was studied by determining the residual glucose in the culture medium. The glucose uptake by the parent was found to be 18.6 µg/g/h while the glucose uptake by the mutant was 10.4 µg/g/h (Data not shown). The documents that report the relationship between glucose uptake capacity and catabolite repression are very few. Fickers et al. (2003) have reported enhanced lipase production by *Y.lipolytica* mutant which exhibited lipase production uncoupled from catabolite repression from glucose and the glucose uptake capacity by the mutant was reduced 2.5 fold compared to the wild type strain. Christiansen and Nielsen (2002) have reported decreased protease production by *B.clausii* due to enhanced glucose uptake.

Increase in enzyme synthesis was observed when the phosphate concentration was increased from 0 to 0.5% (w/v) (Data not shown). Thus 0.5% (w/v) was the optimum concentration for both protease and lipase production. Phosphate plays a vital role as an effector of a large number of enzymatic reactions of primary metabolism, including the synthesis of DNA and RNA, protein and carbohydrate metabolism, cellular
respiration and control of ATP levels. The optimal phosphate requirement by the mutant was the same as that of the parent.

**Km and Vmax of protease and lipase.** The protease and lipase produced by the parent and mutant strains were purified and the purity of the enzymes was confirmed as reported earlier (Sangeetha et al., 2010). The Km and Vmax values were determined for the protease and lipase produced both by the parent and the mutant *B. pumilus* strains. The protease of both SG2 and SGMM8 had a Vmax value of 120 U/ml of protein. The Km values of the protease produced by the wild and mutant were similar, 0.9 and 0.88 mg/ml respectively. The lipase produced by the parent and the mutant strains exhibited slightly different Km and Vmax values. The SG2 lipase had a Km value of 2.265 mg/ml and a Vmax of 158 U/ml while the values of Km and Vmax of SGMM8 lipase were 1.619 mg/ml and 159 U/ml respectively (Figure 2). The decreased Km value exhibited by SGMM8 lipase indicates that the enzyme had increased affinity for its substrate when compared to the lipase of the wild strain.

![Figure 2. Line-weaver Burk plot for the determination of Km and Vmax of SG2 and SGMM8 lipases.](image)

**Protease and lipase genes.** The coding regions of protease and lipase of the parent and the mutant strain were compared to determine whether the enhancement in enzyme production in the mutant could be attributed to the alterations in the nucleotide sequences. The coding regions of SG2 and SGMM8 protease and lipase were amplified. We had earlier reported the molecular characterization of the lipase produced by *B. pumilus* SG2 (Sangeetha et al., 2014). The coding regions of the enzymes were sequenced and the sequences were submitted to GenBank under the following accession numbers: *B. pumilus* SG2 Protease: GQ398415; *B. pumilus* SG2 Lipase: GQ398414; *B. pumilus* SGMM8 Protease: GU143024; *B. pumilus* SGMM8 Lipase: GU143025.

A nucleotide sequence comparison between the *alp* gene of SG2 and SGMM8 revealed two base substitutions. The gene encoding the protease of the improved strain SGMM8 exhibited two G-C to A-T transversion mutations; the first mutation was at nucleotide 5 near the start region while the second was at nucleotide 785 in the coding region (Figure 3). Translation studies of the amplified genes revealed that the cysteine 2, present next to the first amino acid methionine in the signal peptide, was replaced by tyrosine. Also, the glycine 262 present before the oxyanion residue asparagine was replaced by glutamate. Comparison of nucleotide sequences of the *lip* gene of SG2 and SGMM8 indicated a GC-AT transversion mutation at nucleotide 500 in the coding region (Figure 4). The amino acid at position 167 was valine in SG2 lipase and was substituted by isoleucine in the mutant enzyme. This mutation was present near the active site aspartate 164 and thus probably contributed to enhanced lipase activity.

The alterations in the specific activity of the SG2 protease and lipase may reflect the effect of physical and chemical mutagenesis. The specific activity of protease remained unaltered and thus the two transversion mutations had no impact on the catalytic activity of the enzyme. However, specific activity of SGMM8 lipase showed a marginal increase when compared to SG2 lipase. This could be attributed by the base substitution in the coding region which resulted in a neutral mutation by replacing the residue valine 136 with isoleucine. Interestingly, Christiansen et al. (1994)
have patented *B. lentus* alkaline protease variants with enhanced stability. Two of these variants have valine residues replaced with isoleucine. The signal peptide of the protease of SGMM8 had a base substitution at the amino acid position -30 (with respect to the first amino acid of the pro-peptide) present on the N-terminal region of the peptide. There exists a strong preference in the amino acid residues around the signal peptide cleavage site. Enhanced secretion of *B. subtilis* β-lactamase was observed when the amino acids at positions 27 and 28 were substituted with alanine using site directed mutagenesis (Nakamura et al., 1988).

Thus the enhanced proteolytic and lipolytic activity observed in the mutant SGMM8 could be attributed to the enhanced secretion of protease following a mutation in the signal peptide and the enhanced activity of lipase as a consequence of a mutation in the coding region.

**CONCLUSION**

The study involved the analysis of factors which contributed to the improved production of protease and lipase by the improved strain *Bacillus pumilus* SGMM8. The mutant was moderately relieved off the repressive effects of glucose and phosphate. Thus de-repression could be highlighted as the possible reason for the enhanced enzyme production. Also, the decreased Km value exhibited by SGMM8 lipase indicates that the enzyme had increased affinity for its substrate when compared to the lipase of the wild strain. The *alp* and *lip* genes encoding the protease and lipase enzymes of both the parent and the mutant strains were sequenced. The enhanced enzyme activities exhibited by the hyper-producing mutant could be attributed to a transversion mutation induced in both the genes.

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Production of protease and lipase in B. pumilus SG2 mutant


Identification of polyketide synthase gene clusters in a phage P1-derived artificial chromosome library of a Philippine strain of *Streptomyces* sp. PCS3-D2

Aileen Bayot Custodio* and Edwin Plata Alcantara*

*National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Laguna, Philippines 4031

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Abstract. A phage P1-derived artificial chromosome (PAC) library was constructed from genomic DNA of *Streptomyces* sp. PCS3-D2. Polymerase chain reaction (PCR) screening of the PAC library revealed two clones, PAC16D and P222O, which were positively identified to harbor polyketide synthase (PKS) Type I and PKS Type III gene clusters, respectively. Restriction enzyme digestion showed that PAC16D and PAC222O contained a 130 kb and a 140 kb insert, respectively. Results of sequencing and bioinformatics analyses revealed that PAC16D comprised of a full-length PKS type I bafilomycin gene cluster while PAC222O harbored truncated siderophore and putative gene clusters as well as a complete PKS III biosynthetic gene cluster. The PKS III gene cluster had three genes similar to alkyl resorcinol biosynthetic genes, however majority of the novel gene cluster had little similarity to known PKS Type III gene clusters. The successful cloning and identification of these gene clusters from *Streptomyces* sp. PCS3-D2 serve as the jump off point to further genetic manipulation in order to produce the insecticidal natural product in a heterologous host.

Keywords: polyketide synthase gene clusters, *Streptomyces* sp. PCS3-D2, phage P1-derived artificial chromosome (PAC) library, natural products

INTRODUCTION

Actinobacteria are ubiquitous Gram-positive, filamentous bacteria that could be easily isolated from various soil and marine environments. They are well known prolific producers of bioactive compounds including natural insecticides (Kirst, 2010). The use of natural products as insecticides for crop protection is encouraging. Recent reports indicate that the use of natural products and natural product-derived insecticides continue to increase (Dayan et al., 2009). In 2012, natural products and natural product-inspired products comprised approximately 30% of the global agrochemical sales, while an additional 22% of synthetic agrochemical compounds could have been inspired by natural products under different circumstances (Gerwick and Sparks, 2014).

The strain *Streptomyces* sp. PCS3-D2 produces insecticidal compounds active against important agronomic insect pests (Bayot-Custodio et al., 2014). The development of potentially beneficial natural products from this strain is hindered by the lack of proper genetic characterization which when ignored could lead to large missed opportunity for developing effective solutions for mitigating problems in insect resistance to insecticides. Recent statistics showed that at least 30 cases of insecticide resistance have been reported for the Philippines (www.pesticide
The bottom-up approach for natural product discovery utilizes genome sequencing and bioinformatics to identify gene clusters predicted to synthesize important bioactive compounds. Upon identifying a target gene cluster, genetic manipulation techniques are employed to activate the gene clusters either in native or heterologous hosts for the production of the corresponding natural product (Luo et al., 2014). Given that the size of some gene clusters can exceed 100 kb, artificial chromosome-based vectors have been constructed for the maintenance and genetic manipulation of natural producers in Streptomyces (Alduina and Gallo, 2012).

Phage P1-derived artificial chromosomes (PACs) can carry large 100–300 kb DNA fragments in E. coli cells (Shizuya et al., 1992). However, because E. coli is not suitable to heterologously express high GC% actinomycete genes, shuttle vectors have been designed to allow library construction and manipulation in E. coli, while the expression of actinomycete genes are done in Streptomyces host strains. An example of this is the pESAC13 vector, which contains the phage P1 origin of replication, phiC31 integrase gene and a phiC31 attP site, an oriT site that allows transfer into Streptomyces by conjugation (Sosio et al., 2000) and a BamHI cloning site for the insertion of large DNA fragments (Jones et al., 2013).

In this paper, we first report the construction of a phage P1-derived artificial chromosome library and the in silico characterization of two cloned polyketide gene clusters from a Philippine strain of Streptomyces sp. PCS3-D2 using a bioinformatics approach.

Materials and Methods

Genome Mining of Streptomyces sp. PCS3-D2 for Biosynthetic Gene Clusters. The previously annotated genome sequence of Streptomyces sp. PCS3-D2 (Bayot-Custodio et al., 2014) was re-analyzed using the antiSMASH 4.0 software (Blin et al., 2017) to determine both the presence and location of biosynthetic gene clusters in the JDUZ01 genome sequence.

Cell Culture for Construction of Phage P1 Artificial Chromosome (PAC) Library. For preparing mycelia for PAC library construction, Streptomyces sp. PCS3-D2 was grown in YMB (100 mL) that was inoculated with mycelia from a 10 mL TSB starter culture. The mycelia were grown for 72 hours with shaking at ambient temperature and centrifuged (4°C, 12000 rpm, 15 min) to remove the supernatant. The mycelial pellets (approximately 7 mL of concentrated mycelia) were flash frozen in liquid nitrogen and shipped in dry ice to BioS&T (Montreal, Canada) for custom construction of a phage P1 artificial chromosome (PAC) library using a modified E. coli-Streptomyces Artificial Chromosome (ESAC) vector (Fig. 1). The constructed Streptomyces sp. PCS3-D2 PAC library generated a total of 2,688 clones with an average insert size of 125 kb.

Screening of PAC Library for PKS I and PKS III Gene Clusters. PCR primers (Table 1) were designed to screen the Streptomyces sp. PCS3-D2 PAC library for clones containing PKS I and PKS III gene clusters. These PCR primers were initially tested using Streptomyces sp. PCS3-D2 genomic DNA as template for PCR. PKS I-specific primers were expected to amplify a 700 bp amplicon corresponding to the 5-aminolevulinate synthase gene of the bafilomycin gene cluster. On the other hand, a 450 bp PCR amplicon was expected to be amplified using PKS III-specific primers corresponding to the stilbene synthase.

Figure 1. Vector map of PAC pESAC13.
gene. Each PCR amplicon was later sequenced to verify that the desired region of the gene clusters was amplified.

The *Streptomyces* sp. PCS3-D2 PAC library was PCR screened by BioS&T (Montreal, Canada) using the designed PKS I and PKS III-specific primers. The positively identified clones were subjected to restriction enzyme digestion using *N*de to estimate the size of the cloned inserts. Positively identified clones were subjected to next generation sequencing to determine if they contained the full-length PKS I and PKS III gene cluster.

### Table 1. Bacterial strains, vectors and PCR primers used in the study.

<table>
<thead>
<tr>
<th>Items</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces</em> sp. PCS3-D2</td>
<td>Wildtype strain producing insectidal compounds</td>
<td>This work</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH10B</td>
<td>Cloning strain; F- mcrA Δ(mrr-hsdRMS-mcrBC) q80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pESAC13 Vector</td>
<td>PAC vector (P1-phage replicon) for genomic library construction; RP conjugative, ΦC31 integrative tsr, neo, orfT, attP and int from ΦC31, P1 rep, sacB</td>
<td>Sosio et al., 2000</td>
</tr>
<tr>
<td>BatBIII-F</td>
<td>TCCGAGATCACGGTGTGGTC</td>
<td>This work</td>
</tr>
<tr>
<td>BatBIII-R</td>
<td>GCSGTGTTGAGATGAAAGGGRGC</td>
<td>This work</td>
</tr>
<tr>
<td>STS-F</td>
<td>TTCCCCGCCCCAACCACCGCATTTCCCA</td>
<td>This work</td>
</tr>
<tr>
<td>STS-R</td>
<td>ACACRGCTCGGTGGACAGCAGCAG</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Sequencing, assembly, annotation and analysis of PAC clones.

To ensure that the positive PAC clones contained the entire PKS I and PKS III gene clusters, they were subjected to Ion Torrent single ended sequencing with >200 coverage depth using the Ion PGM™ System by BioS&T (Montreal, Canada). The resulting raw sequence reads were quality checked using FastQC (Andrews, 2018). The sequences were processed by trimming low quality ends using the Trim Ends algorithm of the Geneious R11 software (Kearse et al., 2012). The sequence reads were also screened against the UniVec database to trim off contaminating vector and adapter sequences at the 5’ and 3’ ends. The filtered and trimmed sequences were subjected to reference guided mapping and assembly using the Geneious R11 software. The Bowtie2 version 2.3.0. plugin (Langmead and Salzberg, 2012) was used in reference guided assembly to align the sequencing reads to the reference sequence. The reference sequence used was scaffold 1 (scf1) of *Streptomyces* sp. PCS3-D2 JDUZ01 genome sequence. The consensus sequence from reference guided mapping was annotated using the Prokka Galaxy Tool Version: 1.12.0 (Seemann, 2014). The annotated gene clusters were then analyzed using antiSMASH 4.0 for the identification, annotation and analysis of secondary metabolite biosynthesis gene clusters (BGC).

### RESULTS

**Genome mining of Streptomyces sp. PCS3-D2 for biosynthetic gene clusters.** The antiSMASH 4.0 software enables bacterial genome mining for biosynthetic gene clusters that code for chemical classes of secondary metabolites (Blin et al., 2017). This is done by comparing each gene product in the uploaded DNA sequence against a manually curated collection of profile hidden Markov models (pHMMs) which describe key biosynthetic enzymes of the secondary metabolite classes. The key enzymes encoded in each gene cluster are assigned to secondary metabolite-specific clusters of orthologous groups (smCOGs). Depending on the class of secondary metabolite gene cluster detected (i.e. multimodular polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), etc.), further detailed analyses are performed (Blin et al., 2013). KnownClusterBlast is also integrated in antiSMASH which allows the identification of gene clusters against a repository of known and experimentally characterized secondary metabolite biosynthesis gene clusters called MIBiG (Minimum Information on Biosynthetic Gene Cluster) for comparative gene cluster analysis (Blin et al., 2017).
The antiSMASH 4.0 analysis revealed that JDUZ01 genome sequence has a total of 26 BGCs which includes polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), NRPS hybrids, terpenes, siderophores, bacteriocin, butyrolactone, lantipeptides, ectoine, nucleoside and other BCGs as summarized in Table 2. The % similarity from antiSMASH results was based on how similar the query sequences were with known and characterized gene clusters in the MIBiG repository. Among the PKS gene clusters, two were identified as type I PKS: cluster 14 which has high similarity (100%) to the bafilomycin gene cluster and cluster 3 that has low homology (11%) to PKS-Cf saccharide herboxidiene BGC. Cluster 19, which is probably involved in the synthesis of spore pigments, was classified as a type II PKS, while cluster 13 has strong similarity (100%) to type III PKS alkyl resorcinol gene cluster.

Table 2. List of predicted secondary metabolite biosynthetic gene clusters in Streptomyces sp. PCS3-D2 based on antiSMASH 4.0 analysis.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Type</th>
<th>Predicted Products</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacteriocin</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Terpene</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Type I PKS-Cf saccharide</td>
<td>Herboxidiene</td>
<td>11%</td>
</tr>
<tr>
<td>4</td>
<td>Terpene-NRPS</td>
<td>Hopene</td>
<td>61%</td>
</tr>
<tr>
<td>5</td>
<td>NRPS</td>
<td>Streptolydigin</td>
<td>10%</td>
</tr>
<tr>
<td>6</td>
<td>NRPS</td>
<td>Bleomycin</td>
<td>6%</td>
</tr>
<tr>
<td>7</td>
<td>NRPS</td>
<td>Streptothricin</td>
<td>87%</td>
</tr>
<tr>
<td>8</td>
<td>Ectoine</td>
<td>Ectoine</td>
<td>100%</td>
</tr>
<tr>
<td>9</td>
<td>Thiopeptide-Terpene-NRPS</td>
<td>Coelichelin</td>
<td>100%</td>
</tr>
<tr>
<td>10</td>
<td>Terpene</td>
<td>2-methylisoborneol</td>
<td>100%</td>
</tr>
<tr>
<td>11</td>
<td>Melanin-Terpene</td>
<td>Istatimyin</td>
<td>4%</td>
</tr>
<tr>
<td>12</td>
<td>Siderophore</td>
<td>Kedarcidin</td>
<td>1%</td>
</tr>
<tr>
<td>13</td>
<td>Type III PKS</td>
<td>Alkylresorcinol</td>
<td>100%</td>
</tr>
<tr>
<td>14</td>
<td>Type I PKS</td>
<td>Bafilomycin</td>
<td>100%</td>
</tr>
<tr>
<td>15</td>
<td>Butyrolactone</td>
<td>Neocarzinostatin</td>
<td>6%</td>
</tr>
<tr>
<td>16</td>
<td>Lantipeptide</td>
<td>Galbonolides</td>
<td>6%</td>
</tr>
<tr>
<td>17</td>
<td>Lantipeptide</td>
<td>Venezuelin</td>
<td>100%</td>
</tr>
<tr>
<td>18</td>
<td>NRPS</td>
<td>Tambromycin</td>
<td>100%</td>
</tr>
<tr>
<td>19</td>
<td>Type II PKS</td>
<td>Spore pigment</td>
<td>66%</td>
</tr>
<tr>
<td>20</td>
<td>NRPS</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Terpene</td>
<td>Carotenoid</td>
<td>63%</td>
</tr>
<tr>
<td>22</td>
<td>Siderophore</td>
<td>Desferrioxamine B</td>
<td>100%</td>
</tr>
<tr>
<td>23</td>
<td>Nucleoside</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Siderophore</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>NRPS</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>NRPS</td>
<td>Enduracacidin</td>
<td>6%</td>
</tr>
</tbody>
</table>

**PCR screening of Streptomyces sp. PCS3-D2**

**PAC library.** The PKS I primers amplified a 700 bp fragment of the 5-aminolevulinate synthase gene of the bafilomycin gene cluster (BafBIII) while PKS III primers amplified a 450 bp portion of the stilbene synthase gene (STS) which represents PKS III gene clusters. PCR-based screening of the PAC library identified a clone in plate 1-6D (designated as PAC16D) to harbor the PKS I gene cluster and another in plate 2-22O (designated as PAC222O) to contain the PKS III gene cluster. Figure 2a illustrates the PCR screening of PAC16D amplifying a 700 bp PKS I gene product while PAC222O produced the expected 450 bp PKS III band. A 130 kb and 140 kb digest were observed from the *NotI*-treated PAC16D and PAC222O, respectively (Figure 2b).
Polyketide synthase gene clusters in phages

Figure 2. PCR screening of the Streptomyces sp. PCS3-D2 PAC library for (A) PKS I (PAC16D) and PKS III (PAC222O) gene clusters and (B) PAC clone insert verification by NotI restriction enzyme digestion. Lane 1: 130 kb PAC16D insert; Lane 2: 140 kb PAC222O insert; M: molecular weight markers.

PAC clone sequencing and bioinformatics analysis. Genome sequence analysis of JDUZ01 scf1 showed that it contained 14 gene clusters as illustrated in Figure 3. PKS III and PKS I BGCs were determined to be located near the 3' end of JDUZ01 scf 1. The PKS III gene cluster was estimated to be located at 1,457,140 to 1,492,845 bp, having a total size of approximately 35.7 kb. On the other hand, the PKS I gene cluster was located at 1,621,560 bp to 1,713,412 bp with a total length of about 91.8 kb.

Next Generation Sequencing generated 246,357 and 181,775 raw read sequences for PAC16D and PAC222O, respectively. Based on the assembly results, PAC222O had an insert length of about 132 kb which spanned a portion of the neighboring siderophore gene cluster, full length PKS III gene cluster and a putative gene cluster downstream of PKS III (Figure 3, green line). This shows that PAC222O contained the full length PKS III gene cluster.

On the other hand, PAC16D had a 130 kb insert which included a portion of a putative gene cluster upstream of the PKS I gene cluster as well as the full length PKS I bafilomycin gene cluster (Figure 3, red line). This proves that PAC16D also contains the entire PKS I gene cluster.

Bioinformatics analysis using the antiSMASH 4.0 software revealed that PAC16D had high similarity with the PKS I bafilomycin gene cluster. PAC16D contained the modular polyketide synthases which were responsible for the synthesis of bafilomycin's polyketide backbone, bafilomycin regulatory genes, and other genes responsible for the production and conversion of bafilomycin derivatives (Figure 4a). PAC16D was also compared with known bafilomycin gene clusters from Streptomyces griseus DSM 2608, S. lohii JCM 14114 and Kitasatospora setae KM-6054. Pairwise alignment revealed that PAC16D had 83.9% similarity with S. lohii JCM 14114, 81.6% with S. griseus DSM 2608 and 77.2% with K setae KM-6054 bafilomycin gene clusters. This proves that PAC16D contains the full length PKS I bafilomycin gene cluster. The best way to confirm that PAC16D contains a bafilomycin gene cluster that is capable of producing bafilomycin is to transform the PAC16D clone into a Streptomyces heterologous host and check for the production of bafilomycin by HPLC or LC-MS.

PAC222O, on the other hand contained three gene clusters: a truncated siderophore gene cluster (red box), the PKS III gene cluster (tan box) and a putative gene cluster (navy blue box) (Figure 4b). The truncated siderophore gene cluster was about 12 kb in length and included nine genes (Figure 4c). Downstream from the siderophore gene cluster was the full-length PKS III gene cluster (Figure 4d). A neighboring putative gene cluster that overlapped with the 3' end of the PKS III gene cluster was also observed in PAC222O. The PKS III gene cluster has three genes that has high similarity (100%) with S. griseus subsp. griseus NBRC 13350 alkyl resorcinol genes (monooxygenase, methyltransferase and stilbene synthase genes) (red box). This proves that PAC222O contains a PKS III alkyl resorcinol gene cluster. However, the genes surrounding the PAC222O PKS III gene cluster itself were not similar with other known alkyl resorcinol gene clusters and could therefore be a novel producer of resorcinol derivatives.
Figure 3. Mapping of biosynthetic gene clusters in JDUZ01 scaffold 1 of *Streptomyces* sp. PCS3-D2. The green line represents the location of the PAC222O insert while the red line represents the insert of PAC16D. (Cf-saccharide: Putative saccharide cluster; NRPS: Nonribosomal peptide synthetase; PKS I: Polyketide synthase Type I; PKS III: Polyketide synthase Type III).

Figure 4. antiSMASH prediction of biosynthetic gene clusters of *Streptomyces* sp. PCS3-D2 PAC clones (A) PAC16D and (B) PAC222O; (C) truncated siderophore gene cluster in PAC222O; and (D) segment of PAC222O showing the PKS III gene cluster and an overlapping putative gene cluster. The red box represents genes with high similarity to alkyl resorcinol biosynthetic genes.

**DISCUSSION**

In this study, genome mining and construction of a PAC library was carried out in order to gain insight into possible ways of exploiting the diversity of BGCs present in *Streptomyces* sp. PCS3-D2. The whole genome sequence of *Streptomyces* sp. PCS3-D2 served as a blueprint to identify which gene clusters should be prioritized for screening in the PAC library. Indeed, more than twenty-five BGCs were discovered some of which might be responsible for production of novel insecticidal secondary metabolites in *Streptomyces* sp. PCS3-D2. The discrepancy in the present number of predicted BGCs as compared to our
previous results (Bayot-Custodio et al., 2014) might be due to modifications made in the antiSMASH 4.0 software (Blin et al., 2017) used in this study.

The PKS I and PKS III BGCs were screened out from the PAC library because from the list of predicted BGCs (Table 2), we infer that there was a high possibility that these two types of PKS might be responsible for production of insecticidal compound in Streptomyces sp. PCS3-D2. Several variants of bafilomycin have been reported to have insecticidal activity (Kretschmer et al., 1985). To the best of our knowledge, insecticidal alkyl resorcinol has not been reported from any Streptomyces strains although insecticidal counterpart had been reported from plant origin (Kwon et al., 1996). Other previously discovered insecticides from Actinomycetes are also produced by PKS gene clusters (Waldron et al., 2000; Ikeda et al., 1999; Sun et al., 2003). The PKS type II gene cluster in Streptomyces sp. PCS3-D2 was identified to be involved in spore pigment synthesis and was therefore not included in the PCR screening.

Closer analysis of the PKS III BGC showed that only three of the PKS III genes (monoxygenase, methyltransferase and stilbene synthase) had high similarity to the alkyl resorcinol biosynthesis genes. The rest of the PKS III gene cluster did not show similarity to any known PKS Type III gene cluster, making it a potentially novel PKS Type III gene cluster.

Screening of the Streptomyces sp. PCS3-D2 PAC library resulted to the identification of desired clones with insert size up to 140 kb. Cloning of large DNA fragments of Actinomycetes BGCs with sizes up to 150 kb has been reported previously (Alduina et al., 2005; Dai et al., 2011). Because the complete PKS I and PKS III gene clusters were present in PAC16D and PAC222O, they are good candidates for heterologous expression to increase yield of insecticidal compound.

CONCLUSION

Genome mining facilitated the identification of 26 biosynthetic gene clusters including type I and type III polyketide synthases (PKSs) which corresponds to a bafilomycin and alkyl resorcinol gene cluster, respectively. These two PKS gene clusters were successfully screened out from a constructed PAC library of Streptomyces sp. PCS3-D2. Heterologous expression will allow confirmation of insecticidal activity being conferred to Streptomyces sp. PCS3-D2 by either or both these two PKS gene clusters.

ACKNOWLEDGEMENT

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REFERENCES


Biosafety of RNA silencing and genome editing technologies in crop plants: Malaysian and Australian research perspectives

Jennifer Ann Harikrishna a, Rofina Yasmin Othman a, Muhammad Shakirin Mispan a, Sadia Iqbal b, Yong Han b, Michael G. K. Jones b

a Centre for Research in Biotechnology for Agriculture and Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Western Australian State Agricultural Biotechnology Centre, College of Science Health, Engineering and Education, Murdoch University, Perth, Western Australia 6150, Australia

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Abstract. Research in agricultural biotechnology can produce novel solutions to address the ever growing demand for food, feed, renewable materials and renewable energy using increasingly limited resources. Yet research is expensive with long timelines before implementation can disseminate the benefits to society, so there is a need to maximise co-operation and communication between scientists, stakeholders and their governments, to optimise research, its development and the implementation of research outcomes, into mainstream applications. Recognising the impacts of regulations on biosafety, biosecurity and intellectual property policy on strategies for research, senior and early career researchers from two research intensive universities in Malaysia and Australia, held a workshop to identify and to deliberate over two key areas of technology that offer much promise for agriculture, namely RNA silencing and genome editing. A major focus of the workshop was the regulation of new breeding technologies, and how the regulations need to take into account these new technologies. Themes discussed were the need for harmonisation of international legal frameworks and careful use of terminology, standards and guidelines; and the need for good communication and consensus within and between groups of stakeholders and law-makers. This mini-review highlights the deliberations and recommendations from the workshop.

Keywords: Australasia, biosafety, biosecurity, genetic modification, RNAi, new breeding technologies

INTRODUCTION

Agriculture has consistently adopted new technologies to maximise crop productivity, quality and yield. There is little contention that the best available knowledge and resources should be harnessed to address the challenges to agriculture posed by population growth, urbanisation and climate change, while maintaining regard for the environment, and the need for long term sustainability and increased production of food. However, recent increases in fundamental knowledge and the pace of development of new technologies that can be used to manipulate genes and gene expression that are needed to help meet future food needs has outstripped the capacity and related knowledge to support the formulation and enactment of appropriate guidelines and policies to regulate research, development and its implementation in new crop varieties, in particular in relation to biosafety and biosecurity policy. One obstacle is that there is variation in the terminology used by various national and international agencies and authorities and in the
ways which they approach regulation of gene technology. Such differences between regulatory agencies, including different stages of maturity of national regulations can also act as a non-tariff barrier to trade, and this has slowed the adoption of new technologies in some jurisdictions.

Agriculture, climate and socio-economic factors differ widely across, and in some cases within, countries and territories. As a result different crops are grown in different regions, and this is reflected in the spectrum of pests and diseases present, which in turn leads to variation in agricultural practices and requirements from new breeding technologies including biotechnology. In the research sphere, there is also wide variation in the capacity, facilities and stage of development of “biotech crops” and technologies, including RNA silencing methods and genome editing (with or without plant genetic modification). The result of these differences has led to the development of inconsistent regulatory frameworks across the Asia-Pacific region, and includes countries with no legal framework for biosafety, some with regulatory frameworks but little or poor enforcement and those with both strong frameworks and good enforcement and compliance.

In response to these challenges and under the auspices of a “IRU-MRUN” joint research programme under the Innovative Research Universities (IRU, Australia) and Malaysian Research University Network (MRUN, Malaysia), senior and early career researchers from the University of Malaya, Kuala Lumpur, Malaysia and Murdoch University, Perth, Australia, held a joint workshop on “Processes for Biosafety of RNA silencing and Genome editing technologies in crop plants: Malaysian and Australian perspectives” by teleconference on the 12th October 2016. The workshop aimed to address issues relating to new breeding technologies, in particular gene silencing and genome editing, from both research and implementation perspectives, and comprised six lectures each followed by a question and answer session, and concluded with a roundtable discussion. The workshop participants included Jennifer Ann Harikrishna, Rofina Yasmin Othman, Muhamad Shakirin Mispan, Teo Chee How, Katharina Mebus, Tan Boon Chin, Purabi Mazumdar, Pooja Singh, Lee Wan Sin, Umaiyal Munusamy, Lau Su Ee and Tan Yew Seong from the University of Malaya; and Michael G K Jones, Steve Wylie, Sadia Iqbal, Yong Han, John Fosu-Nyarko, Maria Maqsood, Sharmin Rahman, Jebin Akter, Fareeha Naz and Doug Hall from Murdoch University, and Peter Waterhouse from the Queensland University of Technology, Australia. This mini-review provides a summary of the discussions and recommendations from the workshop.

Harmonisation of vocabulary for biosafety legislation and guidelines.

The meeting discussed the differences in vocabulary and terminology used in the various legal frameworks, standards and guidelines for biosafety and biosecurity between different countries and territories. It was suggested that this is a barrier to compliance and collaboration, particularly in relation to transfer of materials across boundaries and which can impact the effective use and development of technology, especially where it is perceived as a barrier to trade or to commercialisation, and which represents a real but unquantified “cost of business”. It was noted that the rapid development of new breeding technologies has led to lack of clarity in the specific terminology among legislators as well as scientists, for example “GMO” (genetically modified organism) and “LMO” (living modified organism) are used to describe the same thing (under different legislations) in Australia and Malaysia respectively (Gene Technology Act 2000; Malaysian Biosafety Act 2007). There is also some uncertainty over the terms and definitions to be used for the new breeding technologies, including the definition of “foreign DNA” and technologies such as RNA interference or RNAi (also often termed “RNA silencing” and “post transcriptional gene silencing” for plants), “genome editing” and “synthetic biology”. It is thus important to have regular dialogue between scientists and legislators both within and between different countries both for clarity and for regional harmonisation.

The meeting also discussed the lack of harmonisation between some of the information requested by biosafety guidelines (or what is “nice to know”) and information that represents the real risks based on scientific evidence from study of about 20 years of experience and safe use of genetically modified crops (what we actually “need to know”). A requirement for extensive but
unnecessary information, or duplication of required information, inflates costs and can discourage the use of new technologies.

To clarify terms, the following diagram was used to provide a comparison of breeding technologies, comparing a simplified conventional plant breeding protocol, with transgenesis (in which a gene from and unrelated organism is transferred), cisgenesis (in which gene from a related or sexually compatible species is transferred) and intragenesis (gene or gene component for the same species).

![Comparison of breeding technologies](image)

**Figure 1.** Comparison of breeding technologies.
(Source: M. G. K. Jones, expanded from original by J. Dunwell, University of Reading).

It can be seen that there is no difference in the mechanism of transgenesis, cisgenesis and intragenesis, the differences lie in the source of the introduced genetic material. In cisgenesis and intragenesis the transferred genes or parts thereof already exist in the gene pool for that genus or species, and could be introgressed in evolutionary time or by conventional breeding. What these approaches do is to widen the gene pool available for conventional breeding, akin to making crosses from wild relatives or land races in conventional breeding. The benefit of the gene transfer approach is its precision, which enables exclusion of unwanted sequences of unknown and possibly undesirable function, and so could well be regarded as less risky than conventional breeding using wide crosses.

The meeting also discussed industry-agreed definitions of different types of New Breeding Technologies. When discussing genome editing technologies, it is useful to consider that classical mutagenic approaches (chemical/radiation) have been used for many years to develop a range of crop varieties. Classical mutagenesis generates randomly multiple mutagenised plants, from which undesirable genotypes are excluded and plants with desired characteristics may be selected. Plants selected in this way are grown widely, and include seedless oranges and ruby red grapefruit. In contrast, Oligonucleotide Directed Mutagenesis (ODM) makes use of a specific oligonucleotide to produce a single DNA base change in the plant genome, which similarly does not contain introduced DNA. Site Directed Mutagenesis (SDNs) make use of specific dsDNAses (eg Fok1, Cas9) and peptides (e.g. ZFNs, TALENs) or more recently oligonucleotides (eg CRISPR/Cas9) that guide
cleavage of both DNA strands at exact sites in the host DNA, and therefore can generate site specific mutagenesis, in a much more precise way than the random breaks cause by classical mutagenesis. This is because the natural process of DNA repair makes mistakes in repairing dsDNA breaks. There are variants of SDN technology, in which when oligonucleotides with ends homologous to each side of the dsDNA break are included, then one or more bases may be inserted at the repair site. These SDNs are sub-classified as: SDN-1 – non-homologous end joining (NHEJ), in which natural repair mechanisms can result in small nucleotide deletions, additions or substitutions; SDN-2 – in the presence of an oligonucleotide template with ends homologous to each side of the double-stranded break, homologous end joining (HEJ) can occur, such that one or more bases can be included in the repaired sequence; SDN-3 – as for SDN-2, but with a longer DNA insert, for example up to a full gene expression cassette.

The question which arises, is where to draw the line in terms of defining what is and what is not a GMO.

In discussing these consequences of new breeding technologies, including the use of guide RNA to modify gene expression or introduce miss-sense/deletion mutants using genome editing, it is clear that some modified plants will not be defined as “transgenic”. From a scientific and safety point of view, crops modified by these methods are no different from plants which would not be regulated under existing biosafety regulations, for example, crops developed using chemical- or radioactivity-induced mutations or where gene expression is modified by environmental factors. Indeed, it can be argued that the new plant varieties developed with these methods present very much lower risks than those produced by random mutation, especially where no new genetic material has been added to the new variety. Indeed, transgenic soybean plants have much less genetic variation from the wild type compared to that between different varieties of soybean or to plants that had been mutagenized (Anderson et al., 2016).

However, there will need to be clarity provided in updated guidelines to cover the new breeding technologies; for example the meeting suggested that no additional methods were required to assess risk for new technologies, as current protocols are sufficient. Two general principles were agreed, these were: (i) It is not desirable or necessary to develop a “third” class of crop products as a result of genome editing and (ii) It was agreed that plant varieties developed using the new breeding methods should not be differentially regulated if they are similar or indistinguishable from varieties that could have been produced by established breeding methods.

As the new technologies are considered by regulators, existing guidelines should be examined and where there are no safety concerns, technologies now regulated should be added to the list of exclusions/exempted methods and materials, based on their track record of safe usage or equivalence to accepted breeding methods. As it is likely that all countries will revise their gene technology regulations to take account of new breeding technologies, such revisions present a real opportunity for dialog and harmonisation of regulations across the Asia-Pacific region with respect to biosafety and biosecurity for crops. The more coherence and congruent the regulations between countries, the more likely risk assessments are to be accepted by other countries, removing the need to repeat assessment studies, which would be especially beneficial to the developing countries in Asia.

The meeting agreed that new strategies of analysis should also be incorporated into the risk assessment process, such as deploying bioinformatics tools that can leverage on the increasing amount of sequence and other biological data that is amassing for many crop plants and varieties to reduce the need for some of the “wet lab” validation. Here again, a regional consensus on the use of risk assessment tools and technology can benefit all parties by sharing of expertise and information.

**Importance of clear communication**

The meeting agreed that good communication in matters relating to biosafety and biosecurity is vital to ensure and assist with regulatory compliance and for the reassurance of consumers and stakeholders. Currently there is much inaccurate and misleading information in the press and online which has adversely impacted on the public perception of biotech crops: there are legitimate concerns for farmers in understanding
new technologies, and in particular organic farmers have often adopted an anti-technology stance, even though organic production yields on average 20-50% less than conventional crops, and others at the food production and preparation end often do not understand the technologies. The different groups of stakeholders require different kinds of information, and different approaches are needed to engage these groups in dialog.

The meeting felt that the general public would mostly have concerns over what may be on their food table, what may be grown near to them and how the crops affect the environment. Since much of the more easily accessed information on GMO is negative and inaccurate, the public needs to be provided with science-based information to correct such misperceptions. Consumer acceptance can have a great influence on the successful introduction of products from biotech crops, although consumer surveys in Asia have also shown that there is generally poor awareness of gene modification technology (ISAAA 2002; Amin et al., 2011; Ismail et al., 2012). Upstream of this, the farmers will have the most exposure to and influence over the choice of crop varieties grown, so they should be engaged both by academic researchers and industry as primary stakeholders.

The meeting suggested that a good model for communication within stakeholders is to consider the approach used in Australia where the public sector research/developers work together at the initiative of private sector stakeholders to find a consensus which is practical, realistic and meets high scientific standards, to assist legislators. The discussion included aspects of avoidance of risk to the environment, avoidance of risk to food supply, ensuring innovation is not restricted and to avoid stifling the development of biotech and trade in biotech goods, especially by small and medium sized industries.

Conclusion and Recommendations

All stakeholders are important for the practical and effective use of new technologies in crops, but scientists can play key roles in this process. Firstly, it is important to have regular dialogue between scientists and legislators both within and between different countries, both for clarity and for regional harmonisation of vocabulary and terminology. Scientists can be a driver for such dialogue as they are already well networked. Industry should be engaged to form strong consensus before bringing issues and suggesting amendments to lawmakers. At present legislation on GMOs usually encompasses all forms of genetic manipulation, but then excludes those which have been used conventionally and have a history of safe usage. A review of existing guidelines to consider additional exclusions and exempted methods should be carried out and based on the track records of safe usage as well as comparisons with knowledge of genetic variation and horizontal gene transfer which occurs naturally. The periodic revision of gene technology legislation being undertaken now or in the future presents a great opportunity for dialogue and harmonisation across the Asia-Pacific region with respect to legislation on biosafety and biosecurity for crops. With better agreement in regulations between countries, the use of such legislation as potential barriers to trade will be reduced, and this would be of great benefit across the Asia-Pacific region. Finally, as indicated above new approaches should be incorporated where appropriate into the risk assessment process (e.g. bioinformatics tools), reducing the need for some of the “wet lab” validation. Here again, a regional consensus on the use of risk assessment tools and technology can benefit all parties by sharing of expertise and information. Ultimately, the aims are to deploy the best tools to provide the most productive crop varieties to farmers, aided by sensible, evidence-based legislation.

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Genetic identification of Javan hawk-eagle (*Nisaetus bartelsi*) from Indonesia using mitochondrial COI gene

Suhadi*, Dwi Listyorini, Riri Wiyanti Retnaningtyas, Fima Rizki Eka Putri, Dina Ayu Valentiningrum

Department of Biology Faculty of Mathematics and Natural Sciences State University of Malang, Indonesia

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Abstract. *Nisaetus bartelsi* is a native hawk from Java which its genetic information is not commonly understood yet. As a part of the conservation effort to maintain genetic diversity of this endangered species, this research aimed to obtain COI gene sequences from five individuals of *N. bartelsi* to confirm its position in the phylogenetic tree. DNA isolation from 5 *N. bartelsi* blood sample was performed and its COI gene sequence was amplified, sequenced, and used to reconstruct phylogenetic tree using MEGA6 with several other members of *Nisaetus*, *Aquila*, and *Saggitaridae* family. Furthermore, the intraspecific distance between 5 *N. bartelsi* samples and interspecific distance with other species were calculated using MEGA6. The result suggested that all five individuals belonged to the species Javan hawk-eagle (*N. bartelsi*) and were closely related to the Blyth’s hawk-eagle (*Nisaetus alboniger*). The DNA barcoding of the Javan hawk-eagle conducted in this study is a stepping stone to conservation efforts for the Javan hawk-eagle.

Keywords: conservation, cytochrome-c oxidase subunit I, DNA barcoding, Javan hawk-eagle

INTRODUCTION

The Indonesian archipelago is renowned for its enormous species endemicity due to its geographical condition. One of the endemic species of Indonesia is the Javan hawk-eagle (*Nisaetus bartelsi*) (Nijman & Sözer, 1998), a diurnal bird of prey living solely in the tropical rainforest of Java (Van Balen *et al.*, 2000). This species mostly inhabits primary rainforest (Van Balen *et al.*, 2001) and is also frequently seen in secondary or even production forests in Java (Syartinilia & Tsuyuki, 2008) ranging from sea level to high mountains, with the most frequent appearance found at 500-1000 m. Furthermore, the estimated average home range of one pair is approximately 400 ha (Gjershaug *et al.*, 2004). The preferred diet of Javan hawk-eagles varies from small mammals to birds and reptiles (Prawiradilaga, 2006).

Javan hawk-eagles’ resemblance to the mythical creature Garuda, which is also the national emblem of Indonesia, has made this species a prominent focus for avian conservation in Java (Van Balen *et al.*, 2000). Furthermore, since the declaration of this species as a National Rare/Precious Animal of Indonesia in 1992 (Widyastuti, 1993), public awareness about the need to preserve their habitat, along with natural resources that support it, has increased. However, this rising awareness has also increased its demand in illegal wildlife trade since in Javanese culture, bird keeping, especially the keeping of expensive and rare birds, is a way to exhibit one’s social status (Nijman, 2009).

The population of this species is decreasing despite the legal status of the Javan hawk-eagle and the ongoing conservation efforts (IUCN, 2016; BirdLife International, 2016). The estimated
population of Javan hawk-eagles throughout the island of Java is around 600-900 individuals, which roughly equals 300-500 mature individuals (IUCN, 2016). The reason for this decrease, besides illegal wildlife trade, is deforestation (Sozer et al., 1998; Collar et al., 2001; Nijman, 2009), which eventually leads to habitat fragmentation (Van Balen et al., 2000). As a result, Javan hawk-eagle populations throughout Java are severely fragmented and, most likely, isolated from each other (Van Balen et al., 2001). Meanwhile, isolated populations are vulnerable to inbreeding depression, which affects birth weight, survival, reproduction and disease resistance due to the loss of genetic diversity within the population; populations with these conditions are prone to extinction (Keller & Waller, 2002).

In response to the race against extinction caused by human disturbance and inbreeding depression, conservation efforts at the molecular level are necessary in order to save any records of the species, including their genetic data, and to maintain the phylogenetic diversity of the Javan hawk-eagle (Frankham, 2003). Taxonomic or phylogenetic distinctiveness is one determining factor in establishing conservation priorities for threatened species, like the Javan hawk-eagle (IUCN, 1980).

In the previous phylogenetic study based on mtDNA sequences Cyt-\(b\) and non-coding control region, Nisaetus bartelsi is sister species to Nisaetus alboniger forming a distinct clade apart from Nisaetus kelaarti and Nisaetus nipalensis; both Nisaetus bartelsi and Nisaetus alboniger belong to the Asian hawk-eagle monophyletic cluster apart from their relatives Spizaetus genus in South America (Haring et al., 2007). Due to the short Cyt-\(b\) sequence, the phylogenetic tree in the previous study resulted in low bootstrap values in the phylogenetic tree especially in the deeper nodes, indicating that further genetic analysis using higher resolution genetic markers is necessary (Haring et al., 2007). Further study based on mitochondrial genes Cyt-\(b\) and 5 nuclear loci also support that Nisaetus bartelsi is sister species to Nisaetus alboniger and both taxa form a separate clade from Nisaetus nipalensis (Lerner et al., 2017). Nevertheless, to clarify the phylogenetic distinctiveness of Javan hawk-eagles, a database of the COI gene is needed.

The cytochrome-c oxidase subunit I (COI) gene is a short sequence of mitochondrial DNA (648-780 bp) that serves as a standardized molecular marker, or commonly known as a DNA barcode, for biological identification with precision up to the species level due to its low variability (1-2%) and low rate of amino acid changes (Hebert et al., 2003; Hebert et al., 2004; Hajibabaei et al., 2007). DNA barcodes can provide a clarification of the genetic distinctiveness of a species by confirming the species name of the taxa based on its unique COI gene sequence (Hajibabaei et al., 2007). To date, however, there is still a lack of reports in GenBank on the genetic data, especially the COI gene, of Javan hawk-eagles living in the primary forests on the island of Java.

A thorough phylogenetic analysis is needed to trace the genetic diversity of this species and to address the lack of genetic data for the Javan hawk-eagle. This is needed to inform conservation programs that aim to preserve the genetic diversity of distinguishable taxonomic units (Lerner & Mindle, 2005). This should be done since concerns about the evolutionary information loss of a species are one of the basic motivations of nature conservation itself (Winter et al., 2012). The COI gene sequence of the Javan hawk-eagle can provide deep insight into the phylogenetic diversity of this species, since it is used as a base to reconstruct a phylogenetic tree and is highly useful in estimating the relationship amongst species, as well as its evolution (Hajibabaei et al., 2007; Winter et al., 2012). Furthermore, the phylogenetic diversity that is defined with DNA barcode sequence data may serve as the most important measure for comparing genetic diversity and establishing a protected habitat across the landscape of Javan rainforests. Additionally, as the DNA barcode database library contains genetic data of species from across the globe, a comparative measure of phylogenetic diversity can serve as a standard metric for conservation assessment (Kress et al., 2015).

The Javan hawk-eagle belongs to the family Accipitridae, along with the other members of genus Nisaetus, such as N. nipalensis, N. alboniger, N. cirrhatus, N. kelaarti, and N. floris. By comparing the COI gene sequence of N. bartelsi with COI genes from the rest of the genera Nisaetus and Spizaetus, we can determine the position of N.
Nisaetus bartelsi genetic identification using COI gene

MATERIALS AND METHODS

Collection of samples. This research was conducted from December 2016 to February 2017 in the Genetic Regulation Laboratory and Biotechnology Division of Central Laboratory of Mineral and Advanced Material, State University of Malang. Javan hawk eagle’s individual which used in this study were from Eco Green Park in Batu City (two individuals; encoded as FM01 and FW01), Cikananga Wildlife Centre in Sukabumi District (two individuals, encoded as RR21 and RR24), and Animal Sanctuary Trust Indonesia (ASTI) in Bogor City (an individual; encoded as RR04). The morphological data was based on morphometry and some supporting profiles provided by Cikananga Wildlife Centre, ASTI and Eco Green Park.

Isolation of DNA and amplification of COI gene. Materials of DNA which used in this study were blood samples taken from pectoralis subclavian veins of each Javan hawk-eagle individual, which were stored in 1000 µl of absolute alcohol. Each blood sample was collected by veterinarians in Eco Green Park, Cikananga Wildlife Center, and ASTI respectively. Samples were sent to the laboratory mentioned above for DNA isolation and COI gene amplification.

Isolation of DNA was performed using DNeasy Blood and Animal Tissue Kit (Qiagen, Cat. No. 69504). Amplification of COI gene was performed using COI primers: (1) forward 5’-TTC TCC AAC CAC AAA GAC ATT GGC AC-3’ and (2) reverse 5’ ACT ACA TGT GAG ATG ATT CCG AAT-3’ (Zein & Prawiradilaga, 2013). COI gene was amplified through polymerase chain reaction (PCR) which consisted of an initial denaturation of 95°C for 5 minutes, followed by five cycles of 94°C denaturation for 1.5 minutes, 57°C annealing for 1.5 minutes, 72°C extension for 1.5 minutes, then the final extension at 72°C for 5 minutes (Zein & Prawiradilaga, 2013). Amplification results were then visualized by performing electrophoresis in 1% agarose gel. After the targeted band of approximately 700 bp was visible, the samples were then sent to First Base Laboratories Malaysia for sequencing.

Analysis of the sequencing result. Sequencing results (in the form of chromatograms of forward and reverse sequence for each sample) were read using FinchTV software (©Digital World Biology LLC), trimmed using MEGA6 based on the read peaks, and combined using DNA Baser to create a consensus sequence (Heracle BioSoft SRL). Multiple alignments were performed using ClustalX2 among forward and reverse sequence for each sample and among encoded samples to produce consensus sequence. Multiple alignment analysis was also used to find the similarity index of consensus sequence from samples against N. alboniger as the same genus from BOLDsystem database (using BOLD system alignment browser) and S. alboginer (accession code: AP008239.1) and S. nipalensis (accession code: AP008238.1) as one family from GeneBank (using Basic Local Alignment Search Tool or BLAST analysis).

Pairwise genetic distance and similarity index were conducted among five encoded samples, S. alboniger as the closest relative and Sagittarius serpentarius as outgroup species. Intrasppecific and interspecific genetic distance in comparison with the members of the Nisaetus genus was conducted using MEGA 6 (Tamura et al., 2013).

The phylogenetic tree analysis was conducted using MEGA 6 with the neigh-borjoining (NJ) (Nei & Saitou, 1987) method according to Kimura 2-parameter distance with five encoded samples and some species provided in GeneBank of National Center for Biotechnology Information (NCBI) for comparison. The species that included for the reference species were in the Nisaetus genus (N. Alboniger accession AP008239.1, N. cirrhatus ROMC331-07, N. tyrranus JQ176245.1, N. melanoleucus JQ176244.1, N. philppensis HM639912.1, N. nipalensis AP008238.1, AB843766.1, AB843173.1, AB843767.1, AB843172.1, AB843171.1, AB843170.1), Aquila
genus (*Aquila chrysaetos* GU571264.1 and GU571738.1), and outgroup from Sagittaridae family (*Sagittarius serpentarius* U83776.1).

### RESULTS AND DISCUSSION

The similarity of encoded samples' species and another. The morphological study conducted on the sample FW01 and FM01 showed that the individuals were 2-3 years old Javan hawk-eagles (*Nisaetus bartelsi*) or were categorized as juveniles along with the sample RR04, RR21, and RR24. The COI gene amplification and electrophoresis results suggested that the targeted gene of approximately 700 bp was obtained. The sequencing results of the five samples, with the code names FM01, FW01, RR04, RR21, and RR24, showed 744, 742, 734, 724, and 741 base pairs, respectively (Figure 1). The identification results from BOLDSystem indicated that each sample was closely related to *N. alboniger*, with a similarity above 98% (Table 1) as there was no similarity at the species level to any sample available in the system due to the lack of genetic information on this species, especially for COI gene. The tree-based identification performed in BOLDSystem also revealed the same results. FM01, FW01, RR04, RR21, and RR24 each formed a sister species clade with *N. alboniger*. However, further analysis using pairwise genetic distance amongst the five samples and *N. alboniger* were necessary to gain further insight into the similarity between the two closely related taxa. According to Table 2, BLAST results of the Javan hawk-eagle COI gene sequence with the GenBank database suggests that the COI gene sequence with the highest similarity was *Spizaetus alboniger*, with a query coverage of 99% and a similarity of 98%.

![Figure 1. The consensus of forward and reverse sequence of the COI gene of samples FM01, FW01, RR04, RR21, and RR24, with lengths of 744 bp, 742 bp, 734 bp, 742 bp, and 741 bp respectively.](image)

<table>
<thead>
<tr>
<th>No</th>
<th>Sample code</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FW01</td>
<td>98.35</td>
</tr>
<tr>
<td>2</td>
<td>FM01</td>
<td>98.35</td>
</tr>
<tr>
<td>3</td>
<td>RR04</td>
<td>98.30</td>
</tr>
<tr>
<td>4</td>
<td>RR21</td>
<td>98.35</td>
</tr>
<tr>
<td>5</td>
<td>RR24</td>
<td>98.51</td>
</tr>
</tbody>
</table>

Table 1. The similarity of javan hawk-eagle from samples against *N. alboniger*.

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Table 2. The similarity of *N. bartelsi* from samples against *Spizaetus* genus.

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>Accession code</th>
<th>Query cover (%)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Spizaetus alboniger</em></td>
<td>AP008239.1</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td><em>Spizaetus nipalensis</em></td>
<td>AP008236.1</td>
<td>98</td>
<td>97</td>
</tr>
</tbody>
</table>

The genetic distance of Javan hawk-eagle and other species. According to the pairwise genetic distance of the five samples, with *S. alboniger* AP008239.1 as the closest relative and *Sagittarius serpentarius* U83776.1 as an outgroup, samples FM01, FW01, RR04, RR21, and RR24 had a pairwise genetic distance of 0.000, meaning that all samples had no difference in their COI gene sequences. The genetic distance of 0.000 for each sample thus indicated that all samples belonged to the same species. Furthermore, the pairwise genetic distance of each of the five individuals of
N. bartelsi compared to N. alboniger or S. alboniger AP008239.1 supported the previous result by showing a genetic distance of 0.021; in other words, the similarity of the said taxa was 97.9% (Table 3), meaning that the five samples and S. alboniger AP008239.1 were sister species since genetically distinct but closely related species show low divergences, ranging from 0.6-2.0%, while divergence values between species are ordinarily greater than 3% (0.03) (Hebert et al., 2003).

The confirmed status of the five samples as Javan hawk-eagles (N. bartelsi), according to their pairwise genetic distances, became the ground for the grouping in the intraspecific and interspecific genetic distance analysis. According to the intraspecific genetic distance analysis, the Javan hawk-eagle (N. bartelsi) has no genetic variation within the species with a genetic distance of 0.000 (Table 4). This implied that there was no genetic variation within the species group of Nisaetus bartelsi consisting of the five samples; thus, it did not only confirm the species label as Javan Hawk-Eagle (Nisaetus bartelsi) but also the fact that the five individuals COI gene sequences were conserved regardless the habitat fragmentation that isolates the population. However, to confirm this, more data on COI gene sequences of Javan hawk-eagle is needed.

Table 3. Pairwise genetic distance amongst N. bartelsi, S. alboniger (ingroup; accession code: AP008239), and Sagittarius serpentarius (outgroup; accession code: U83776).

<table>
<thead>
<tr>
<th></th>
<th>RR24</th>
<th>FW01</th>
<th>FM01</th>
<th>RR21</th>
<th>RR04</th>
<th>AP008239</th>
<th>U83776</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR24</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.007</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.007</td>
<td>0.027</td>
</tr>
<tr>
<td>FM01</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.007</td>
<td>0.027</td>
</tr>
<tr>
<td>RR21</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.007</td>
<td>0.027</td>
</tr>
<tr>
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<td>0.027</td>
</tr>
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<td>0.021</td>
<td>0.021</td>
<td>0.021</td>
<td>0.021</td>
<td>0.007</td>
<td>0.026</td>
</tr>
<tr>
<td>U83776</td>
<td>0.217</td>
<td>0.217</td>
<td>0.217</td>
<td>0.217</td>
<td>0.217</td>
<td>0.217</td>
<td>0.209</td>
</tr>
</tbody>
</table>

Table 4. The intraspecific genetic distance of N. bartelsi against few species from Genebank.

<table>
<thead>
<tr>
<th>Genebank species</th>
<th>Distance</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. tyrannus</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>N. bartelsi</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>S. alboniger</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>N. nipalensis</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Sagittarius serpentarius</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>Aquila chrysaetos</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>S. melanoleucus</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>N. cirrhattus</td>
<td>n/c</td>
<td>n/c</td>
</tr>
<tr>
<td>S. philippensis</td>
<td>n/c</td>
<td>n/c</td>
</tr>
</tbody>
</table>

n/c: not covered and genetic distance's value was very high

Interspecific genetic distance was analyzed to disclose the relationship amongst the species N. bartelsi with the other closely related taxa. The data revealed that Nisaetus alboniger had a genetic distance of 0.013 compared to N. bartelsi, which supports its status as the sister species of the Javan hawk-eagle (N. bartelsi). Moreover, the next closest relative of N. bartelsi was Nisaetus nipalensis, with a genetic distance of 0.021. The furthest relative of N. bartelsi, according to the interspecific genetic distance, was S. tyrannus, with a genetic distance of 0.111 (Table 5).
Table 5. Interspecific genetic distance of few genus in Accipitridae family.

<table>
<thead>
<tr>
<th></th>
<th>C. tyrrannus</th>
<th>S. bartelsi</th>
<th>N. alboniger</th>
<th>N. nipalensis</th>
<th>Sagittarius serpentarius</th>
<th>Aquila chrysaetos</th>
<th>S. melanoleucus</th>
<th>N. cirrhatus</th>
<th>S. philippensis</th>
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<tbody>
<tr>
<td>S. tyrrannus</td>
<td>0.017</td>
<td>0.016</td>
<td>0.016</td>
<td>0.074</td>
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</tr>
<tr>
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<td>0.013</td>
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<tr>
<td>S. alboniger</td>
<td>0.108</td>
<td>0.013</td>
<td>0.006</td>
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<td>0.014</td>
<td>0.013</td>
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<td></td>
</tr>
<tr>
<td>N. nipalensis</td>
<td>0.103</td>
<td>0.021</td>
<td>0.021</td>
<td>0.069</td>
<td>0.015</td>
<td>0.014</td>
<td>0.013</td>
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<td></td>
</tr>
<tr>
<td>Sagittarius serpentarius</td>
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<td>0.850</td>
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<td>0.850</td>
<td>0.072</td>
<td>0.072</td>
<td>0.071</td>
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<td></td>
</tr>
<tr>
<td>Aquila chrysaetos</td>
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<td>0.088</td>
<td>0.088</td>
<td>0.086</td>
<td>0.850</td>
<td>0.015</td>
<td>0.015</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>S. melanoleucus</td>
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<td>0.014</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N. cirrhatus</td>
<td>0.102</td>
<td>0.081</td>
<td>0.076</td>
<td>0.850</td>
<td>0.091</td>
<td>0.089</td>
<td>0.009</td>
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<tr>
<td>S. philippensis</td>
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<td>0.089</td>
<td>0.083</td>
<td>0.863</td>
<td>0.101</td>
<td>0.081</td>
<td>0.037</td>
<td></td>
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</tr>
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</table>

Phylogenetic tree of Javan hawk-eagle. According to the neighbor-joining tree reconstruction, we confirmed that the five samples, FM01, FW01, RR04, RR21, and RR24 belonged to the same species since they formed a single clade with a bootstrap value of 97. The tree also confirmed that the reference sequence, S. alboniger AP008239.1 and N. bartelsi were sister species, with a bootstrap value of 88. They formed a monophyletic branch along with N. nipalensis, with a bootstrap value of 97. This monophyletic group split from the other Spizaetus groups, consisting of S. tyrrannus, S. melanoleucus, N. cirrhatus, and S. philippensis (Figure 2).

Figure 2. Phylogenetic tree of Javan hawk-eagle and the other species in Accipitridae family.
The Javan hawk-eagle used to be grouped in the genus Spizaetus, which distributes in South America, due to their shared morphological characteristics, including long tails and feathered tarsi. The latest studies at the molecular level suggest that all hawk-eagle species in Asia separated earlier from genus Spizaetus and formed a monophyletic branch named Nisaetus (Gamauf et al., 2005; Helbig et al., 2005; Lerner et al., 2017). However, the new phylogeny and taxonomy did not necessarily change the registered sequence names in the Genebank of the NCBI database. This explains why several reference sequences in this study still bear the genus name Spizaetus.

Genus Nisaetus comprises all species of hawk-eagles distributed in Asia, including Southeast Asia, with some species endemic to particular areas (Lerner et al., 2017). The geographical condition of southeast Asia is the major factor for its high rate of species endemity, with the Javan hawk-eagle as an example (Gamauf et al., 2005). The Javan hawk-eagle particularly inhabits the primary rainforests of Java and shares the habitat generically with the changeable hawk-eagle (N. cirrhatus) (Nijman, 2004). The two species prefer similar prey. Thus, they also share the same ecological role as top-order predators. Despite the similarities in their ecological roles on the island of Java, according to this study, the two species are not closely related; in fact, the two species sit on different clades.

On the other hand, according to this study, the Javan hawk-eagle was at the same clade with the Blyth’s hawk-eagle (N. alboniger or S. alboniger AP008239.1), which is distributed in the closed forests of Sumatra and Borneo, as sister species. Morphologically, the Javan hawk-eagle shares many characteristics with the Blyth’s hawk-eagle (N. alboniger), such as the feathered tarsi, plumage pattern and distinct crest, other than their color. N. bartelsi has cinnamon brown plumage that grows darker, barred breast and stripes that grow clearer as the bird ages. Meanwhile, its sister species, the Blyth’s hawk-eagle (N. alboniger), has black and white plumage with a spotted breast and barred underparts (IUCN, 2016). This is because of the Javan hawk-eagle (N. bartelsi) along with the Blyth’s hawk-eagle (N. alboniger) came from different lineages of the changeable hawk-eagle (N. cirrhatus) (Gjershaug, 2006).

The changeable hawk-eagle (N. cirrhatus) has a much wider distribution, ranging throughout southeast Asia, specializing in forest edges and open woodlands (Ferguson & Christie, 2001). In Java, it can also be found in primary rainforests, along with the Javan hawk-eagle (Nijman, 2004). However, the interspecific genetic distance data reveals that the two species are not closely related, despite their similar habitat preferences and hunting behaviors. The high interspecific genetic distance between N. bartelsi and N. cirrhatus (0.081) indicates that the two groups had already split even before the South American and African hawk-eagle lineages separated (Lerner & Mindell, 2005; Haring et al., 2007); a close interspecific genetic distance of 0.013 with N. alboniger shows that the Javan hawk-eagle (N. bartelsi) split from their ancestral population due to the rising sea level, which separated Java from Sumatra and Borneo and isolated the Javan hawk-eagle population (Gamauf et al., 2005; Haring et al., 2007; Lerner et al., 2017).

Moreover, the Javan hawk-eagles and the changeable hawk-eagles in Java are segregated by habitat, by the topography of the terrains, and partially by altitude. N. bartelsi occurs over a smaller range of habitats than N. cirrhatus. The once dense continuous rainforest of Java has been fragmented into numerous small patches or has been replaced by more open habitat types, to which Javan hawk-eagles are not well adapted (Nijman, 2004). This condition enhances the significant genetic divergence with the changeable hawk-eagle and generates this species’ endemity as it prevents gene flow from one population of Javan hawk-eagles to the others (Gamauf et al., 2005).

A reliable diagnosis of the taxonomic status of populations is essential for conservation because unrecognized species may become extinct due to a lack of information, including genetic information, which can lead to a lack of protection (Gamauf et al., 2005). A taxonomic unit as detailed as the species level is usually prioritized in conservation measures as an evolutionarily significant unit (Frankham, 2002). As a result, the phylogenetic species concept affects conservation more than any other disciplines (Collar, 1997).
CONCLUSION

The COI gene sequences obtained from the five samples with the code names FM01, FW01, RR04, RR21, and RR24 had 744, 742, 734, 724, and 741 base pairs, respectively. According to the phylogenetic analysis, Javan hawk-eagles FM01, FW01, RR04, RR21, and RR224 was confirmed as one species, *N. bartelsi*, a sister species of *Nisaetus* (*Spizaetus*) alboniger AP008239.1. Thus, the DNA barcoding of the Javan hawk-eagle conducted in this study is a stepping stone to conservation efforts for the Javan hawk-eagle.

REFERENCES

Investigating anti-neuroinflammatory mechanism of orientin in lipopolysaccharide-induced BV2 microglia cells

Pei Hong Gan¹, Erna Laere², Anna Pick Kiong Ling²*, Kenny Gah Leong Voon², Rhun Yian Koh², Ying Pei Wong¹

¹Division of Applied Biomedical Sciences and Biotechnology, School of Health Sciences, International Medical University, Bukit Jalil 57000 Kuala Lumpur, Malaysia
²Department of Pathology, School of Medicine, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

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Abstract. Chronic neuroinflammation in central nervous system (CNS) can lead to neurodegenerative diseases (ND). This was due to the over-activated microglia, which releases excessive pro-inflammatory mediators. The molecular mechanisms of orientin as anti-neuroinflammatory are yet to be fully elucidated. In order to investigate the effect of orientin on LPS-stimulated BV2 microglial cells, the cells were pre-treated with orientin at maximum non-toxic dose (MNTD) (15 µM) or half MNTD (½ MNTD) (7.5 µM) for 3 hours, followed by incubation with 0.1 µg/mL of LPS for 24 hours. The LPS-stimulated cells were then subjected to three series of studies, including the determination of ROS level using 2',7'-dichlorofluoresceindiacetate (DCFH-DA) methods and the determination of mRNA of nuclear factor (NF)-κB, Signal transducer and activator of transcription 1 (STAT1), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) via real-time PCR (qPCR). The findings from this study demonstrated the probable mechanism of orientin in treating neuroinflammation via the downregulation of ROS level, STAT1, NF-κB, iNOS and COX-2 whilst upregulating HO-1. Validation of molecular mechanism of orientin suggested that it could be a potential therapeutic agent in treating ND.

Keywords: anti-neuroinflammatory, microglia, neurodegenerative diseases, neuroinflammation, orientin

INTRODUCTION

Neurodegenerative diseases (ND), such as Alzheimer’s disease (AD), Parkinson’s diseases (PD), Huntington disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, etc. are diseases characterized by progressive damage in the nerve cells. The deterioration will gradually result in the loss of motor and also cognitive functions such as memory and decision making (Chen et al., 2016). According to National Institute of Health (NIH), ND affect millions of people worldwide, with AD and PD as the most common types (Katzman, 2008). It was reported that there are more than 5 million American living with AD, while roughly 500,000 living with PD (Katzman, 2008). The cases were expected to increase from 13.5 million in 2000 to 36.7 million in 2050 (Katzman, 2008). Even though the etiologies of ND remain unclear, recent studies indicated that neuroinflammatory processes are closely related to several neurodegenerative pathways leading to ND (Chen et al., 2016; Frank-Cannon et al., 2009, Streit et al., 2004).

* Author for correspondence: Dr. Anna Pick Kiong Ling, Division of Applied Biomedical Sciences and Biotechnology, School of Health Sciences, International Medical University, Bukit Jalil 57000 Kuala Lumpur, Malaysia. Email – anna_ling@imu.edu.my
Microglia cells are said to be the key cellular mediators of neuroinflammatory processes, which result in ND (Frank-Cannon et al., 2009; Streit et al., 2004; Rojo et al., 2008; Rogers et al., 2007). Studies suggested that when there are stimulus such as lipopolysaccharide (LPS), hypoxia, trauma or neurotoxins acting on microglia cells, it will initiate the neuroinflammatory response in central nervous system (CNS) by releasing pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α), and IL-6 (Chen et al., 2016; Frank-Cannon et al., 2009; Nakajima & Kohsaka, 2001). The release of these pro-inflammatory cytokines will eventually lead to the activation of transcription factors, such as nuclear factor kappa-B (NF-κB) and signal transducer and activator of transcription 1 (STAT1) (Lawrence, 2009), with subsequent upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in order to get rid of the intruders and tissue damage, hence promoting the neuronal survival (Chen et al., 2016; Frank-Cannon et al., 2009; Nakajima & Kohsaka, 2001; Hsieh & Yang, 2013). In short, the transmembrane receptor in CNS will firstly recognize the inflammatory stimulus followed by the transmission of the signal to the nucleus for pro-inflammatory gene activations (Ahmed et al., 2015). This activation of pro-inflammatory genes is then controlled by the selective binding of transcription factors to the promoters of these genes (Ahmed et al., 2015).

However, when the cells are left untreated, it will eventually result in sustained activation of the microglia cells, causing uncontrolled release of pro-inflammatory factors that lead to continuous activation of NF-κB and STAT1, which then result in high expression of iNOS and COX-2 and eventually contribute to chronic neuroinflammation. In addition, over-activated microglia cells may also produce excessive amounts of reactive oxygen species (ROS) leading to oxidative stress (Hsieh & Yang, 2013; Yune et al., 2004; Massa et al., 2006). As a normal feedback mechanism, this oxidative stress will trigger the upregulation of heme oxygenase-1 (HO-1), regulating the imbalance ROS levels, hence, exerting an anti-oxidative and anti-neuroinflammatory effects (Hsieh et al., 2013; Chau, 2015). In short, the uncontrolled release of pro-inflammatory factors and the oxidative stress will lead to chronic neuroinflammation, causing cumulative neuronal dysfunction, neuronal death and eventually neurodegenerative disorders (Luu & Block, 2010). Therefore, targeting the microglia cells could be one of the potential therapeutic approaches in treating ND.

The current anti-inflammatory agents are nonsteroidal agents (NSAIDs), such as indomethacin and rofecoxib, which are shown to have anti-neuroinflammatory effects (Kulkarni et al., 2005; Ajmone-Cat et al., 2010). However, these drugs were known to cause adverse effects in long term treatment (Kulkarni et al., 2005). For instance, Kanatani et al. suggested that indomethacin is associated with the damage of gastric mucosal (Kanatani et al., 2004). Besides, rofecoxib was shown to possess cardioxicity which will lead to heart failure (Mamdani et al., 2004). Due to all these adverse effects of NSAIDs, researchers started to divert their focus on some active constituents derived from medicinal plants, particularly the flavonoids. For example, studies have shown that flavonoid quercitin extracted from Juglans mandshurica could be a potent anti-neuroinflammatory agent (Kulkarni et al., 2005). Besides, flavonoid such as cudraflavanone D and paeonol have shown their anti-neuroinflammatory responses in activated microglia cells through the inhibition of nitric oxide (NO), ROS production and the expression of NF-κB, iNOS and COX-2 (Lin et al., 2015; Himaya et al., 2012; Kim et al., 2016), whereas curcumin was found to induce the HO-1 expression in microglia cells, providing an anti-oxidative effects (Parada et al., 2015). In addition, both oroxylin A and luteolin have also shown to suppress the expression of NF-κB and STAT1 in LPS-induced BV2 microglia cells, which resulted in its anti-neuroinflammatory effects (Liu et al., 2012; Kao et al., 2011).

In this study, orientin was chosen among all the flavonoids. Orientin is a water-soluble c-glycoside flavonoids that can be found or isolated from various medicinal plants such as Ocimum sanctum (holy basil), Phyllostachys species (bamboo leaves), Passiflora species (passion flowers), Trollius species (Golden Queen), and Jatrophaossypifolia (Bellyache Bush) (Lam et al., 2016). Orientin are widely reported to have beneficial properties such as anti-oxidative (An et al., 2012), anti-thrombotic
and anti-platelet (Lee & Bae, 2015), anti-viral and anti-bacterial (Li et al., 2004), anti-inflammation (Bae, 2015), vasodilation (Fu et al., 2005), neuroprotective (Law et al., 2014), radioprotective (Nayak et al., 2006), anti-adipogenesis (Kim et al., 2010) and antinociceptive (Da Silva et al., 2010), anti-inflammatory and anti-oxidative properties (An et al., 2012; Bae, 2015; Kim et al., 2010; Yu et al., 2015; Seo et al., 2012) that has stimulated the interest to further study this compound in the present study. At the molecular level, Yu et al. showed that orientin could alleviates oxidative stress in β-amyloid (Aβ)-mediated mouse model with Alzheimer’s disease through the activation of Nrf2/HO-1 signaling (Seo et al., 2012). The activation of this signaling pathway induced the translocation of Nrf2, which then upregulated the expression of HO-1, activating the redox signaling pathway by exerting an anti-oxidative property (Seo et al., 2012). In addition, Seo et al. suggested that orientin isolated from Spirodela polyrhiza ethanolic extract possesses anti-inflammatory effect through the inhibition of iNOS and COX-2 expression by attenuating the expression of NF-kB p65 in LPS-induced RAW264.7 cells (Kim et al., 2010; Matsuo et al., 2005).

Even though orientin has demonstrated various bioactivities, its anti-neuroinflammatory activity is not well studied or elucidated. Thus, this is a worthwhile study as elucidation of molecular mechanism could contribute largely in the development of new therapeutics that are capable of preventing neuroinflammation. As a potential therapeutic agent in treating ND, the bioavailability of orientin in crossing the blood-brain barrier (BBB) is a concern. There has been study suggesting that orientin have difficulty in crossing the BBB (Li et al., 2008). However, studies showed that the function of BBB is altered in ND (Ohtsuki et al., 2010; Jaeger et al., 2009). Therefore, although orientin have difficulty in crossing BBB under normal physiological condition, the BBB permeability of compound might be altered and enhanced in neurodegenerative conditions. Hence, with the objectives to determine the effects of intracellular reactive oxygen species (ROS) and the mRNA and protein expression of iNOS, COX-2, STAT1, NF-κB and HO-1 upon treatment with orientin, these findings could reveal the possibility of orientin to be used as potential therapeutic agent in neuronal diseases.

**MATERIALS AND METHOD**

**Preparation of orientin stock solution.** The pure orientin compound was purchased from Sigma Aldrich, USA. It was then dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) to prepare a 22.3 mM of stock solution. Then, the stock solution was further diluted to the desirable concentrations using complete Dulbecco’s Modified Eagle Medium (DMEM) medium (GIBCO, USA) before treating the cells.

**Cell culture and maintenance.** The BV2 microglia cells used in this study was provided by Dr. Sharmili of University Putra Malaysia (UPM). BV2 cells are a primary form of active immune defense in the central nervous system and had been reported to be a valid model and substitute for primary microglial in many experimental settings (Henn et al., 2009; Ackerman et al., 2015). The cells were cultured in complete DMEM medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, South America), 1% Penicillin and Streptomycin (GIBCO, South America), 0.1% Fungizone (GIBCO, South America) and 0.1% gentamycin (GIBCO, China), and were maintained in a 5% CO₂ incubator (RS Biotech, UK) at 37°C.

For continuous maintenance, the cells were subcultured into new tissue culture flasks (Corning, USA) when the confluency reached 70%. The procedures were initiated with the removal of culture medium, followed by washing with 2 mL of sterile phosphate buffer solution (PBS) (AMRESCO, USA). Next, 1 mL of 0.25% trypsin-EDTA (GIBCO, Canada) was added and incubated for few minutes in the 5% CO₂ incubator at 37°C. As soon as the cells detached, 1 mL of fresh culture medium was added into the flask in order to inactivate the trypsin. Then, the cell suspension was centrifuged at 0.4 g for 5 min. After centrifugation, the supernatant was then discarded and the pellet was resuspended with 2 mL culture medium. Lastly, 1 mL of the cell
suspension was added into each new T-flask containing 4 mL fresh culture medium.

As for storage purpose, the pellet was resuspended with 1 mL of freezing medium consisting of 10% DMSO and 90% FBS after the centrifugation step. It was then transferred to cryovial and stored at -80°C or liquid nitrogen.

**Determination of maximum non-toxic dose (MNTD).** About 100 µL of BV2 microglial cells at the density of 8x10^5 cells per mL were seeded in 96-well plates (Corning, USA). Once the confluency of the cells reached about 70%, the medium was removed from each well and the cells were treated with a range of orientin concentrations (0, 0.032, 0.16, 0.80, 4, 20 and 100 µM) to determine its cytotoxic effect on BV2 microglial cells. After 24 hours of incubation, the viability of the cells was assessed using MTT assay by adding 20 µL of 5 mg/L MTT (Bio Basic, Canada) into the wells containing BV2 microglial cells. After 4 hours of incubation at 37°C, the solution was removed carefully. Then, 100 µL of solvent grade DMSO (Friendemann Schmidt, Germany) was added and mixed well to dissolve the formazan. The absorbance was then measured at 570 nm using microplate reader (Dynex, USA).

Based on the absorbance measured, the percentage of cells viability as well as percentage of cytotoxicity were calculated using the following formulae:

\[
\text{Percentage of viability} (\%) = \frac{\text{Absorbance reading of treated cells} - \text{Absorbance reading of medium}}{\text{Absorbance reading of control cells} - \text{Absorbance reading of medium}} \times 100\%
\]

\[
\text{Percentage of cytotoxicity} (\%) = 100\% - \text{percentage of cell viability} (\%)
\]

**Determination of optimal lipopolysaccharide (LPS) concentration.** A total of 1x10^5 BV2 microglial cells were seeded in each well of 24-well plate (Nuclon, Denmark). At 70% confluent, the cells were treated with 0.0, 0.1, 1.0 or 10.0 µg/mL of LPS (Sigma, USA) and incubated for 24 hours. After 24 hours, the level of nitric oxide (NO) being released was measured. Griess test was used to measure the nitric oxide level, whereby 50 µL of the supernatant was mixed with 50 µL Griess reagent (Sigma, Germany). After 15 minutes, the absorbance reading at 540 nm was measured using microplate reader (Molecular Devices, USA). In this study, fresh culture medium was used as the control. The effects of LPS on the morphology of the cells were observed under inverted microscope (Nikon Eclipse Ti-80, Japan) at 100x magnification and compared with the untreated cells.

**Cell treatment and stimulation.** BV2 microglial cells at density of 5x10^5 cells per well was seeded in 60 mm petri dishes (Thermo Scientific, USA) and incubated for 24 hours. Once the confluency of the cells reach about 70 to 80%, the cells were treated with orientin at MNTD (15 µM as determined above) or half MNTD (7.5 µM) and were incubated for 3 hours. Next, 0.1 µg/mL of lipopolysaccharide (LPS) (Sigma Aldrich, USA) was added to the treated cells to stimulate inflammation and was further incubated for 24 hours. After 24 hours, the cells were subjected to determination of intracellular ROS level, the mRNA expression of iNOS, COX-2, STAT1, NF-kB and HO-1 through real time PCR. An independent set was conducted for the determination of ROS as well as mRNA expression, respectively. In this study, indomethacin (Sigma Aldrich, USA) at 25 µM was used as the positive control. All the studies were conducted with 7 treatment groups: (1) untreated cells, (2) cells treated with ½ MNTD (7.5 µM), (3) cells treated with MNTD (15 µM), (4) cells treated with 0.1 µg/mL LPS, (5) cells treated with ½ MNTD (7.5 µM) and 0.1 µg/mL LPS, (6) cells treated with ½ MNTD (7.5 µM) and 0.1 µg/mL LPS, (7) cells treated with 25 µM Indomethacin and 0.1 µg/mL LPS, (8) cells only.

**Determination of reactive oxygen species (ROS) level.** After 24 hours of incubation with LPS, the medium and cells were collected and centrifuged at 0.4 g for 5 min. After centrifugation, the pellet was resuspended in PBS. Next, 100 µL of each cell suspension was added into 96-well plates (Corning, USA), which then followed by the addition of 100 µL of 40 µM 2',7'-dichlorofluorescindiacetate (DCFH-DA) (Sigma Aldrich, USA). The contents were mixed well and were measured at excitation wavelength of 485 nm and emission wavelength of 538 nm using Tecan Infinite F200 Multifunctional Microplate Reader (Tecan, USA) after 10 min of incubation. Then, the cell concentration was determined using trypan blue exclusion methods, in which 10
μL of the sample was mixed with 10 μL of trypan blue (Thermo Scientific, USA). A total of 10 μL of the suspension was then placed on the haemocytometer (Abcam, USA) for cell counting and the cell concentration of each treatment was calculated. The ROS level in each treatment was expressed as Relative Fluorescence Unit (RFU) per unit of cells.

**Determination of mRNA expression.** After 24 hours of incubation with LPS, RNAs were extracted and purified from the treated and untreated BV2 microglia cell lines by PureLink® RNA Mini Kit (Ambion, USA). Then, cDNA was synthesized from the RNA in Peltier Thermal Cycler-100 (MJ Research, USA) through qPCRBIO cDNA Synthesis Kit (PCR Biosystems, UK) following the manufacturer’s instructions. Measurement of cDNA was then carried out using Tecan Infinite F200 Multifunctional Microplate Reader at Nanoquant filter wavelength of 260 nm. Then, equal amount of cDNA samples (50 μg) with 2X qPCRBIO SYGreen and specific iNOS, COX-2, STAT1, NF-κB (subunit p65), HO-1 and β-actin forward and reverse primers (Table 1) were added and mixed well in semi-skirted 96-well PCR plates (Molecular Bioproducts, USA). Quantitative real-time PCR was performed using iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, US). Lastly, the mRNA expression level of iNOS, COX-2, STAT1, NF-κB, HO-1 and β-actin were analysed and CT values were obtained. In this study, β-actin was used as the housekeeping gene in normalization of data. Relative mRNA expression were determined using Double Delta CT analysis (Kenneth & Thomas, 2002).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
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<td>5'-CACCCAAAGTGGCTTCTAGTCA-3'</td>
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<tr>
<td>COX-2</td>
<td>5'-TGCGTCAAGGCAAAATACAGG-3'</td>
<td>5'-GAAGTGTGGCAAGAAAGATG-3'</td>
</tr>
<tr>
<td>STAT1</td>
<td>5'-CTTACCTTCTCCTCCTGGG-3'</td>
<td>5'-TCCCGTACAGATGTCTCATGAT-3'</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5'-CTGTTGACACATACAGGAAGAC-3'</td>
<td>5'-ATAGGCACTGTCTTTCTCACCTC-3'</td>
</tr>
<tr>
<td>HO-1</td>
<td>5'-AGGCCCAACAGTCCAACAAC-3'</td>
<td>5'-CATCACCTGACCTCTCAAA-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-TCCCTCTGAGCGGAAGTACTCT-3'</td>
<td>5'-GTCAGTAAACAGTCCGCTA-3'</td>
</tr>
</tbody>
</table>

The abbreviations of genes; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; STAT1: signal transducer and activator of transcription 1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; HO-1: heme oxygenase-1; β-actin: Beta-actin.

**Statistical analysis.** The data obtained from three independent experiments with triplicates each were analysed and represented as mean ± SD (standard deviation). Significant differences at $p<0.05$ were examined using One way analysis of variance followed by Tukey’s multiple comparison test using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

**Determination of MNTD.** MNTD is the highest concentration of orientin that did not cause any cytotoxic effect on BV2 microglia cells. It was determined by plotting a graph of percentage of cytotoxicity against concentrations of orientin (Figure 1). From Figure 1, there was a very clear relationship between the orientin concentrations and its cytotoxic effect on the cells. The studies demonstrated that at ≤15 μM of orientin, the concentrations were not cytotoxic to BV2 microglia cells. Thus, the MNTD and ½ MNTD of orientin that were determined in this study were 15 μM and 7.5 μM, respectively. At these concentrations, BV2 cells showed a normal morphology of small soma with distal arborization, characteristic of “ramified” microglia (Dai et al., 2015). This concentration is lower than the MNTD of orientin (20 μM) towards SH-SY5Y cells (Law et al., 2014). By comparing MNTD of orientin in these two cells, it seems to indicate that orientin is more toxic towards BV2 microglia cells compared to SH-SY5Y cells. Based on this comparison, it proved...
that the cytotoxic effect of orientin might depended on the treated cell types, most probably due to structure-cytotoxicity relationship between orientin and the cells (Matsuo et al., 2005). It was proposed that cytotoxic effect of flavonoids will be higher if the permeability of the cells towards them is higher (Matsuo et al., 2005).

Figure 1. Percentage of cytotoxicity of orientin on BV2 microglial cells. After 24 hours of incubation with orientin at the range of 0 to 100 µM, the viability of the cells was measured by MTT assay. At ≤15 µM of orientin, the concentrations were not cytotoxic to BV2 microglia cells. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Meanwhile, at lower concentration, particularly at 0.16 µM, negative cytotoxicity values were recorded that indicated that orientin exhibited promoting growth effects on BV2 microglia cells. At this concentration, the growth of BV2 cells was promoted by 4.19% as compared to the untreated cells. This observation might be due to the antioxidant activity of orientin that helped to scavenge the excessive free radicals produced in normal cells during ATP production in mitochondria, which eventually promote the growth of the cells (Praveena et al., 2014, Middleton et al., 2000; Khan, 2012; Spencer et al., 2012; Droge, 2002). The high antioxidant activity of orientin are known to be due to the presence of ring B, C-glucoside, maximum numbers of hydroxyl group and the ability to form intramolecular hydrogen bonding in its molecular structure (Praveena et al., 2014; Middleton et al., 2000).

However, as the concentration of orientin increased above 15 µM, there was a marked increase in the percentage of cytotoxicity of BV2 microglial cells. For instance, approximately 37% of an increase was recorded in treatment using 100 µM relative to 15 µM. The cytotoxic effect of orientin above MNTD might be related to the generation of intracellular ROS, which was demonstrated by some other flavonoids (Matsuo et al., 2005). The findings by Matsuo and her colleagues proved that at higher concentrations, flavonoids would have an increased ability to be incorporated into the cells thus, increased the intracellular ROS generating ability as well as the levels of few oxidative metabolites, which subsequently contributed to cell death (Matsuo et al., 2005).

**Determination of optimal LPS concentration.**

To determine the concentration of LPS that could induce maximum inflammation on BV2 microglia cells, two parameters were analysed: the release of NO by the cells and cell cytotoxicity. The optimal concentration of LPS that was selected in this study is the concentration that would cause the cells to release a significant amount of NO but insignificantly cytotoxic to the cells.

Under physiological conditions, NO acted as neuromodulator and neurotransmitter in the brain that helped to maintain the function of neuronal and vascular cells (Lowenstein et al., 1994). There are two types of NO synthase (NOS) enzymes involved in the production of NO: neuronal NOS (nNOS) and endothelial NOS (eNOS) (Bredt & Snyder, 1990). They are calcium (Ca²⁺)-dependent enzymes, which are constitutive forms of NOS (Bredt & Snyder, 1990). Nevertheless, NO has been selected as a parameter of neuroinflammation in many studies, including the present study due to its well-known role under pathological conditions in CNS (Ghasemi & Fatemi, 2014). The inflammatory stimuli will induce the expression of iNOS in activated microglial cells and lead to NO production (Dello Russo et al., 2004). Overproduction of NO has been correlated with many neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and amyotropic lateral sclerosis (Yuste et al., 2015).

Findings from this study showed a significant dose-dependent increase in the release of NO by BV2 microglia cells (Figure2A). As LPS concentration increased to 0.1, 1.0, and 10.0
µg/mL, the produced NO were 2.33, 3.98, and 4.10 µM, respectively, which were higher than NO produced by the cells in the control group. It was most probably due to the upregulation of iNOS in LPS-induced BV2 microglia cells through various pathways, such as NF-κB, c-Jun NH2-terminal kinase (JNK) MAPK, and p38MAPK (Lee et al., 2012; Svensson et al., 2010; Oh et al., 2010). On the other hand, the results also demonstrated that even in the absence of LPS, NO was still being produced. Under normal conditions, the neuron cells will produce a low concentration of NO, in which it will act as neurotransmitter that involves in many biological effects (Snyder, 1995).

As for the effect of LPS on the viability of BV2 microglial cells, the present study demonstrated an insignificant decrease in the percentage of cell viability in a dose dependent manner (Figure 2B). Figure 2B illustrates that as the concentration of LPS increased from 0 to 0.1, 1.0, and 10.0 µg/mL, the percentage of cell viability decreased to 10.76, 11.52, and 14.18 %, respectively. In terms of its morphology, the observation was in accordance with the findings by Dai et al., whereby the untreated cells showed small soma with distal arborization, characteristic of “ramified” microglia (Figure 2C) while the 10.0 µg/mL LPS-treated BV2 cells showed fewer branches that were shorter (Figure 2D) and/or appeared to be resorbed into the cell body (Dai et al., 2015). It is postulated that the cytotoxic effect of LPS on BV2 microglial cells could be via co-expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Mander & Brown, 2005). The NADPH oxidase is an enzyme responsible for the production of intracellular ROS, such as superoxide (O2·−), which is important to kill the pathogens that infected the body (Qin & Crews, 2012; Gandhi & Abramov, 2012). However, a prolonged inflammation process will lead to the excessive production of several other potent free radicals, such as peroxynitrite, which will kill all cells nearby the inflammation site through protein oxidation, lipid peroxidation and DNA damage, eventually leading to neuronal death (Mander & Brown, 2005; Wilkinson & Landreth, 2006; Possel et al., 2000).

As illustrated in Figure 2A, a significant inflammation as exhibited through 112% of increase in NO level was observed in cells treated
with 0.1 µg/mL LPS. This same concentration of LPS did not cause any significant cytotoxicity towards BV2 cells (Figure 2B). Thus, 0.1 µg/mL of LPS is considered as the optimal concentration to be used in the following studies.

**Determinaton of ROS level.** ROS is a redox signal that can be produced by various enzymatic reactions and chemical processes (Hsieh et al., 2013). Low concentration of ROS are known to be essential in physiological functions and acted as second messengers (Hsieh et al., 2013). However, excessive production of ROS have implicated the pathogenesis of human diseases, such as ND. This is probably due to the imbalance redox states resulting in oxidative stress. Studies had shown that cumulative oxidative stress can lead to cellular damage, impairment of DNA repair system and mitochondrial dysfunction, which eventually accelerated the aging process and the development of ND (Gandhi & Abramov, 2012; Federico et al., 2012; Patten et al., 2010). Hence, it is important to suppress the release of ROS levels in order to prevent ND.

The release of LPS-induced ROS levels in different treatments were determined in this study. As exhibited in Figure 3, the LPS-stimulated microglia cells showed significant upregulation of ROS levels compared to the untreated cells. This suggested that LPS plays an important role in the upregulation of ROS level. Studies suggested that this could be due to the upregulation of NADPH oxidase (Patten et al., 2010). Han et al. (2012) showed the upregulation of the transcriptional levels of two NADPH oxidase components, p47phox and gp91phox by LPS, suggesting the upregulation of NADPH oxidase upon LPS treatment, which resulted in an increase in the production of ROS levels (Qin et al., 2004).

On the other hand, present studies also suggested that orientin at both MNTD and ½ MNTD showed significant downregulation of LPS-induced ROS levels by 35.75% and 47.04% respectively (Figure 3). Of which, orientin at ½ MNTD was found to be more effective than its MNTD, possibly due to the fact that an increase in the concentration of orientin could also lead to an increase in the intracellular stress of the cells, thus increases the ROS levels. The probable mechanism of orientin in reducing the LPS-induced ROS levels might be through the suppression of the two NADPH oxidase components, p47phox and gp91phox. The study by Ganet al. showed that glaucocalyxin B (GLB) markedly inhibited the expression of p47phox and gp91phox, which resulted in the inhibition of NADPH oxidase with subsequent downregulation of ROS production (Gan et al., 2015). In addition, quercetin and isorhamnetin were shown to inhibit the expression of p47phox subunit, with subsequent decreased in O2− production, suggesting the correlation between NADPH oxidase and the production of ROS levels (Sanchez et al., 2007). Furthermore, there were increasing evidences suggesting the upregulation of HO-1 in response to the oxidative stress as a natural anti-oxidant (Loboda et al., 2016). For instance, orientin was shown to alleviate oxidative stress through the induction of Nrf2 translocation to nucleus, which then upregulated the expression of HO-1 and activated the redox signaling pathway (Shih et al., 2015). Consequently, downregulating the ROS level in Aβ1-42-induced mouse model of AD (Shih et al., 2015). In short, the probable mechanisms of orientin in downregulating the ROS level might be through the inhibition of NADPH oxidase, and the upregulation of HO-1 warrants further study.

**Determinaton of NF-κB expression.** NF-κB is a transcription factor that are responsible for the transcription of genes, such as chemokines, pro-inflammatory cytokines, pro-inflammatory enzymes, and other factors in order to regulate the inflammatory responses upon stimulation (Shih et al., 2015). NF-κB resides in the cytoplasm as a heterodimer formed by p50 and p65, physically linked to an inhibitory molecule, IκB-α. Upon stimulation, NF-κB signaling pathway will be activated firstly through the phosphorylation of IκB-α subunit, with subsequent proteolytic degradation after detachment from the main NF-κB unit (Shih et al., 2015). Then, the NF-κB p65 unit will translocate into nucleus and bind to promoter of pro-inflammatory cytokines and enzymes, which eventually resulted in the transcription into respective cytokines or proteins, such as TNF-α, IL-1β, IL-6 iNOS and COX-2, and hence their upregulation expressions (Shih et al., 2015).
expression of NF-κB might be through the similar pathways. Nevertheless, the activation of these transcription factors was not investigated in the present study and thus, should be validated in the future.

**Determination of STAT1 expression.** Besides NF-κB, STAT1 is also a transcription factor. Stimulation by stimulus such as LPS will eventually lead to the activation of STAT1, which then mediated the transcription of STAT1-responsive genes (Rezao-Zadeh et al., 2008). Binding of LPS-induced interferons (IFNs) had shown to increase STAT DNA binding activity and eventually resulted in the phosphorylation of STAT1 (Rezao-Zadeh et al., 2008). This was then followed by dimerization and nuclear translocation of STAT1, resulting in the transcription of STAT1-responsive genes, such as iNOS, IL-1β, and IL-6 (Rezao-Zadeh et al., 2008).

In this study, STAT1 expression was shown to be upregulated by 4 folds upon LPS stimulation (Figure 4B). Incubation with LPS for 24 hours had resulted in the over-stimulation of BV2 microglia cells and this eventually contributing to the active STAT1, which was responsible for producing pro-inflammatory cytokines or proteins. However, pre-treatment of the BV2 microglia cells with orientin had significantly downregulated the STAT1 expression. The results showed the reduction of STAT1 expression by 71.15% at MNTD of orientin and 60.85% at ½ MNTD of orientin. Up to now, not many studies have been conducted on STAT1. However, based on a study carried out on luteolin, Kao et al. demonstrated the inactivation of STAT1 through the attenuation of upstream stimulatory kinases such as IFN-γ, which then inhibited the downstream pathways and eventually downregulated the production of pro-inflammatory cytokines (Kao et al., 2011). Hence, it was postulated that orientin probably also acted through the same way. Nevertheless, the activation of these transcription factors warrants further study.

**Determination of iNOS expression.** iNOS, an enzyme encoded by Nitric oxide synthase 2 (NOS2) gene, generates nitric oxide (NO) from amino acid L-arginine (Lirk et al., 2002). The production of NO plays an important role in the

In this study, the expression of LPS-induced NF-κB were downregulated significantly after the pre-treatment of orientin at both MNTD and ½ MNTD by 85.29% and 85.87% respectively (Figure 4A). Similar finding was shown in Glaucoalyxin B (GLB) whereby the expression of NF-κB was suppressed. The suppression mechanisms shown by GLB was through the inhibition of IKK phosphorylation, IkB-α phosphorylation and degradation, as well as the inhibition of NF-κB promoter (Gan et al., 2015). Besides, both anthocyanins and dihydromyricetin isolated from *Amelopsis grossedentata* also shown the inhibition of phosphorylation and degradation of IkB-α in suppressing the inflammatory responses (Jeong et al., 2013; Hou et al., 2015). Olajide et al. (2013) also demonstrated that the suppression of NF-κB expression by cryptolepine was through the inhibition of nuclear translocation of NF-κB. Hence, the probable mechanism of orientin in suppressing the
Anti-neuroinflammatory mechanism of orientin (Sharma et al., 2007). In normal physiological conditions, NO production gives an anti-inflammatory effects (Sharma et al., 2007). However, excessive NO production will be considered as pro-inflammatory mediators that induces the neuroinflammation and eventually leads to neuronal cell death (Yuste et al., 2015; Lirk et al., 2002; Sharma et al., 2007). Studies suggested the activation of glia cells expressing the enzyme iNOS produced NO, which then triggered calcium mobilization and activated the release of vesicular glutamate from astroglia cells (Yuste et al., 2015). This eventually resulted in progressive neuronal cell death, contributing to ND (Yuste et al., 2015). Besides, Kroncke et al. showed that high levels of iNOS protein were found in the brains of post-mortem Alzheimer’s and Parkinson’s diseases patients. Similarly, elevated iNOS level was found in the degenerating infarct region in transgenic mouse model of Huntington’s disease (Chen et al., 2000). In addition, strong iNOS immune-reactivity was also detected in activated astrocytes in ALS (Barbeito et al., 2004). All these studies suggested the lethal effects of high iNOS expression. Hence, it is necessary to suppress the expression of iNOS.

In this study, the LPS-simulated BV2 microglia cells showed approximately 4 folds increase in the iNOS expression (Figure 4C). This was known to be highly related to the activation of NF-κB and STAT1 pathway as discussed above, which resulted in the transcription and upregulation of iNOS. However, pre-treatment of the cells with orientin at MNTD showed significant downregulation of the LPS-induced iNOS expression by 50.09% whereas it showed 95.92% at half MNTD of orientin (Figure 4C). Effectiveness of orientin at half MNTD over its MNTD was in accordance with the present findings on ROS level as iNOS expression could be ROS-dependent. Similar findings were obtained in torilin and geniposide, whereby iNOS expression was suppressed in LPS-induced N9 and BV2 microglia cells, respectively (Choi et al., 2009; Zhang et al., 2012). This downregulation of iNOS expression in both torilin and geniposide was known to be due to the inhibition of NF-κB pathway (Choi et al., 2009; Zhang et al., 2012). Study by Zhang et al. showed that geniposide inhibited the IκB-α subunit degradation and subsequent translocation of NF-κB into nucleus (Zhang et al., 2012). This eventually inhibited the production of iNOS and led to the downregulation of iNOS expression. Therefore, the inhibition of NF-κB signaling pathway might be a possible mechanism of orientin in downregulating iNOS expression.

Figure 4. Relative mRNA expression (Fold Change) of (A) NF-κB (B) STAT1 (C) iNOS (D) COX-2 (E) HO-1 in LPS-stimulated BV2 cells upon treatment with ½ MNTD and MNTD of orientin. Cells were treated for 24 hours and gene expression was calculated using Double Delta CT analysis. Bars indicate the means ± standard deviation. ‘*’ indicates that the treatment was significantly different from the untreated cells using one way analysis of variance followed by Tukey’s multiple comparison test at p<0.05. ‘#’ denotes the treatment was significantly different from the LPS-stimulated cells using one way analysis of variance followed by Tukey’s multiple comparison test at p<0.05. LPS, lipopolysaccharide; MNTD, Maximum Non-toxic Dose of orientin (15 µM); ½ MNTD, half value of Maximum Non-toxic Dose of orientin (7.5 µM); Indo, Indomethacin; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; STAT1, signal transducer and activator of transcription 1; NF-κB, nuclear factor-κB; HO-1, hemeoxygenase 1.
**Determination of COX-2 expression.** COX-2 is an enzyme encoded by prostaglandin-endoperoxide synthase 2 (PTGS 2) gene. It involved in the synthesis of prostaglandin H2 from arachidonic acid where overproduction will result in neurotoxicity (Ricciotti & Fitzgerald, 2011; Minghetti, 2004). Studies suggested that COX-2 was expressed in the central nervous system (CNS) under normal condition and it contributed to synaptic activity, long term memory and also helped in functional hyperemia (Minghetti, 2004). When there were pro-inflammatory activities, expression level of COX-2 was known to be elevated in the brain and played a crucial role in inflammatory reactions involving in the pathogenesis of ND (Ricciotti & Fitzgerald, 2011). For instance, there were elevated levels of COX-2 mRNA and protein found in post-mortem spinal cords of ALS patients (Yasojima et al., 2001). Besides that, studies also showed increased expression level of COX-2 in microglia cells from idiopathic PD patients (Knott et al., 2000). These suggested COX-2 as one of the potential targets used in treating ND.

As shown in Figure 4D, LPS significantly upregulated the COX-2 expression by 4-fold as compared to the untreated cells. This drastic upregulation of COX-2 expression was also known to be due to the activation of NF-kB pathway (Rezai-Zadeh et al., 2008). Upon treatment, both MNTD and ½ MNTD of orientin significantly downregulated COX-2 expression in LPS-induced BV2 cells by 85.3% and 78.3%, respectively. These findings were in accordance with various studies that suggested the attenuation of LPS-induced COX-2 expression upon treatment with natural compounds. For instance, astaxanthin showed the inhibition of COX-2 expression in LPS-stimulated BV2 microglia cells (Choi et al., 2008). Besides, Kang et al. (2004) suggested the suppression of LPS-induced COX-2 expression in BV2 microglia cells by curcumin through the inhibition of NF-kB and AP-1 DNA bindings. Another study by Yoon et al. (2016) showed that Cudratricusxanthone A suppressed the expression of both iNOS and COX-2 in LPS-stimulated BV2 microglia cells and this inhibitory effects were known to be due to the inhibition of NF-kB and p38 MAPK pathways. Therefore, the probable mechanism of suppression of COX-2 might be via the inhibition of NF-kB pathway, which was also the probable mechanism for iNOS as discussed in the previous section.

However, it was noted that the COX-2 expression (Figure 4D) in LPS-stimulated BV2 microglia cells at both MNTD and ½ MNTD orientin were independent of iNOS (Figure 4C). There are studies suggesting that the decrease in iNOS would increase the COX-2 activity through the inhibition of NO production (Patel et al., 1999; Clancy et al., 2000; Habib et al., 1997). For instance, study by Patel et al. suggested that inhibition of nitrite accumulation with high concentration of iNOS inhibitor (L-NMMA) in activated murine macrophage (RAW 264.7) showed the upregulation of PGE2 production and the accumulation of COX-2 protein (Patel et al., 1999). Hence, this could be the possible reason that iNOS and COX-2 acted independently in this study.

**Determination of HO-1 expression.** HO-1 as an anti-inflammatory enzyme, is enzyme encoded by hemeoxygenase (decycling) 1 (HMOX1) gene located on chromosome 22q12 (Alzaraz et al., 2003; Schipper et al., 2009). HO-1 are responsible for degrading heme to generate carbon monoxide, biliverdin and free iron. It is a normal mechanism in response to oxidative stress or nitrosative stress caused by the high ROS/RNS levels (Kwon et al., 2017). It will also be induced by cytokines or mediators formed during inflammatory processes in order to exert a negative feedback mechanism for cell activation and mediators production, which eventually regulates the inflammatory response (Kwon et al., 2017).

In this study, orientin at MNTD showed a significant upregulation of LPS-induced HO-1 by approximately 4-fold as opposed to untreated cells (Figure 4E). Similar findings were also demonstrated with flavonolquercetin whereby it showed significant upregulation of Nuclear factor-like 2 (Nrf2) and HO-1 expression after LPS exposure (Kwon et al., 2017). This suggested the induction of HO-1 by orientin most probably occurred via Nrf2 pathway. Besides, Kwon et al. (2017) also suggested the reduction of pro-inflammatory cytokines production via Nrf2/HO-1 signaling pathway by trythanthrin. Another study by Zhou et al. (2014) on Orientin-
2″-O-Galactopyranoside (OGA) in BV2 microglia cells also demonstrated the significant induction in Nrf2 expression, suggesting the induction of HO-1 expression was mediated by Nrf2 activation. Furthermore, xanthohumol was reported to exert anti-inflammatory properties in BV2 microglia cells through Nrf2-ARE signaling and the upregulation of downstream HO-1 (Lee et al., 2011). Thus, it is also hypothesized that the probable mechanism of orientin was via the Nrf2 pathway.

![Probable mechanism of orientin](image)

**Figure 5. Probable mechanism of orientin.** Orientin inhibit the production of pro-inflammatory components such as STAT1, NF-κB, iNOS and COX-2, whereas promote the production of anti-inflammatory component, HO-1, which eventually leads to anti-inflammation.

**CONCLUSION**

In conclusion, this study showed that orientin at both MNTD and ½ MNTD was able to downregulate the ROS level. Orientin was also shown to be able to significantly downregulate the mRNA expression of transcriptional factor, such as NF-κB and STAT 1, as well as the downregulation of pro-inflammatory mediators, such as iNOS and COX-2 significantly. In addition, orientin was also capable of upregulating HO-1. Based on all these findings, the probable mechanism of orientin was summarized and illustrated in Figure 5, which further validates the potential of orientin as an alternative therapeutic agent in treating ND. Nevertheless, additional studies, for instance, detecting the effects of orientin on other key inflammatory cytokines, such as IL-1β and TNF-α, *in vivo* study in evaluating the capacity of orientin to achieve effective concentration in the brain of animal models with neurodegenerative conditions as well as investigating the bioavailability. The neuroprotective effect of orientin in animal models of neuroinflammatory diseases should be further carried out prior to developing orientin as a potential drug in treating ND.

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Anti-neuroinflammatory mechanism of orientin 91


Improved extracellular secretion of β-cyclodextrin glycosyltransferase from *Escherichia coli* by glycine supplementation without apparent cell lysis

Nik Ida Mardiana Nik-Pa\(^{ab}\), Suraini Abd-Aziz\(^c\), Mohamad Faizal Ibrahim\(^a\), Noorjahan Banu Mohamed Alitheen\(^c\), Norhayati Ramli\(^a\)*

\(^a\)Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
\(^b\)Section of Bioengineering Technology, Universiti Kuala Lumpur Branch Campus, Malaysian Institute of Chemical & Bioengineering Technology, Taboh Naning, 78000 Alor Gajah, Melaka.
\(^c\)Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

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**Abstract.** The use of an effective inducer feeding strategy without causing cell lysis presents significant advantage to enhance the secretion of an enzyme to the culture medium of *Escherichia coli*. The *cgt* gene encoding β-cyclodextrin glycosyltransferase (β-CGTase) was cloned into pQE30xa as an N-terminal His-tagged protein and transformed into *E. coli*. The induction strategy was applied towards enhancing the extracellular secretion of the recombinant β-CGTase by increasing permeability of the outer membrane of *E. coli*. The supplementation of 1.2 mM glycine following 2 h of fermentation at 37°C enhanced the activity of β-CGTase to 38.295 U/mL, which was approximately 1.3-fold higher than the control (without induction). Further flow cytometry analysis was adopted as a rapid and highly reproducible approach to determine the effect of glycine supplementation on the viability of *E. coli* cells. The supplementation of glycine did not contribute to apparent cell lysis, with no adverse effects on cell viability, hence indicating the effectiveness of glycine in enhancing the extracellular secretion of β-CGTase. The recombinant β-CGTase was then purified through a combination of diafiltration and Ni-NTA affinity chromatography with 18.4-fold increase in purity. An effective glycine feeding strategy could enhance the extracellular secretion of β-CGTase without adverse effects on cell viability. This strategy could be applied potentially to enhance the secretion of a recombinant protein to the culture medium from *E. coli* cells without having cell lysis.

**Keywords:** cell viability, cyclodextrin glycosyltransferase, extracellular secretion, glycine, inducer, membrane permeability

**INTRODUCTION**

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is a member of the α-amylase family, also known as glycosyl-hydrolases which catalyse the formation of cyclodextrins (CDs) from starch (Han *et al.*, 2014). Among the well-characterised CDs which are α-, β- and γ-CDs, β-CD has been extensively used due to its hydrophobic internal cavity and hydrophilic outer surface which provides water soluble properties (Wang *et al.*, 2012). For the production of β-CD, *Escherichia coli* is the most commonly used host, especially strains that produce a full-length enzyme which is often used for its industrial applications.
The predominant production of β-CD is of interest that can reduce the time and cost of purification (Al-Sharawi et al., 2013; Cheirsilp et al., 2010). β-CD has been frequently employed to form inclusion complexes with a variety of guest molecules, for instance to encapsulate antimicrobial compounds for the development of antimicrobial active packaging materials (Abarca et al., 2016), development of CD nanoparticles to be used in drug delivery systems (Lakkakula and Maçedon Krause, 2014), development of CD-based nanosponges to be used as innovative carriers in cosmetics, pharmaceutical, agrochemistry and environmental protection (Atul and Bhushan, 2017) and many others.

However, large amounts of β-CGTase are necessary for an effective production of β-CD through enzymatic synthesis. The low specific activity of the β-CGTase synthesised by the natural host cells is the main drawback in the production of CD at a larger scale. Therefore, the need for enhancement of the CGTase properties has become increasingly necessary to improve its quantity and quality for efficient use in industrial applications. According to Qi and Zimmermann (2005), about 50% of commercialised CGTases were produced through genetic engineering approach. Although a lot of progress has been made to overproduce the enzyme mainly through the development of secretory expression system in E. coli as the common expression platform (Zhou et al., 2018), further research on the enhancement of extracellular secretion of CGTase is needed to improve CD production.

The use of E. coli as a cell factory is preferred as the genetic properties are well established, simple, inexpensive and fast high-density cultivation (Sorensen and Mortensen, 2005). However, the formation of inclusion bodies, poor growth of host and protein inactivity (Rosano and Ceccarelli, 2014) are the critical issues that need to be tackled in order to produce the recombinant enzymes from E. coli effectively. The formation of inclusion bodies could block the translocation channels of pre-proteins and eventually delaying protein secretion (Li et al., 2014). An inadequate release of β-CGTase across two cell membranes of E. coli is the main challenge in order to achieve higher enzyme secretion (Li et al., 2010). Therefore, it was suggested that a balance between the rate of pre-protein synthesis and translocation needs to be maintained for an efficient secretion of extracellular enzyme from E. coli.

The optimization of the cultivation process has been frequently employed to increase the level of extracellular production of CGTase (Cheng et al., 2011). The use of inducers presents significant advantages in modifying the integrity of the cell wall of E. coli, hence promoting the secretion of recombinant enzyme to the culture medium. The extracellular secretion of the recombinant enzyme from E. coli is preferred over periplasmic and cytoplasmic expression, mainly due to high expression level, the benefit of simple and inexpensive downstream processing (Tesfai et al., 2012), substantial yields of correctly formed proteins and to minimise and avoid protease attack (Choi and Lee, 2004). In the present study, we emphasised on the optimisation of the inducer feeding strategy and attempted to enhance the secretion of recombinant β-CGTase to the culture medium by adding glycine, Triton X-100 or xylose. This study was also intended to address the effect of inducer supplementation on the cell viability where healthy cell growth is required to enhance the secretion of β-CGTase.

**MATERIALS AND METHODS**

**Bacterial strain and plasmid.** E. coli JM109 harbouring pTZCGT-BS from previous study (Ramli et al., 2013) was used for sub-cloning into pQE30xa. E. coli JM109 [endA1, recA1, gyrA96, thi, hsdR17 (rk-,mk+), relA1, supE44, D (lac-proAB)], F’ (tra D36, pro AB, lacIqZ Δ M15)] from Promega (Madison, WI) was used as a host strain that is ideal for propagating pQE plasmids and used as an expression host. Plasmid pQE30xa from QIAGEN (Germany) was used as an expression vector.

**Construction of β-CGTase expression system.** The amplified PCR fragment encoding the β-CGTase gene was ligated into pQE30xa vector, designated as pQECGT-BS. The amplification of β-CGTase gene was performed under standard PCR conditions with 1 µg of genomic DNA, 200 µmol of each forward and reverse primer, 0.01 µL of Taq DNA polymerase in 1× reaction buffer and 0.2 mM of dNTPs in a final volume of 20 µL. The specific primers designed for
amplification of CGTase used for construction of pQE CGT-BS expression system were ProB 5′-TTA GGATCC TTG CTCTAG ATTTG ATC ACA CGA-3′ and P2 5′-GTC AAG CTT TTA CCA ATT AAT CAT AAC CGT-3′ for forward and reverse primer, respectively. The forward and reverse primers were designed to contain the restriction site BamHI and HindIII (underlined), respectively. The amplified DNA fragment was purified after agarose gel electrophoresis using Hi Yield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan) and digested with BamHI and HindIII before ligation into pQE30xa. To study the expression levels, the ligation products were subsequently introduced into E. coli JM109. Luria-Bertani (LB)-ampicillin (100 µg/mL) was used to plate out the transformation mixtures. After overnight growth at 37°C, colonies were chosen for insert confirmation.

**β-CGTase expression in E. coli.** Recombinant E. coli JM109 harbouring pQE CGT-BS was inoculated into LB broth containing 100 µg/mL ampicillin and incubated overnight at 37°C in an incubator shaker agitated at 200 rpm until an optical density of 1.5 at 600 nm was obtained. Terrific Broth (TB) supplemented with 100 µg/mL ampicillin was used as a production medium for the preparation of crude recombinant β-CGTase. The culture was incubated at 37°C, agitated at 200 rpm for 24 h and sampled every 4 h. Cell-free supernatant was collected by centrifuging at 10000 rpm at 4°C for 10 min to determine the profile of enzyme expression. For estimation of cell growth, the pellets were appropriately diluted and the optical density was measured at OD600 nm.

**Effects of the induction strategies on β-CGTase production.** TB media supplemented with either glycine, Triton X-100 or xylose were used to investigate the effects of inducers on the extracellular secretion of the recombinant β-CGTase. Furthermore, the effects of selected inducer supplementation time (0, 1, 2, 3 and 4 h), supplement concentrations (0.1, 0.4, 0.8, 1.2 and 1.6 mM) and post-induction temperatures (25, 30, 47 and 40°C) were determined. The experiments were carried out in triplicates.

**Flow cytometry sample preparation.** The culture broth was diluted at least 1:10 (v/v) in order to obtain a measurement range of less than 1000 cells/second (Falcioni et al., 2008). The diluted broth was filtered using 40 µm Nylon cell strainer (Falcon, NJ, USA) to minimise the possibility of causing a clog in the flow cytometer, followed by vortex for at least five min.

**Fluorescent probes and staining procedure.** A nucleic acid double staining assay (Barbesti et al., 2000) was applied to distinguish between the populations of viable and dead cells. A 42 µmol/L of thiazole orange diluted in dimethyl sulfoxide and 4.3 mmol/L propidium iodide diluted in distilled water (BD™ Cell Viability Kit) were used. The unstained samples were prepared as a control to eliminate the assay background noise. The stained samples were instead prepared by adding 5 µL of each dye into 500 µL of cell suspension to achieve a final concentration of 43 µmol/L for propidium iodide and 420 nmol/L for thiazole orange, respectively. Further to that, both stained and unstained samples were added with 5 µL of EDTA (1 mM, pH 8). The staining process was carried out following the manufacturer’s protocol of the BD™ Cell Viability Kit (Cat. No. 349483, Becton Dickinson, San Jose, CA). The samples were incubated in an ice box on an orbital shaker for one hour. A control for the gating process was prepared by heat treatment (Sharuddin et al., 2018) whereby 50 mL of the samples were heated at 80 to 90°C in a water bath for 45 min.

**Flow cytometric analyses.** The flow cytometric analyses were done using a BD Accuri® C6 cytometer (Becton Dickinson UK Ltd., Oxford, UK) equipped with red (640 nm) and blue (488 nm) lasers, forward-scattered (FSC) and side-scattered light (SSC) and four fluorescence detectors. The propidium iodide fluorescence was collected at the FL3 channel, while the thiazole orange fluorescence was collected at the FL1 channel, with 20,000 cells counted for each sample. The differentiation between viable and dead cells was done through a gating on a dot plot of red versus green fluorescence after excluding the auto-fluorescence. The visualisation of the plots and the gating process were done using the built in CFlow Plus software. The repeated measurements Analysis of Variance (ANOVA) were conducted to test for significant differences of viable and dead cells between no induction, 0 h and 2 h induction with glycine.
Purification of β-CGTase. The crude β-CGTase was washed with equilibrium buffer (10 mM imidazole) with molecular weight cut-off (MWCO) of 30 kDa using AKTA Flux tangential flow filtration system (GE Healthcare, USA). Affinity purification was performed on a Ni-NTA column on an AKTA Avant chromatography system (GE Healthcare, USA) according to the manufacturer’s instructions. The sample was incubated for 30 min in the column, followed by washing with buffer containing 20 mM imidazole (pH 8.0). Elution buffer containing 250 mM imidazole (pH 8.0) was used to elute the bound enzyme. The fractions with maximum activity were pooled together and concentrated using Amicon ultrafiltration membrane kit (30 kDa MWCO membrane). The samples were assayed for β-CGTase activity and protein concentration.

Determination of protein concentration and size. Protein concentration was measured using Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard. The protein size was analysed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at constant voltage of 100 V for 1.5 h at room temperature until the band was migrated sufficiently. Gel (8.3 cm X 7.3 cm) was run according to the method of Laemmli (1970). The concentrations of the stacking and resolving gels were 5% and 12%, respectively. Appropriately diluted protein samples (30 µg) with 4× marker containing 50 mM Tris–HCl (pH 6.8), 40% (w/v) of glycerol, 10% (w/v) of SDS, 5% (v/v) of mercaptoethanol and 0.05% (w/v) of bromophenol blue were boiled for 5 min before applied into each lane. The lane was formed by a 10-well comb (Mini-PROTEAN®) and the molecular weight marker (PageRuler™ Unstained Protein Ladder) from Thermo Scientific, USA was used as a standard. The gel was stained with Coomassie Brilliant Blue R-250.

β-CGTase activity. The β-CGTase activity was measured using phenolphthalein assay (Kaneko et al., 1987). Reaction mixture containing 1 mL of 40 mg of soluble starch in 100 mM phosphate buffer (pH 6.0) and 0.1 mL of β-CGTase solution was incubated at 60°C for 10 min in a water bath. The reaction was stopped by the addition of 3.5 mL of 30 mM NaOH. Subsequently, 0.5 mL of 0.02% (w/v) phenolphthalein in 5 mM Na₂CO₃ solution was added to the reaction mixture and mixed well. After 15 min, the reduction in colour intensity was measured at 550 nm. Blank lacking the CGTase was analysed simultaneously with each batch of samples. As a standard, the soluble starch and enzyme were replaced with 1 mL of β-CD solution and 0.1 mL of water, respectively. A calibration curve was made using 0-0.5 mg/mL of β-CD in 100 mM phosphate buffer, pH 6.0. One unit of enzyme activity was defined as the amount of enzyme that formed 1 µmol of β-CD per min under the conditions defined above.

Production of cyclodextrin and kinetic parameters. The kinetic parameters, $K_m$ and $V_{max}$ of the recombinant β-CGTase were analysed by linear regression with the linear transformation (Hanes-Woolf plot) of the Michaelis-Menten equation using an Excel curve-fitting program (Microsoft Excel 2010, San Francisco, CA, USA). The $K_m$ and $V_{max}$ values for the recombinant β-CGTase were determined by incubating 0.5 U/mL of β-CGTase with 2-10 mg/mL of soluble starch at 60°C for 10 min in 1 mL of 0.1 M phosphate buffer (according to standard β-CGTase assay) (Kaneko et al., 1987). The concentrations of β-CD were determined by high performance liquid chromatography (HPLC) using a Lichrospher 100 NH₂ column (Merck, USA) eluted with 65:35 acetonitrile:water mixture at 1 mL/min. β-CD was detected by a refractive index detector (Shimadzu, Japan). The experiments were carried out in triplicates and standard deviations were calculated.

RESULTS AND DISCUSSION

Construction of recombinant plasmid and production of β-CGTase. The cgt gene was constructed in pQE30xa and in frame with 6x-His tag to assist in protein purification using affinity chromatography. The expression vector was successfully constructed and verified by sequencing before it was transformed into E. coli JM109. The resulting strains exhibited 30.371 U/mL of β-CGTase activity from the supernatant without any inducer (Figure 1), a 2.6-fold increment of β-CGTase activity as compared to
wild type producer (Ramli et al., 2010). The same cgt gene was previously constructed in pTZ57R/T and expressed in E. coli JM 109 with the activity of 37.480 U/mL (Ramli et al., 2013). A low enzyme activity detected in the culture medium of E. coli without inducer might be due to the accumulation of protein in the periplasmic space (Li et al., 2010). However, the disruption of outer membrane to recover the target protein is not preferred as it may cause the intracellular proteolysis by periplasmic proteases (Choi and Lee, 2004). Moreover, it could also result in cell death and injury, for instance by introducing high pressure to E. coli cells to recover proteins (Hauben et al., 1996). The disruption of outer membrane of E. coli for the recombinant protein production needs to be handled with care so as not to compromise cellular integrity.

**Effects of inducers on extracellular secretion of β-CGTase.** Three types of inducers were selected to enhance the extracellular secretion of β-CGTase from E. coli which were glycine, Triton X-100 and xylose, in comparison to control culture without any inducer. The glycine, Triton X-100 and xylose were known to have different effects on the integrity of the cell wall of E. coli (Li et al., 2014, 2010; Lo et al., 2007). The supplementation of inducers during cultivation offers advantages as compared to the physical methods including osmotic shock, sonication and freezing and thawing where they can only be applied after harvesting cells. Therefore, by adopting the inducer feeding strategy, the additional steps of recombinant proteins recovery after the fermentation process are not necessary.

The induction by glycine increased the extracellular secretion of β-CGTase activity at 12 h of fermentation by 1.1-fold, while the Triton X-100 and xylose induction showed negative effects (Figure 1A). ANOVA test was conducted in this study and demonstrated significant differences of β-CGTase activities between the use of different types of inducers at a confidence level of 0.05 (p<0.05). Glycine’s capability to act as a permeability enhancer, resulted in the increased activity of recombinant β-CGTase. The glycine could replace the alanine residues in the peptide component of the peptidoglycan of E. coli cell wall, resulting in a more loosely cross-linked peptidoglycan and therefore, enhancing the permeability of outer membrane of E. coli (Yang et al., 2017). The glycine was previously shown to increase the secretion of extracellular α-CGTase from E. coli by 11-fold to achieve maximum activity of 28.5 U/mL (Li et al., 2010). However, the supplementation of Triton X-100 and sucrose might have compromised the cellular integrity and the growth of E. coli, which resulted in the reduced activity of extracellular β-CGTase. The glycine was thus selected to be used in the optimisation of inducer feeding strategy by single factor design to further increase the extracellular secretion of β-CGTase.

**Effect of glycine feeding strategy on extracellular secretion of β-CGTase.** The effect of glycine supplement time was conducted to enhance the extracellular secretion of recombinant β-CGTase from E. coli (Figure 1B). The addition of 0.1 M glycine at 2 h fermentation has resulted in the 1.1-fold increment of enzyme activity at 12 h of incubation as compared to the induction at the beginning of the culture. The induction at 2 h during early log phase might have increase the leakage of periplasmic proteins to the culture medium, which resulted in the increased activity of β-CGTase. According to Li et al. (2010), cell growth impairment caused by glycine could be controlled by delaying the addition of glycine to the culture medium. However, reduction of β-CGTase activities was recorded by increasing the timing of induction to 3 and 4 h of fermentation. The induction at high cell density during stationary phase is not recommended due to the bacterial cell membrane rigidity (Bao et al., 2016).

The amount of glycine necessary to enhance the permeability of outer membrane of E. coli was 1.2 mM and showed enhancement of β-CGTase activity by 1.3-fold as compared to the culture without inducer (Figure 1C). However, the supplementation of 1.6 mM glycine inhibited the β-CGTase activity and an excessive amount of glycine is unfavorable to the cell and might inhibit the bacterial growth (Zou et al., 2014). In addition, an approach to increase the production of recombinant proteins by lowering the induction temperature is frequently reported (Lu et al., 2018; Zheng et al., 2016). Although the enzyme expression could be significantly enhanced at lower induction temperature to increase the formation of correctly folded protein, this study
found opposing results where the incubation at 25°C caused a reduction of enzyme activity by 2.4-fold as compared to the incubation at 37°C (Figure 1D). Low incubation temperature at 25°C might contribute to the impaired growth of *E. coli* (Chang *et al.*, 2017), hence reducing the activity of β-CGTase.

![Graphs showing effects of different types of inducers, glycine supplement time, glycine concentrations, and post-induction temperatures on extracellular secretion of β-CGTase from *E. coli*](image)

**Figure 1.** Effects of different types of inducers (A); glycine supplement time (B); glycine concentrations (C); post-induction temperatures (D) on the extracellular secretion of β-CGTase from *E. coli* harbouring pQECGT-BS. Each value represents the mean of three independent measurements and the deviation from the mean is below 5%.

**Effect of glycine supplementation on cell viability.** To confirm the secretion of β-CGTase is not due to the cell lysis, the live and dead cells were quantified by using nucleic acid double staining assay based on flow cytometry. The secretion of enzyme into the culture medium via cell lysis is undesirable due to the release of proteins and other contaminants from the intracellular space (Shin and Chen, 2008), which might interfere in the purification process. Although different methods were reported to indicate for cell lysis, for instance by determination of cytoplasmic proteins; β-galactosidase (Bao *et al.*, 2016) and glucose-6-phosphate dehydrogenase (Li *et al.*, 2010) that are expected to be present in very low concentration in the culture medium, they differ in respect to sensitivity and accuracy. In this study, a flow cytometry method was adopted to quantify the concentrations of live and dead cells where it considered as a rapid, sensitive and highly reproducible approach (Fontana *et al.*, 2017). This
approach could also replace the cells quantification method under microscopy that is time-consuming and lacks reproducibility.

As shown in Figure 2, the addition of 1.2 mM glycine at 2-h of fermentation did not show any apparent effect on the viability of the *E. coli* cells where the percentage of viable cells recorded was more than 97%, comparable with the viable cells in the culture without any inducer. Only a slight reduction of the percentage of viable cell was recorded with the addition of 1.2 mM glycine at the beginning of the culture. A higher percentage of live cells recorded following glycine induction indicates the release of recombinant β-CGTase was due to the interference of glycine with the membrane integrity and not due to cell lysis.

**Purification and kinetic parameters for determination of the recombinant β-CGTase.** The recombinant β-CGTase was collected and purified from the 12-h culture induced with 1.2 mM glycine at 2 h induction time at 37°C. The recombinant β-CGTase carried an N-terminal fusion peptide containing a 6x-His sequence that provides metal-binding affinity. Therefore, it was purified through a combination of diafiltration and Ni-NTA affinity chromatography, which resulted in increased purity to 18.4-fold. Ultrafiltration was then applied to concentrate the protein where the purity was increased to 21.6-fold (Table 1). To evaluate the purity of protein, the diafiltrated and purified proteins were resolved by SDS-PAGE. The results showed the expected size band of the purified protein of 78.6 kDa (Figure 3).

Furthermore, a different range of soluble starch concentrations was tested to produce β-CD by β-CGTase. A maximum β-CD of 16.671 mg/mL was achieved by using 4 mg/mL of starch (Figure 4). However, the production of β-CD was reduced and attained almost a similar concentration with increased starch concentration. At these points, the enzyme could probably has reached saturation with the substrate (Robinson, 2015). The cyclisation reaction of β-CGTase was then described by Michaelis-Menten kinetic parameters (Figure 5) to evaluate the performance of recombinant β-CGTase for β-CD production (Chen *et al.*, 2018; Rakmai and Cheirsilp, 2016). The *V*<sub>max</sub> and *K*<sub>m</sub> values were evaluated to be 1.3 mg/mL/min and 0.025 mg/mL, respectively. A small value of *K*<sub>m</sub> could indicate a high affinity for the substrate (Ong *et al.*, 2008). Hence, it was suggested that the recombinant β-CGTase was specific towards...
starch and could achieve its maximum catalytic efficiency at low starch concentrations.

**Table 1** Purification yield of recombinant β-CGTase from *E. coli* following induction with 1.2 mM glycine at 2 h of fermentation at 37°C.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>β-CGTase activity (U/mL)</th>
<th>Protein concentration (mg/mL)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>38.585 ± 1.513</td>
<td>16.175 ± 0.702</td>
<td>2.385 ± 0.197</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Diafiltration</td>
<td>31.354 ± 0.877</td>
<td>10.189 ± 0.456</td>
<td>3.077 ± 0.052</td>
<td>1.290</td>
<td>81.26</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>19.953 ± 0.143</td>
<td>0.455 ± 0.025</td>
<td>43.882 ± 2.821</td>
<td>18.395</td>
<td>51.71</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>8.361 ± 0.088</td>
<td>0.163 ± 0.029</td>
<td>51.408 ± 3.021</td>
<td>21.550</td>
<td>21.67</td>
</tr>
</tbody>
</table>

![Figure 4](image-url)  
*Figure 4.* The production of β-cyclodextrin by purified β-CGTase using different starch concentrations. Standard deviations are shown as bars and the deviation from the mean is below 5%.

![Figure 5](image-url)  
*Figure 5.* The Hanes-Woolf plot to identify *K*<sub>m</sub> and *V*<sub>max</sub> which were used in the Michaelis-Menten equation. S: substrate, v: velocity.

**CONCLUSION**

As a conclusion, the present study demonstrated that the supplementation of glycine with correct feeding strategy could enhance the secretion of β-CGTase into the culture medium of *E. coli* without any adverse effect on the viability of *E. coli* cells. This feeding strategy could be applied to enhance the extracellular secretion of a recombinant protein from *E. coli* system at the cultivation process.

**ACKNOWLEDGEMENT**

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Phylogenetic patterns in the tribe Acacieae (Caesalpinioideae: Fabaceae) based on rbcL, matK, trnL-F and ITS sequence data

Aramide Dolapo Igbari* and Oluwatoyin Temitayo Ogundipe

Molecular Systematics Laboratory, Department of Botany, University of Lagos, Akoka, Yaba, Lagos, Nigeria

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Abstract. The tribe Acacieae is one of the three tribes of the distinct mimosoid clade nested within the re-circumscribed sub-family Caesalpinioideae. Many uncertainties exist with the taxonomic status of tribe Acacieae in relation to tribe Ingeae and genus Acacia. To unravel the phylogenetic patterns within Acacieae, nine members of the tribe were phylogenetically analysed employing both parsimony and Bayesian methods. Six data matrices (ITS, rbcL, matK, trnL-F, rbcL+matK+trnL-F and ITS+rbcL+matK+trnL-F) representing 46 sequences, and 2 outgroup taxa were used for the analysis. Our results are in support to some previous studies on the phylogeny of the Acacieae. It supports the polyphyly of tribe Acacieae. The monophyly of Vachellia, Senegalalia and Faidherbia taxa were strongly supported at >70% bootstrap support values and >0.90 bayesian inference. An unresolved basal paraphyletic clade of Acacia auriculiformis with the outgroup taxa was shown in all the datasets, at mostly low support values. Faidherbia albida was nested within the Senegalia grade while A. auriculiformis (Acacia s.s.) was the closest taxon to the outgroup taxa. A key finding of this study is the polyphyly of Albizia and its close association with A. auriculiformis.

Keywords: African Acacias, molecular phylogeny, Ingeae, cp DNA, nr DNA

INTRODUCTION

The tribe Acacieae is one of the three tribes of the distinct mimosoid clade (Mimosoideae) nested within the re-circumscribed sub-family Caesalpinioideae (LPWG 2017). Tribe Acacieae was formally grouped with many genera that are today classified in tribe Ingeae Benth (Bentham 1842). Initially, Bentham (1875) restricted the genus Acacia Mill. to the Acacieae as the only member of the tribe. Today, tribe Acacieae is now regarded to comprise just two genera, the very large cosmopolitan genus Acacia s.l. and the monotypic genus Faidherbia A. Chev. (Chappill and Maslin 1995; Miller and Bayer 2000; Miller et al., 2003; Murphy et al., 2010). However, many uncertainties still exist, not only with the status of tribe Acacieae, especially in relation to tribe Ingeae and to a lesser extent tribe Mimoseae, but also with the definition, classification, and phylogeny of genus Acacia (Luckow et al., 2000; Maslin et al., 2003; Miller et al., 2003; Murphy et al., 2010). More recently, Miller and Seigler (2012) noted that the tribal relationships within the mimosoid clade are now in disarray, and new phylogenetic data is imperative for establishing an acceptable tribal and generic system of classification of the Mimosoideae.

Members of tribe Acacieae are generally trees or shrubs, they are distinguished by an indefinite number of stamens (more than 10) that are usually free or united only at the base and valvate calyx (Elias, 1981; Vassal, 1981). Individual flowers are arranged in inflorescences that may be either globular heads or cylindrical spikes. However, these features are not only unique to the tribe. In
Fact, no single morphological character distinguishes Acacieae from other tribes in subfamily Mimosoideae, and this has called into question the monophyly of Acacieae and the other tribes in the Mimosoideae (Chappill and Maslin, 1995). The main character that distinguishes the Acacieae from the Ingeae, are free filaments of the stamens while the Ingeae have united filaments. However, this is not entirely maintained in all taxa, some Acacia species have filaments shortly united at the base (Vassal, 1981; Miller et al., 2003; Murphy et al., 2010). Additionally, numerous stamens and eight polyads per anther are other characters also shared between the two tribes (Chappill and Maslin, 1995). The close relationship of the Ingeae and Acacieae has already been observed (Guinet, 1981; Vassal, 1981) and the conflicting character states have made classification that is solely based on morphological characters difficult.

Furthermore, the relationship of the Faidherbia genus with Acacia is controversial. The genus has shortly united stamens and pollens similar to some taxa of the Ingeae but was placed in the tribe Acacieae (Guinet, 1981). Hence, ensuing some ongoing debate as to whether Faidherbia is better placed in the tribe Ingeae or Acacieae (Elias, 1981; Guinet, 1990; Lewis and Rico-Arce, 2005). The non-monophyly of Acacieae and Ingeae are particularly problematic, with the recognition of monophyletic taxa requiring the generic revision of Acacia s.l. (Miller et al., 2003b). This has contributed to a highly controversial debate about the application of the name Acacia (Pedley, 1986; Maslin et al., 2003a; b, Luckow et al., 2005b).

Albeit, because the tribe Acacieae constitute a large and morphologically heterogenous group, an infrageneric classification based on phyllogenetic relationships has become imperative and cardinal for other studies such as: host-parasite co-evolution (Crespi et al., 2004; McLeish et al., 2007); plant physiology (Pohlman et al., 2005; Warwick and Thukten, 2006); atmospheric nitrogen fixation in relation to rhizobial interactions (Brockwell et al., 2005); and insects’ radiations associated with Acacia (Austin et al., 2004) amongst others.

Therefore, the objective of this study is to elucidate on the phylogenetic relationship of the tribe Acacieae in order to test the monophyly of the tribe and establish relationships between its two genera in relation to tribe Ingeae. Similarly, the former, broadly circumscribed genus Acacia Mill. is polyphyletic and has been segregated into five genera, Acacia s.s., Acaciella Britton & Rose, Mariosousa Seigler & Ebinger, Senegalia Raf. and Vachellia Wight & Arn (Maslin, 2015). This study also tests the monophyly of the two groups of Acacia s.l. (Senegalia and Vachellia) that abound in Africa employing molecular systematic techniques and data from three chloroplast regions (rbcL, matK, trnL-F) and the nuclear ITS region.

**MATERIALS AND METHODS**

**Plant material.** Thirty-two samples of Acacia species representing six species were randomly collected from the arid and semi-arid region of Nigeria while sequences of two other species (A. karroo & A. tortilis) were downloaded from GenBank. Two outgroup species Albizia lebbeck and Albizia zygia were included. These outgroup taxa were selected based on results of previous studies, which indicate members of the Ingeae as sister to Acacia (Brown et al., 2008). Collected samples were identified and authenticated at the University of Lagos Herbarium (LUH). The voucher number, GenBank and other information about samples are given in Appendix 1.

**DNA extraction and amplification.** Total genomic DNA was isolated from approximately 0.0300 g of silica-gel dried and 0.0180 g of herbarium plant material following a modified 2X CTAB protocol of Doyle and Doyle (1987). Herbarium samples were precipitated for one week while silica dried for 1hr. Extracted DNA was stored at -20°C prior subsequent use. Polymerase chain reaction (PCR) was performed in 50 µl reaction mixtures containing 25 µl biomix, 1 µl BSA, 2 µl DMSO, 1.75 µl of 10 µM of each primer, 17.5 µl of Millipore H₂O and 1 µl of 30-50 ng template DNA. The Kim matK primers were used to amplify matK region while primers according to Sun et al. (1994), Olmstead et al. (1992) and Taberlet et al. (1991) were used for ITS, rbcL and trnL-F regions respectively. PCR profiles run for each region are given on Table 1. Amplifications were run on a Veriti® 96 well
thermal cycler. Each PCR product was run on 1% agarose gel stained in ethidium bromide and successful amplified products were sent to Source Bioscience (UK) for bidirectional sequencing using the same primer used in PCR.

**Phylogenetic analysis.** Chromatographic traces and contiguous alignments were edited using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, Michigan). Any uncertain base positions, generally located close to the priming sites, and regions of uncertain alignment were excluded from the phylogenetic analysis. Sequences were aligned and edited in Bioedit (Hall, 1999). Informative insertion/deletion events (indels) were identified and coded as binary characters, and gaps were treated as missing data. All four regions were analyzed separately, a combined data matrix of three chloroplast regions and the combined data matrix of the four regions were also analysed. Less than 1% of the data were scored as missing.

Parsimony analyses were performed on the aligned sequences using heuristic search in PAUP 4b10 (Swofford 2002) with nucleotide substitutions equally weighted and unordered. Heuristic search was used with tree-bisection-and-reconnection and random sequence addition. Bootstrap analysis was also performed to test the robustness of each clade with random addition of sequences 1000 replicates. A Bayesian analysis (Ronquist et al., 2011) was carried out by first determining the optimal substitution model using MrModeltest v2.3 (Nylander, 2004) and the Akaike information criterion. The general reversible model with a gamma shape (GTR + G) was selected for the trnL-F region; Hasegawa–Kishono–Yano with a proportion of invariable sites (HKY+I) for matK; Hasegawa–Kishono–Yano with a gamma shape and a proportion of invariable sites (HKY+I+G) for rbcL region whereas Hasegawa–Kishono–Yano with a gamma shape (HKY+G) was specified for the nuclear ITS region. Four discrete states were used for the gamma substitution. The data were therefore partitioned into two for the Bayesian analysis and the correct substitution model as specified by MrModeltest was specified for each partition. The partitions were unlinked so that each parameter could be specified separately. Analysis was run for 30,000,000 generations with sampling every 30,000 generations. Metropolis coupling with four chains, one cold and three heated were used with the two independent runs running simultaneously. The runs, however stopped (split standard deviation set at 0.01) after 7705000 generations and 7705 sampled trees in each run. Approximately 24.7% (7,410,000) trees were used as burnin in summarizing the parameters and tree. The robustness of each clade was ascertained based on the posterior probability as an inference on the validity of the tree.

### Table 1. Amplification profiles.

<table>
<thead>
<tr>
<th>Region</th>
<th>Initial denaturing Temp./time</th>
<th>Denaturation Temp./time</th>
<th>Annealing Temp./time</th>
<th>Extension Temp./time</th>
<th>Final extension Temp./time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>97°C/2:00</td>
<td>97°C/1:00</td>
<td>55°C/0:45</td>
<td>72°C/0:45</td>
<td>72°C/7:00</td>
<td>30</td>
</tr>
<tr>
<td>matK</td>
<td>94°C/5:00</td>
<td>94°C/0:40</td>
<td>48°C/0:40</td>
<td>72°C/0:40</td>
<td>72°C/7:00</td>
<td>30</td>
</tr>
<tr>
<td>rbcL</td>
<td>96°C/0:50</td>
<td>96°C/0:50</td>
<td>53°C/0:50</td>
<td>72°C/2:00</td>
<td>72°C/7:00</td>
<td>30</td>
</tr>
<tr>
<td>trnLF</td>
<td>94°C/2:00</td>
<td>94°C/1:00</td>
<td>55°C/1:00</td>
<td>72°C/2:00</td>
<td>72°C/10:00</td>
<td>30</td>
</tr>
</tbody>
</table>

**RESULTS**

In this present study, eight *Acacia* s.l. and two outgroup (*Albizia*) species were examined for phylogenetic patterns employing 3 chloroplast regions and the nuclear ITS region. A total of 29 taxa were successfully sequenced for *matK* and *trnLF* regions, 26 taxa for *rbcL* region while 23 taxa for the nuclear ITS region. Results of parsimony analysis and tree description for each examined gene region is summarized on Table 2. The strict consensus tree of all combined chloroplast region (Figure 1) revealed an unresolved basal clade of *A. auriculiformis* with outgroup taxa but at high support value of 99%. It also depicted a closer relationship of *Faidherbia albida* with the outgroup taxa, the monophyly of *Senegalia* and *Vachellia* was also observed. The topology of all the combined data (Figure 2) is
congruent with the combined chloroplast regions. An unresolved basal entity and close relationship of *A. auriculiformis* and *F. albida* with the outgroup species is revealed. The strict consensus tree of ITS region Figure 3 revealed the base of the tree is well resolved with a paraphyletic *F. albida* and distinct clades of *Senegalia* and *Vachellia* species. There are good bootstrap support values of $>70\%$ for each monophyletic group. The strict consensus *matK* gene tree (Appendix 2a) revealed an unresolved paraphyletic clade of outgroup taxa and *A. auriculiformis* at a low support value of 51.7%. *Vachellia*, *Senegalia* and *Faidherbia* taxa exhibited a monophyletic relationship at high bootstrap value. However, some low support values were recorded. *rbcL* strict consensus gene tree (Appendix 2b, Supplementary Data) revealed an unresolved paraphyletic base at low support value of 35.11%, *A. auriculiformis* was clustered with the outgroup taxa whereas *Vachellia*, *Faidherbia* and *Senegalia* taxa were monophyletic. Relationships were mostly depicted at low support values. The last chloroplast *trnL*-F strict consensus gene tree depicted an unresolved basal clade (Appendix 2c). Other Acacieae species, *Senegalia*, *Vachellia* and *Faidherbia* are monophyletic whereas *A. auriculiformis* was clustered with the outgroup taxa at low support value of 30.13%.

Robustness of most clades were at high bootstrap support values of $>70\%$.

Bayesian analysis showed a higher resolution of species cluster at distinct node with a quite high posterior probability. A similar topology was observed with the parsimony results. Although the trees from the Bayesian analysis are somewhat similar in topology from the parsimony analysis. Bayesian phylogram of the combined chloroplast and all combined dataset (Figures 4 and 5) established a closer association of *A. auriculiformis* with the outgroup taxa at 0.6 BI and likewise a close relationship of *F. albida*. ITS gene tree Bayesian analysis (Figure 6) revealed a well resolved basal taxa and a close relationship of *F. albida* with the outgroup taxa. *matK* gene tree (Appendix 2d) revealed a monophyletic *Senegalia* and *Vachellia* at high bayesian inference of 1.00. However, an unresolved basal entity was observed. Bayesian phylogram of *rbcL* region (Appendix 2e) showed an unresolved basal relationship of *A. auriculiformis* and the outgroup taxa at 0.92 BI. However, members of the *Vachellia* taxa were closer to the outgroup taxa than *F. albida*. *trnL*-F bayesian phylogram (Appendix 2f) also revealed an unresolved basal entity at 0.50 BI, and a close relationship of *F. albida* with the outgroup clade.

### Table 2. Parsimony profiles and tree description.

<table>
<thead>
<tr>
<th>Gene region</th>
<th>Total number of characters</th>
<th>Constant characters</th>
<th>Variable characters</th>
<th>Parsimony informative characters</th>
<th>Tree length</th>
<th>Restriction index (RI)</th>
<th>Homoplasy index (HI)</th>
<th>Consistency index (CI)</th>
<th>Rescaled consistency index (RC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>783</td>
<td>486</td>
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Figure 1. Maximum parsimony phylogram of the original tree (\textit{matK}+\textit{rbcL}+\textit{trnL}–\textit{F}); numbers at node indicates bootstrap support values.
Figure 2. Maximum parsimony phylogram of the original tree (ITS+matK+rbcL+trnL-F); numbers at node indicates bootstrap support values.
Figure 3. Maximum parsimony phylogram of the original tree (ITS); numbers at node indicates bootstrap support values.
Figure 4. Phylogram inferred by Bayesian analysis (matK+rbcL+trnL-F); numbers at node indicates posterior probability values.
Figure 5. Phylogram inferred by Bayesian analysis (ITS+matK+rbcL+trnL-F); numbers at node indicates posterior probability values.
DISCUSSION

The phylogenetic pattern of the tribe Acacieae based on three chloroplasts (matK, rbcL, trnL-F) region and the nuclear ITS region was elucidated in order to test the monophyly of the tribe and establish relationships between its two genera in relation to tribe Ingeae. Combined data matrix of the three chloroplast regions and a combined data matrix of the four regions were also analyzed. Based on both parsimony and bayesian methods, results revealed the polyphyly of tribe Acacieae. An unresolved basal paraphyletic clade of *A. auricaliformis* with the outgroup taxa was shown in
all the datasets, at mostly low support values. Results support the monophyly of \textit{Vachellia}, \textit{Senegalia} and \textit{Faidherbia} taxa. Our results are in support to some previous studies on the phylogeny of the Acacieae. The polyphyly of the tribe Acacieae were equally found in some previous studies: Luckow \textit{et al.} (2000, 2003) based on plastid \textit{trnL}, \textit{trnK} intron and \textit{matK} gene sequences; Miller \textit{et al.} (2003) based on \textit{trnK}, \textit{matK}, \textit{psbA-trnH}, and \textit{trnL-F} sequence data. Considerable attention has also been devoted in recent phylogenetic studies to unravel the non-monophyly of \textit{Acacia} s.l. Numerous analyses have established clear support for at least five independent lineages scattered widely across the mimosoid clade: \textit{Vachellia}, \textit{Acacia} s.str., \textit{Acaciaella}, \textit{Senegalia} and \textit{Mariosousa} (Miller and Bayer, 2001, 2003; Luckow \textit{et al.}, 2003; Maslin \textit{et al.}, 2003; Seigler \textit{et al.}, 2006b; Bouchenak-Khelladi \textit{et al.}, 2010; Miller and Seigler, 2012). These studies suggest a non-monophyletic \textit{Senegalia}. However, based on our dataset, \textit{Senegalia} is monophyletic. It exhibited a close association with \textit{Faidherbia albida}.

The affinities of the monotypic genus \textit{Faidherbia} has been controversial. Grimes (1999) found \textit{Faidherbia} as sister to the \textit{Acacia} subg. \textit{Acacia} whereas, Maslin (1995), presented two conflicting relationships of \textit{Faidherbia} nested within either the Ingeae or \textit{Acacia} subg \textit{Aculeifera} grade. Polhill (1994), Maslin and Stirton (1997), Robinson and Harris (2000), and Maslin \textit{et al.} (2003) classified \textit{F. albida} to be closer to the \textit{Ingeae}. However, in our analysis \textit{F. albida} is nested within the \textit{Senegalia} grade while \textit{A. auriculiformis} (\textit{Acacia} s.s) was the closest taxon to the outgroup taxa. Results from these analyses support the current classification of \textit{F. albida} within the tribe \textit{Acacieae}.

In our study, the polyphyly of \textit{Albizia} was observed. This support some previous studies of Grimes (1999), Polhill (1994), Luckow \textit{et al.} (2000, 2003), Sulaiman \textit{et al.} (2003), Lewis \textit{et al.} (2005), and Shinwari \textit{et al.} (2014), which found a lack of resolution and support in the \textit{Acacia} \textit{sstr} and \textit{Albizia}. Although the genus \textit{Albizia} was selected as the outgroup taxa, the unresolved cluster of the basal outgroup taxa with \textit{Acacia} \textit{sstr} (\textit{A. auriculiformis}) could likely be attributed to the polyphyly of the \textit{Albizia} (Luckow, 2003). This could probably be as a result of our limited taxon sampling of \textit{Acacia} s.s. A powerful solution will likely be found in a denser species sampling and highly variable character selection (Doyle \textit{et al.}, 1997; Wojciechowski \textit{et al.}, 2004; Lavin \textit{et al.}, 2005). A key finding of this study is the polyphyly of \textit{Albizia} and its close association with \textit{Acacia} s.s. \textit{Acacia}.

In summary, the phylogeny based on both chloroplast and nuclear DNA confirms the polyphyly of Acacieae and paraphyly of tribe Ingeae. Our results show that Ingeae and \textit{Acacia} s.s. together form an unresolved basal entity. Furthermore, the monophyly of \textit{Vachellia} and \textit{Senegalia} taxa at high support values were also indicated. However, the relationship between the Ingeae and \textit{Acacia} s.s. still remain unresolved. This study is a maiden attempt to resolve the intergeneric relationships of tribe Ingeae and \textit{Acacia} s.s. however, based on traditional generic concepts and recent molecular phylogenetic findings, some large genera e.g. \textit{Albizia} are potentially polyphyletic. Phylogenetic patterns of additional gene regions should be explored as well as a more detailed taxon sampling of morphologically variable genera.

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REFERENCES


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*LUH= University of Lagos Herbarium, Nigeria
*RNG= University of Reading Herbarium, Reading, United Kingdom
Appendix 2a: Maximum parsimony phylogram of the original tree (matK); numbers at node indicates bootstrap support value
Appendix 2b: Maximum parsimony phylogram of the original tree (rbcL), numbers at node indicates bootstrap support value.
Appendix 2c: Maximum parsimony phylogram of the original tree (trnL-F), numbers at node indicates bootstrap support value
Appendix 2d: Phylogram inferred by Bayesian analysis (matK), numbers at node indicates posterior probability value.
Appendix 2e: Phylogram inferred by Bayesian analysis (rbcL), numbers at node indicates posterior probability value
Appendix 2f: Phylogram inferred by Bayesian analysis (trnL-F), numbers at node indicates posterior probability value
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