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CHARACTERIZATION OF *Enterobacter Spp*. ISOLATED FROM THE GUT OF RICE WEEVIL

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ABSTRACT

Rice weevil is the most widely distributed and destructive insect pest found in cereal products. The insects usually harbour some biologically important bacteria in their guts which may considerably affect various aspects of their ecology, behaviour, and physiology. The aim of this research was to characterize the *Enterobacter* sp. previously isolated from rice weevil and identified molecularly. These bacteria was investigated based on (i) morphological properties: (ii) biochemical; its ability to utilize different type of carbon and nitrogen sources (glucose, sucrose, lactose, starch, casamino acid, nitrate, nitrite and ammonium), (iii) tolerance towards various heavy metal ions (copper, zinc, manganese, aluminium) and antibiotics. This *Enterobacter* sp. was found to be Gram negative, rod-shaped, non-motile bacterium, and showed positive reaction with catalase, urease and citrate. Based on carbon and nitrogen experiments, sucrose and casamino acid were found to be suitable carbon and nitrogen source for the growth of *Enterobacter* sp. The isolate was also tolerant to aluminium with maximum tolerance concentration of 800 mg/L with growth rate (μ) of 0.240 h^{-1}. Besides that, this bacterium was able to grow in 400 mg/L of copper and exhibit intolerance towards zinc at concentration of 200 mg/L to 800 mg/L. In addition, the isolate was able to tolerate well up to 800 mg/L (growth rate: 0.244 h^{-1}) of manganese. The bacterium was also found resistant to erythromycin, chloramphenicol and ampicillin. Considering the ability of the bacteria to tolerate various types of heavy metals, they could potentially be used for bioremediation of heavy metal from environment

Key Words: Bioremediation, *Enterobacter* sp., Rice weevil, Heavy metal,

Introduction

Rice weevils *Sitophilus oryzae (L).* is a destructive pest found in stored grains, processing plant, rye, barley, rice, oats, wheat and corn which seriously affecting the growth and productivity of respective agricultural products (Olotuah, 2014). The weevils are distributed worldwide especially in warm climate regions, such as North America and south Asia including Malaysia (Chen *et al.*, 2013). The weevils are most species-rich and associated with large group of bacterial community. Nevertheless, little knowledge is known, regarding weevils from rice and bacterial communities in weevils and their associations with hosts (Lu *et al.*, 2013).

As other types of insects, rice weevil is reported to contain prokaryotic symbiotes harboured in a specialized organ-like structure called bacteriocytes (Lu *et al.*, 2013). These symbiotes, even though are not essential for the reproduction and survival of the host, but may considerably affect various aspects of the ecology, behavior, and physiology of their host, such as traits associated with plant utilization, protection against natural enemies, or responses to climate changes (Sugio *et al.*, 2014). Generally, as for all animals, the microorganisms are prominent in the digestive tract or gut system of the insects' host. Bacteria in the insect guts system are diverse in nature, their diversity and abundance is usually affected by varied lifestyle of host organisms which includes environmental factors such as temperature and nutrient uptake (Douglas, 2009). The bacterial symbiotic relationship in insect guts play a crucial role in regulating the host's metabolism, efficient digestion for extraction of maximum energy from ingested foods and protect the host from other potentially harmful microbes (Yun *et al.*, 2014).

Besides, the bacteria from gut system can be genetically manipulated, then reintroduced into insects to negatively modify specific biological features (Lu, *et al.*, 2013). For instance, an interesting research was conducted on *Dermolepida albohirtum* larvae, where a subset of the bacterial community from scarab hindgut, was characterized. The bacterium was then served as a potential candidate for genetic manipulation strategies targeting the feeding activity of the beetles (Pitman *et al.*, 2010).

Isolation of bacterial community from rice weevil and other insects gut system is still a major challenge for the researchers due to the resistance of the microbes to be cultivated under laboratory conditions. *Less* than one percent of the insect gut microbiota is being isolated and identified via the culture dependent method (Colman *et al.*, 2012). Thus, limited studies have been demonstrated on the isolation and characterization of bacteria from the gut of rice weevil. This study aimed at characterization of bacteria from rice weevil previously identififed as *Enterobacter* sp. obtained via culture dependent method, this could possibly reveal some important application of the bacteria itself.

Several studies have been conducted on the isolation of bacteria from the guts of various rice weevils (Brucker and Bordenstein, 2012; Sanchez-Contreras and Vlisidou, 2008; Weiss and Aksoy, 2011). These bacteria are usually harboured in a specialized organ called bacteriocytes. The insect gut offers a conducive environment where microbes can develop good conditions with plentiful nutrients resources (Rinke *et al.*, 2011). Moreover, the host and the bacteria normally share closely biochemical and genetics interactions. The symbiont supplies the host insects with vitamins which subsequently interfere with the host metabolisms. On the other hand, these endo-symbionts, even though are not essential for the reproduction and survival of the host, but may considerably affect various aspects of the ecology, behaviour, and physiology of their host. They found to be associated with plant utilization, protection against natural enemies, or responses to climate changes (Sugio, *et al.*, 2014). Beside, some of these microbes are able to secrete enzymes capable of degradation of compounds found in the insect diet, helping them in the digestion process and decreasing the toxicity of plant defense compounds (Dillon and Dillon, 2004).

In the recent years, the understanding of the role of symbiotic bacteria in the insect gut system emerged as an important step in the process of using microbes in the biocontrol of pest species. For example, gut bacteria *Bacillus megaterium, Alcaligenes faecalis* and *Proteus vulgaris,* isolated from *Hylesia metabus* (Cramer) larvae, have been reported to cause larval mortality (Thakur *et al.*, 2015). Similarly, *Bacillus licheniformis*, *Serratia marcescens, Enterobacter hormaechei, Paenibacillus* sp., and *Enterobacter* sp. isolated from the gut of *Rhynchites bacchus* were found to cause mortality in the same host (Thakur *et al.*, 2015). In addition, *P. aeruginosa*, *Serratia* sp., *Variovorax paradoxus and S. marcescens* associated with the gut of *Ostrinia nubilalis* (Hubner) have been reported to be pathogenic against their host (Thakur *et al.*, 2015). The findings reveal the application of the bacteria isolated from the gut system of insects which can act as microbial control for insect pests.

Methodology

Media Preparation

In this research, nutrient agar (NA) and nutrient broth (NB) were used to grow the bacteria.

Background of Isolate

In this current study, previously isolated bacterial strain from rice weevil which was molecularly characterized and designated as *Enterobacter* sp. was used. The strain was retrieved from glycerol stock from enzyme research laboratory in Faculty of Biosciences and Medical Engineering (FBME) Universiti Teknologi Malaysia.

Characterization of the bacterial strain

Morphological Tests

Gram staining of the isolate was performed in order to determine the Gram reaction of the bacterial strain.

Biochemical Tests

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To determine the metabolic ability of the bacteria, some biochemical tests were conducted, these include catalase test which was done by droping 3% (v/v) hydrogen peroxide added over the test smear as described by Atta and Radwan, (2012). Indole test by adding few drops of Kovac's reagent to the culture tubes as described by Khalifa *et al.*, (2015). Citrate test was conducted using the method described by Atta and Radwan, (2012). Oxidase test using drops of oxidase reagent (N,N,N′,N′-tetramethyl-p-phenylenediamine) on top of smeared pure colony of the bacteria, urease test using Christiansen's urea agar, starch hydrolysis test using Iodine reagent as described by Khalifa, *et al.*, (2015), triple sugar iron test, motility test was done by adding 0.43g of meat extract, 1.5g peptone, 0.72g of sodium chloride and 0.72g of agar powder in 200 mL of distilled water. Autoclaved transferred to test tubes, the test tubes were incubated for 24 hours at 37°C. Oxidative fermentation test using OF medium, lactose utilization test using Mac Conkey agar and citrate utilization was done by streaking of colony of the bacterial strains on universal bottles containing Simmon citrate agar slants and incubated at 37°C. After 48 hours, the colour change and growth of the microorganisms was examined. MacWilliams, M. P. (2009).

Determination of carbon and nitrogen source

Preparation of chemically defined medium (CDM)

To determine the best carbon and nitrogen source for the growth of the bacteria, a chemically defined medium was prepared and the pH of the solution was adjusted to 7.0 ± 0.2 using sodium hydroxide (NaOH) and autoclaved at 121ºC, 101.3kPa for 15 minutes. Each of the selected carbon sources was added to CDM at final concentration of 0.1% (v/v) using filter sterilization.

The selection of the carbon source was done based on literature and biochemical tests observation. The carbon source includes sucrose, glucose, lactose and starch. While the nitrogen source are casamino acids, nitrate, nitrite and NH_4. In order to determine the best nitrogen source, the best carbon source earlier determined (sucrose) was used for the growth of the bacteria.

Determination of Bacterial Growth in Selected Carbon and Nitrogen Sources by Growth Profiling

The utilization of selected carbon and nitrogen source was determined by measuring the growth of bacterial strains. The absorbance of bacterial strains was measured using spectrophotometer at optical density $OD_{600nm.}$

Strain tolerance towards heavy metals
Preparation of heavy metal stock solution

To test for heavy metal resistance, different type of heavy metals stock solution (Aluminium, Copper, Manganese and Zinc) at different concentration (200mg/L, 400mg/L, 600mg/L and 800mg/L) was prepared separately and filter-sterilize into 50 mL falcon tubes and keep it for further used.

Antibiotic susceptibility test

For antibiotic susceptibility test, disc diffusion method was used (Bauer *et al*. 1966). Each Muller Hinton (MH) agar plates were divided into four sections where three containing different antibiotic site and one served as control. The antibiotics that were used in this study are ampicillin, tetracycline, erythromycin, bacitracin, kanamycin and chloramphenicol. Negative control was prepared using sterile distilled water. The inoculated plates were then sealed and incubated for 18 hours at 37ºC.

Results and discussion

Colony Morphology

The *Enterobacter* sp. that was previously isolated from rice weevil, was successfully sub cultured onto Nutrient agar (NA) at 37°C for 24 hours from glycerol stock. The growth of the bacterial strain was examined on nutrient agar plates and the colony morphology was summarized as in Table 1.

Table 1 Colony morphology of bacteria strains

Characteristics	*Enterobacter* sp.
Pigmentation	White
Shape	Circular
Size	Small
Appearance	Shiny
Texture	Smooth
Optical property	Translucent
Elevation	Convex
Gram staining	Negative

Biochemical tests

The results of biochemical tests conducted on *Enterobacter* sp. is compared with strains from previous studies and summarized in the Table 2. In this research, *Enterobacter* sp. exhibit almost the same biochemical properties with the previous research of (Madhaiyan, *et al.*, 2010), where they isolated a Gram negative, motile *Enterobacter* sp. from rhizosphere soil of groundnut. Their results indicates positive for catalase, oxidase, starch hydrolysis and MacConkey, but negative for indole, urease, however, differences are observed in the motility and oxidase test, which was because of the absence of flagella and enzyme cytochrome c oxidase as earlier stated.

Effect of different carbon sources on growth of the bacteria

The effect of different carbon sources on the growth of both *Enterobacter* sp. was determined. The four carbon sources used include glucose, lactose, sucrose and starch. The results are presented in the form of growth curve and bar charts.

Table 2 Summary of biochemical test

Characteristics	*Enterobacter* sp.	*Enterobacter arachidis* sp. (Madhaiyan, *et al*., 2010).	*Enterobacter helveticus* sp. (Stephan *et al*., 2007)
Catalase Test	(+)	(+)	(+)
Oxidase	(-)	(+)	(+)
Urease	(+)	(-)	(-)
Citrate	(+)	(+)	(+)
Mac Conkey	(+)	(+)	ND
Starch Hydrolysis	(-)	(+)	(+)
Oxidative Fermentation	(+)	ND	ND
Indole test	(-)	(-)	(-)
Motility	(-)	(+)	(+)
Triple Sugar Iron Test	(+)	ND	ND

Legends: (+) Positive, (-) Negative, (ND) Not determine

The growth of *Enterobacter* sp. in a chemically defined medium (CDM) containing glucose, lactose, sucrose or starch as the sole carbon sources were presented in Figure 1. Among the carbon sources tested, sucrose was found to be the best carbon source for the growth of the strain, with a growth rate by the logarithmic derivative of the OD in micron (μ) of $0.1688h^{-1}$, followed by glucose with growth rate of $0.1578h^{-1}$. This slightly slow growth of the bacteria on glucose may be due to suboptimal level of cAMP, leading a situation called reversed diauxic shift, usually occur as a result of poor nitrogen sources (Bren, A. *et al.* 2016*)*. While the sufficient utilization of the sucrose by the bacteria is due to the presence of an enzyme called sucrose phosphorylase. This enzyme catalyzes phosphorylation of sucrose to α-D-glucos-1-phosphate and fructose (Schwab *et al.,* 2007). Such system and the metabolic pathway has been previously described for Gram positive bacteria *Bacillus subtilis* sp. and Gram negative *Klebsiella pneumonia (*Bockmann *et al.,* 1992).

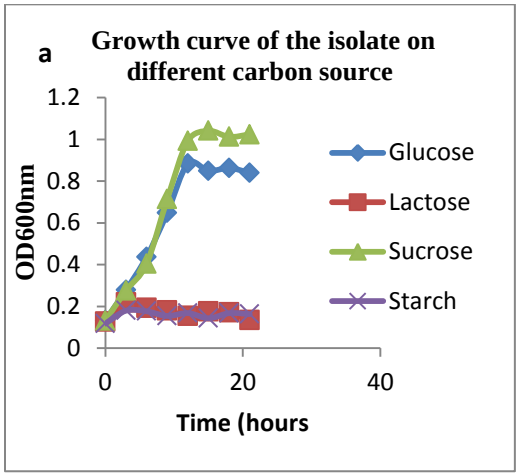
Bacteria showed poor growth when lactose was supplied as sole carbon sources in the medium. This indicates the absence of enzyme lactase (β-galactosidase) to break down lactose. Hence, lactose cannot be broken down into smaller glucose subunits which are subsequently required to enter pentose phosphate pathway and the Krebs cycle.

As presented in the Figure 1. *Enterobacter* sp. showed zero growth rate in the CDM containing only starch as a sole carbon and energy source, this is due to the absence of an enzyme alpha-amylase which use to break down starch.

Effect of different nitrogen sources on growth of the bacteria

The effect of different nitrogen sources on the growth of the *Enterobacter* sp. was

determined. The four nitrogen sources used include casamino acid, ammonium, nitrate and nitrite. The results are presented in the form of growth curve and bar charts.



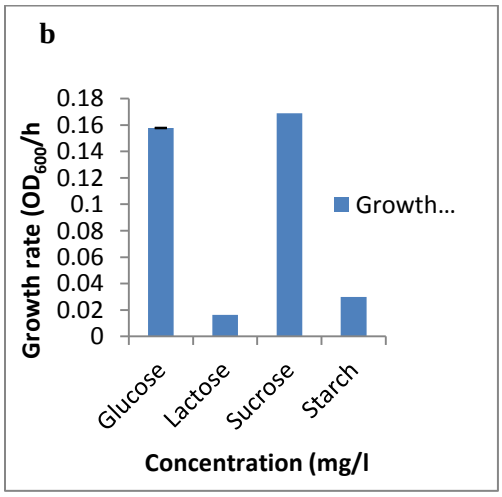


Figure 1: The Growth of *Enterobacter* sp. on Different Carbon Sources.(a) Line graph, (b) Bar chart

Effect of different nitrogen sources on growth of the bacteria

The effect of different nitrogen sources on the growth of the *Enterobacter* sp. was determined. The four nitrogen sources used include casamino acid, ammonium, nitrate and nitrite. The results are presented in the form of growth curve and bar charts.

Nitrogen Sources and the Growth of *Enterobacter* sp.

Figure 2 illustrate the effect of different nitrogenous compounds (casamino acid, ammonium, nitrate and nitrite) on the growth of *Enterobacter* sp. The result indicates that, casamino acid appeared to be the superior nitrogen source of choice relative to ammonium, nitrate and nitrite in supporting growth of the strains (Figure 2). However, the remaining nitrogen sources showed growth with significant decrease in the growth rate as compared with the growth obtained in the casamino acid. Casamino acid is a hydrochloric acid hydrolysate of casein, which supplied a completely hydrolyzed protein nitrogen source (Williams *et al.*, 2001). Its ability to remarkably sustain the growth of *Enterobacter* sp. was because it can easily break down small amino acid sub units.

In general, the order of nitrogen preference on growth of *Enterobacter* sp. is observed as casamino acid > ammonium > nitrate > nitrite, with growth rate of 0.1586, 0.1062, 0.0517 and 0.0494h^{-1} respectively.

Antibiotic susceptibility test

The antibiotic susceptibility of *Enterobacter* sp. was tested against six antibiotics, which include ampicillin, tetracycline, erythromycin, bacitracin, kanamycin and chloramphenicol.

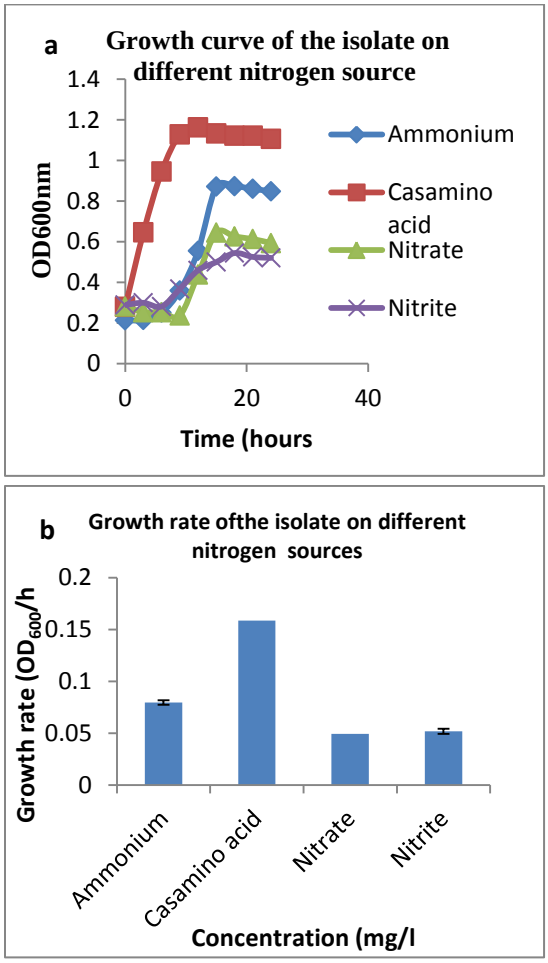


Figure 2: Growth of *Enterobacter* sp. on different nitrogen sources. (a) Line graph (b) Bar chart

The results were measured based on zone diameter, and classified as susceptible, intermediate, or resistant (Table 3).

Ampicillin is usually bactericidal, which acts as an irreversible inhibitor of the enzyme transpeptidase that is needed by bacteria to make their cell wall (Kabir, 2013). In this research, *Enterobacter* sp. showed susceptibility towards ampicillin with a clear zone of 1.30 ± 0.10mm. Erythromycin is another antibiotic which interferes with aminoacyl translocation of tRNA from the A site to the P site of the rRNA, thereby inhibiting the formation of cell wall (Wilson, D. N. 2014). However, *Enterobacter* sp. showed resistance against erythromycin with no clear zone of inhibition around the disc, Tetracycline which acts in the same way with erythromycin indicated to be susceptible for the strain with clear zone diameter of 2.10 ± 0.00mm. Chloramphenicol usually binds reversibly with the large ribosomal subunit of bacteria thereby inhibiting their growth (Xaplanteri *et al.*, 2003). However, the isolate was found to have an intermediate resistance against chloramphenicol with zone of inhibition of 0.90 ± 0.03mm. Both bacitracin and kanamycin function as inhibitors of cell wall biosynthesis (Garrett and Won, 2007). They were indicated susceptibility for *Enterobacter* sp. with clear zone diameter of 1.80 ± 0.10mm.

Tolerance to heavy metals

Heavy metal tolerance of the bacteria *Enterobacter* sp. was tested on four metal ions, which include aluminum, copper, manganese and zinc. The test was conducted at different concentrations of 200 mg/l, 400 mg/l, 600 mg/l and 800 mg/l. Based on heavy metals screening, the growth pattern of isolate was further investigated on three metals, which include aluminum, manganese and copper. The growth of both isolates was completely inhibited in medium containing zinc.

Table: 2 Antibiotics Susceptibility of *Enterobacter* sp.

Antibiotics	Inhibition zones' diameters (mm) after 24 hours incubation at 37°C.	
	***Enterobacter* sp.**	**Distilled H_2O**
Ampicillin	1.30 ± 0.10	No zone inhibition
Erythromycin	No zone inhibition	No zone inhibition
Tetracycline	2.10 ± 0.00	No zone inhibition
Chloramphenicol	0.90 ± 0.03	No zone inhibition
Kanamycin	1.80 ± 0.10	No zone inhibition
Bacitracin	1.80 ± 0.10	No zone inhibition

The growth of *Enterobacter* sp. on Aluminum

The growth of *Enterobacter* sp. on aluminum at various concentrations is presented in Figure 4. The strain illustrated significant tolerance towards aluminum at high concentration of 800 mg/l with maximum growth rate (μ) of 0.240h^{-1}. However, the best growth of the bacteria was seen on the aluminium concentration of 200 mg/l with the maximum growth rate of 0.404h^{-1}. Therefore, it is indicated that, at lower concentrations, the cells were exposed to less toxicity of heavy metals as compared to the higher concentrations. Thus, the order of tolerance for *Enterobacter* sp. to aluminum concentrations can be presented as 200 mg/l > 400 mg/l >600 mg/l >800 mg/l.

The ability of the strains to tolerate the aluminum even at the high concentration of 200 mg/l may be associated to the binding of the metal to certain natural carriers such as the catecholamines transferrin or, simply, to the amino acids, in the transport mechanism (Bragadin *et al.*, 2004).

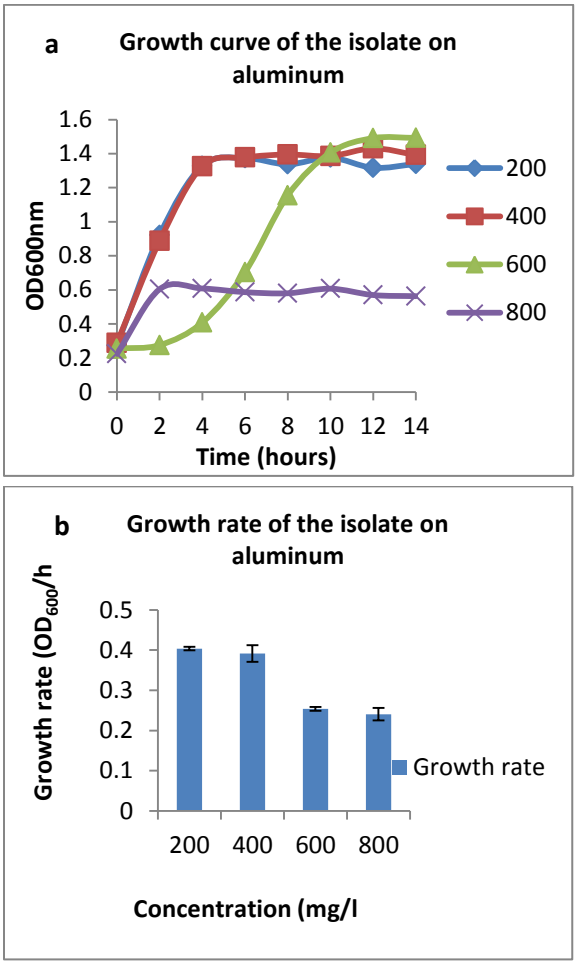


Figure 4: The growth of *Enterobacter* sp. On different concentrations of Aluminum at 37°C for 14 hours. (a) Line graph, (b) Bar chart

The growth of *Enterobacter* sp. on Copper

The growth of *Enterobacter* sp. in the presence of copper observed is shown in Figure 5. The strain was able to tolerate the toxicity of copper in relatively high concentrations as compared to many previous researches. (Luna *et al.*, 2015; Farkas *et al.*, 2015). However, in this research the growth of *Enterobacter* sp. on copper was relatively weak as compared with the other metals tested. This isolate displayed great level of tolerance in the copper concentrations of 200 and 400 mg/l. But at 600 mg/l and 800 mg/l the growth was completely inhibited (during screening). The result obtained at 200 mg/l showed a slight decrease as compared to the control. Further increase in the concentration to 400 mg/l decreases the growth with long lag phase. Growth rate for the bacteria in the absence of metals was compared with the growth on copper at 200 mg/l and 400 mg/l and the result obtained was $0.167h^{-1}$, $0.166h^{-1}$ and $0.158h^{-1}$ respectively. Figure 5.Copper may disrupt enzyme structures, and functions by binding to sulfur or carboxylate-containing groups and amino groups of proteins, it may also interact with lipid, causing their peroxidation and opening holes in the cell membranes, thereby compromising the integrity of cells (Manzl *et al.*, 2004). The ability of both strains to tolerate copper may be link to the ATPase pump functions in the organisms' ability to adapt to a copper-rich environment (Ohno *et al.*, 2015).

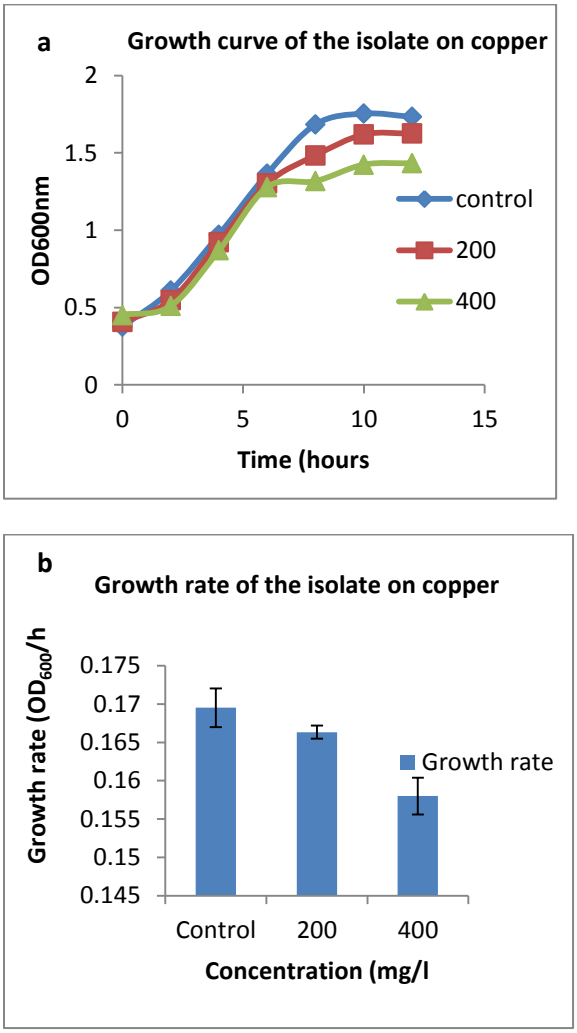


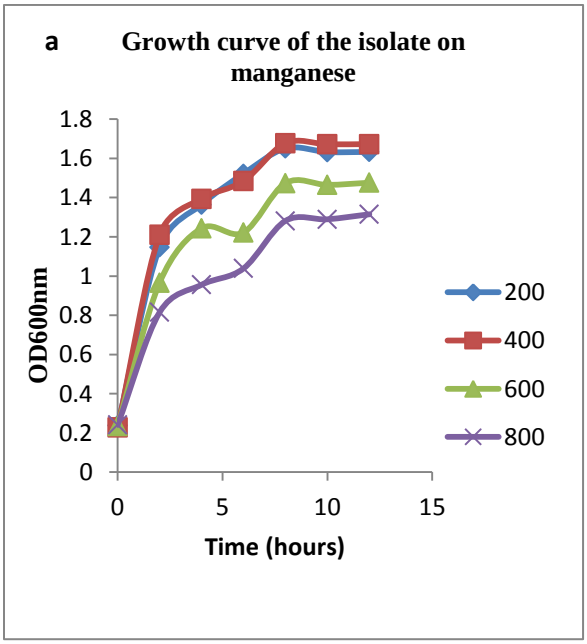
Figure 5: The growth of *Enterobacter* sp. On different concentrations of Copper at 37ºC for 14 hours. (a) Line graph, (b) Bar chart

The growth of *Enterobacter* sp. on Manganese

As shown in the Figure 6, the growth of *Enterobacter* sp. on manganese at various concentrations was observed. The isolate grow well in high concentrations of manganese at 800 mg/l, with maximum growth rate of $0.244h^{-1}$. However, when the concentration of manganese was 600 mg/l,

the growth rate of the bacteria improved. Further decrease in the manganese concentration to 400 and 200 mg/l, significantly improved the rate of the growth. Hence, the order of toxicity of manganese on *Enterobacter* sp. is 200 mg/l > 400 mg/l >600 mg/l >800 mg/l with the growth rate of (0.310, 0.312, 0.275 and 0.244 g/l) respectively.

The variations between Mn^{2+} concentration and the decrease in the growth rate for the strain at Mn concentrations >200 mg/l, may reflect the fact that, excess Mn may interfere with the bacterial transport system. The ability of the strain to tolerate the high concentration of manganese may be as a result of direct transport of Mn in to the cell via the plasma membrane Ca^{2+} channels, which subsequently allow the uptake of Mn^{2+} (Pittman, 2005).



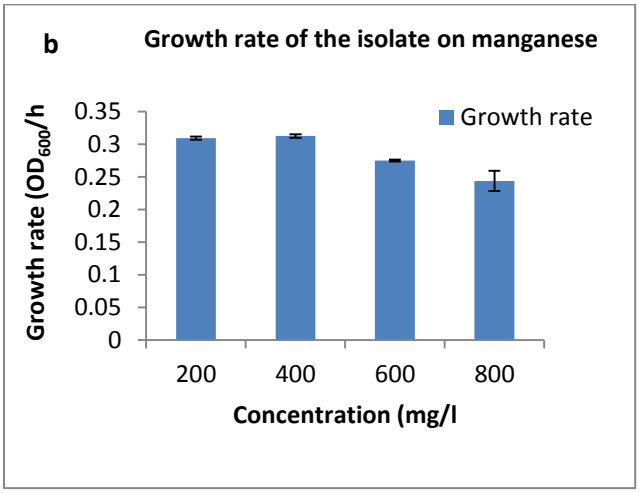


Figure 6: The growth of *Enterobacter* sp. on different concentrations of Manganese at 37ºC for 14 hours. (a) line graph (b) Bar chart

Conclusion

This bacterial strain designated as *Enterobacter* sp. was characterized based on its biochemical, morphological and substrate utilization. Different carbon and nitrogen source were utilized by strain for its growth, in which sucrose and casamino acids were found to be the best combination of carbon and nitrogen sources suitable for its growth. Antibiotic susceptibility tests are normally used to characterize most of bacteria isolated from insect guts. In this research, the strain have shown resistance to some antibiotics, therefore, this information confirmed the potential use of this strain as a bio-control agent for rice weevil. Ability of strain to grow in the presence aluminium, copper and manganese signifies their functional roles in the biodegradation of heavy metal compounds. Thus, the bacterial strain could be useful for degradation of toxic heavy metals ions in the environment.

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