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Isolation and Characterization of a 2,4-Dinitrophenol-degrading Bacterium

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ABSTRACT

2,4-dinitrophenol (2,4-DNP) is utilized in the production of wood preservatives, dyes, and also as a pesticide. Human acute (short-term) exposure to 2,4-DNP in humans by means of oral exposure are nausea or vomiting, sweating, headaches, dizziness, and weight reduction. Thus, the removal of this compound is highly sought. A 2,4-DNP-degrading bacterium (isolate 1) was isolated from a sample soil from Terengganu. This bacterium (isolate 1) was characterized as a rod Gram positive, non-sporulated, and non-motile bacterium. The bacterium is oxidase negative and had catalase positive activity and was able to grow aerobically on 2,4-dinitrophenol as the sole carbon source. This bacterium showed maximal growth on 2,4-DNP at the temperature optimum of 30 °C, pH 5.0 and was tolerant to 2,4-DNP concentration of up to 0.5 mM (0.092 g/L). This bacterium prefers to use urea as the nitrogen source in addition to yeast extract for mineral source and vitamin precursors.

INTRODUCTION

Nitrophenols are one of the most abundant xenobiotic compounds present in nature. Nitrophenols or nitroaromatic compounds are present everywhere in the environment. These compounds are usually the building blocks for the synthesis of pesticides, dyes, explosives, herbicides and drugs [1] and precursors of arylamines that are widely used in industry [2]. Therefore, nitrophenols occur as contaminants in waste water, rivers, groundwater (in pesticides-treated soils) and the atmosphere [3]. Nitroaromatic compounds such as 2,4-dinitrophenol (2,4-DNP), 2,4,6-trinitrophenol are dangerous contaminants. These compounds pose significant health risk since they are carcinogenic.

The U.S Environmental Protection Agency has listed 2-nitrophenol, 4-nitrophenol, and 2,4-DNP as " Priority Pollutants" and recommended restricting their concentrations in natural water to < 10 mg/l [4]. Their toxic effects include damage of liver and kidneys and also can damage blood cells that can cause anemia. This has been reported on workers engaged in large scale manufacturing and handling operation in concentrations greater than 2 mg/l [5]. To achieve high level of nitrophenol removal, special systems and analytical techniques are required. Even though 2,4-dinitrophenol has been band nowadays but their persistence in environment still have to be cleaned to have a better environment system. Although a variety of physicochemical are available for clean up the surface water, interest in use of microbial degradative is growing [3]. Bioremediation is most popular ways of microbial degradation process nowadays.

Bioremediations can be defined as the cleaning up techniques that transform the hazardous chemical to less hazardous chemicals by biological systems [6] Degradation of 2,4-DNP is part of biodegradation where is one of bioremediations process. Biodegradation is nature's way of recycling wastes or breaking down the organic matter that can later be used as nutrients by the other organisms. Microorganisms such as bacteria, fungi cynobacteria or organisms can carry out this process. Biodegradation of nitrophenols compounds are very important in order to reduce the contaminants; rapid, cost effective, environmentally friendly. Nitrophenols are widely used as intermediates in many industrial manufacture. Therefore, nitrophenols occur as contaminants in waste waters, river groundwater and in our

atmosphere also. It has been shown that phenols are organic carcinogens and mutagens [3]. Commercial dinitrophenol is primarily used in industrial organic compounds. They are frequently used as raw materials or intermediates in the manufacture of explosives, pharmaceuticals, pesticides, dyes, wood preservatives, and rubber chemicals [4]**.** The discharge 2,4-dinitrophenol as effluents are xenobiotics to the environment. In addition, they are also recalcitrant due to additional stability that nitro substituents confer to the aromatic ring that make the physicochemical breakdown and microbial degradation of these compounds occur very slowly in natural environment. Some anaerobic biodegradation studies of nitrophenols have shown that the compound is not easily biodegraded and is inhibitory to methanogenic microorganisms at high concentration [4]. Moreover, nitrophenols is well known as an uncoupler of the electron transport phosporylation [2].

2,4-dinitrophenol can across membranes in its protonated form, acting as an H^+ carrier, and dissipate the electrochemical gradient across the cell membranes [7]. This condition will cause the uncoupling of the oxidative phosphorylation pathway without blocking oxygen consumption [8]. Consequently, the U.S. Environmental Protection Agency listed 2,4-dinitrophenol on its " Priority Pollutants List" and recommends restricting its concentrations in natural water [8]. The emission standard for phenol (precursor of 2,4-dinitrophenol) in industrials effluents after wastewater treatment is set at 5 mg/l in Japan [9]. However, nitrophenols have been reported to be toxic to aquatics organisms at concentrations lower than the emission standard. Therefore, it is important to remove 2,4-dinitrophenol at low concentrations from industrials wastewaters for aquatic environment protection [9]. Hirooka *et al.,* have investigated the 2,4-dinitrophenol degrading ability of *A. variabilis* and clarified that 2,4-dinitrophenol is reduced to 2-amino-4-nitrophenol (2-ANP) by this strain. Nitrophenols are known to be reduced to nitroamines or amines during the microbial biodegradation. The 2-ANP, which is a degradation product of 2,4-dinitrophenol is a potent mutagen [9].

Other than that, some anaerobic biodegradation studies of nitrophenols have shown that the compound is not easily biodegraded and is inhibitory to methanogenic microorganism at high concentrations [10,11]. Other studies have shown that nitrophenols are readily reductively detoxified in methanogenic consortia to their respective aminophenols, which are several orders of magnitude less toxic [12–14]. In some anaerobic toxicity tests, data have shown that 2,4-dinitrophenol caused obvious inhibition to methanogenesis [4].

In the atmosphere, 2,4-dinitrophenol may exist in the gaseous or particulate form. It may be removed by direct photolysis, by settling or washout in precipitation, or it may react in the gas-phase with photochemically produced hydroxyl radical. In this case, probable routes of human exposure to 2,4-dinitrophenol are by inhalation or dermal contact (at site). 2,4-dinitrophenol is exposure orally in humans has resulted in nausea, vomiting, sweating, dizziness, and headache as the acute toxicity of but for the chronic oral exposure in humans and animals has resulted in formation of cataracts and skin lesions and has caused effect on bone marrow, central nervous system, and cardiovascular system. Other than that, toxic effects of 2,4-dinitrophenol also include liver damage and anemia that have been reported on workers engaged in large scale manufacturing and handling operation in concentrations greater than 2 mg/l [5].

MATERIALS AND METHODS

Microorganisms

The 2,4-dinitrophenol-degrading bacterium used in this study was isolated from a soil sample from Kertih, Terengganu in 2007.

Media preparation

A modified mineral salts medium (MS medium) was used for the isolation and maintenance of the isolated bacterium. A modified MS medium containing (7 g/L) Na_2HPO_4, (1 g/L) KH_2PO_4, (10 mg/l) $CaCl.2H_2O$, (20 mg/l) $MgSO_4.7H_2O$ and (0.046 g/L) 2,4-dinitrophenol. All of these then were dissolved and bring the volume to 1 L [15]. Soil sample, previously diluted in tap water (1 g in 10 mL tap water) was added to the medium for growth and incubated at room temperature on the orbital incubator shaker. The bacterium was grown in the liquid medium until the color of the 2,4-DNP disappeared. 2,4-DNP media was used for the isolation of the 2,4-DNP-degrading bacterium. Bacterium from the liquid 2,4-DNP was spread on the solid 2,4-DNP media plates at pH 7 [2].

Single colonies obtained from the plates were transferred to liquid 2,4-DNP broth (pH 7). After being subcultured in fresh medium, single colonies were isolated by repeatedly plating on 2,4-DNP agar and incubated in liquid culture on the shaker (120 rpm) for 24 hours at room temperature. The 2,4-DNP-degrading bacterium was maintained in MS medium broth supplemented with 0.25 mM (0.046 g/l) of 2,4-DNP and incubated for 24 hours on rotating shaker in room temperature. Working cultures were maintained by subculturing every two weeks to prevent possible plasmid loss.

Effect of 2, 4-dinitrophenol concentration

A serial dilution technique was performed to determine the optimum 2,4-dinitrophenol concentration by counting the colony-forming unit (CFU). 2,4-DNP broth media was prepared which contain different 2,4-dinitrophenol concentrations from 0.1 to 1.0 mM (0.1841 g/L) of 2,4-DNP. After 120 hours of incubation at room temperature, the procedure for single colonies enumeration was carried out. This experiment was carried out in triplicate.

Effect of pH

An overlapping buffer system of pH 4.0-6.0 (citrate buffer) and 6.0-8.0 (phosphate buffer) was employed. The isolate culture was suspended in 2,4-DNP broth media made from the components of the corresponding buffer with reduced amounts of $KH_2\ PO_4$ and K_2HPO_4, before inoculation. The concentration of PO_4^{3-} in the original medium was reduced 100x to prevent interfere when other buffer systems with the pH range beyond the buffering capacity of PO_4^{3-} were intended; but not totally emitted as minute amount of PO_4^{3-} was still needed for the nucleic acid synthesis during bacterial growth.

Effect of temperature

To study the effects of the temperature, bacterial culture was incubated at 10°C, 20°C, 30°C, 37°C, 40°C, 50°C, 60°C. Each temperature media was prepared in triplicate. After 120 hours incubation, the sample was diluted to 10^8 times by serial dilution and was then spread onto 2,4-DNP agar. The single colonies formed were counted.

Effect of various nitrogen sources

Preparation of 2 mg/L of various sources of nitrogen which included ammonium sulphate [$(NH_4)_2SO_4$], sodium nitrate ($NaNO_3$), ammonium chloride ($NH_4\ Cl$), tyrosine, glycine, urea,

leucine, cysteine, and alanine were added into 2,4-DNP broth media, respectively. The medium was prepared in triplicate. Samples were then inoculated for 120 h at room temperature in a constant stirring condition. The growth of the isolate was observed by counting the single colonies forming after a serial dilution was done.

Effect of yeast extract

2,4-DNP broth media with various concentration of yeast extracts were prepared. As usual, the samples were incubated for 120 hours at room temperature in a constant stirring condition. 100 µl of bacteria was taken out and a serial dilution was performed. The aliquots were then spread onto the 2,4-DNP agar. Single colonies formed were then enumerated. Each of the medium was done in triplicate.

2, 4-Dinitrophenol degradation standard curve

To get a standard curve for the rate of degradation use, a range of 2,4-DNP concentrations from 0-0.3 mM were observed under spectrophotometer at absorbance value 546 nm. A straight line standard curve was plotted using absorbance on axis-y and 2,4-DNP concentration on axis-x.

Rate of degradation

Five hundred µl of bacteria culture were suspended in the 2, 4-DNP broth media at room temperature in continuous stirring condition. Two mL of bacteria were taken out for degradation observation. The 2 mL of bacteria suspend then centrifuged for 5 minutes at 13 rpm at room temperature to pellet down the bacteria. The supernatants then were observed were observed under spectrophotometer at absorbance value 546 nm. This experiment was done in triplicate.

RESULTS AND DICUSSION

Dinitrophenols effluents are generally removed via physical, chemical or biological treatments. Although the physicochemical means of remediation are widely effective, nevertheless they suffer from shortcomings such as high cost of implementation, low efficiency, and formation of hazardous by products and high energy requirements. Thus, there is a need to find alternative treatments that are effective in removing 2,4-dinitrophenol from larger volume of effluents and economical. Biological approach is the most environmental friendly as it does not require large amount of energy is cost effective and does not generate toxic substances. Nevertheless, the effectiveness of microbial treatment depends on the survival, adaptability and activities of the selected microorganisms.

The use of biological means to circumvent crucial catabolic steps in xenobiotic degradation has been subject of leading interest in many laboratories. Effort to isolate bacterial cultures capable of degrading 2,4-dinitrophenol started long time ago and after some considerable periods of time passed, before an upsurge of the same interest took place in the western world. Reports about decolourisation of 2,4-dinitrophenol were aplenty among a wide range of microorganism either in an aerobic or an anaerobic condition.

Although a number of organisms that utilize mononitroaromatic compounds as a carbon source have been described, only a few bacteria which mineralize dinitrophenols [16] are known. Unsuccessful attempt to isolate bacteria that grow on dinitroaromatic compounds as sole carbon source prompted researchers to enrich for organisms which utilize these xenobiotic compounds as a nitrogen source. Aerobic decolourisation of 2,4-dinitrophenol by bacteria cultures, on the other hand, is limited and long periods of adaptation in chemostat conditions is necessary to isolate strain that is able to degrade 2,4-dinitrophenol aerobically. However, aerobic co-metabolic decolourisation of 2,4-dinitrophenol in the presence of external carbon source is common [15]. Several bacteria of the *Actinomycetales* family (notably of the genera *Rhodococcus* and *Nocardioides*) grow aerobically on trinitrophenol and dinitrophenol and utilize the compounds as sole of nitrogen, carbon, and energy source [17]. This capacity can be harnessed for bioremediation.

Nitroaromatic compounds are very toxic for living cells, making the biodegradative processes more difficult. 2,4-dinitrophenol produces its toxic effect on animals, plants, and microorganisms by uncoupling the oxidative phosphorylation process in mitochondria. 2,4-dinitrophenol can cross the membranes in its protonated form, acting as H^+ carrier, and dissipate the electrochemical gradient across the cell membranes without blocking the oxygen consumption [8]. Nevertheless, some bacteria degrade mononitrophenols by several pathways.

Thus, monooxy-genases produce benzoquinones that are subsequently reduced to catechol or hydroquinones as a first step of the mineralization of mononitrophenols [1]. In contrast, the polynitrophenol ring is even more deactivated and is not a good substrate for oxygen attack. Therefore, dinitro- or trinitrophenols are usually nitrophenols graded through reductive pathways that include: (i) the reductive degradative pathway of polynitroaromatic compounds the initial reduction of the nitro groups yielding the corresponding amino or hydroxylamino derivatives [3,18]. (ii) The oxidative pathway prior to fission of the aromatic nucleus the nitro group are replace by hydroxyl group [19]. The type of metabolic pathway is depended on culture and substrate [20].

Other than that, sometimes several strains of bacteria such as *Rhodococcus opacus* was shown that to encode the tautomerase, catalyzing a proton shift between the aci-nitro and the nitro form the dihydrade Meisenheimer complex of 2,4,6-trinitrophenol. The product of nitrite released was hydride Meisenheimer complex of 2,4-dinitrophenol, which was hydrogenated to the dihydride to the Meisenheimer complex of 2,4-dinitrophenol by the hydride transferase I and the NADPH-dependent F_{420} reductase from the strain [17].

Characterization of isolate 1

Several taxonomic and biochemical tests were carried out to identify isolate 1 based on the results obtained in **Table 1**. Isolate 1 was determined to be Gram positive cocci and non-motile bacterium as shown in **Fig. 1**. This bacterium is not able to produce cytochrome oxidase that catalyses the oxidoreduction of cytochrome c. In the presence of oxygen, the cytochrome c system can reduce the whole series of organic substances. The presence of this enzyme is observable with the formation of blue violet colouration on oxidase test. This bacterium also, showed positive result with the catalase indicating that these bacteria can rapidly degrade hydrogen peroxide using enzyme superoxide dismutase.

Numerous dinitrophenol-degrading microorganisms are reported in the literature such as *Trichosporon cutaneum*, *Burkholderia* sp. strain KU-46, *Nocardioides simplex*, *Sphingomonas* sp. UG30 and several *Rhodococcus* spp. such as *Rhodococcus imtechensis*, *Rhodococcus erythropolis*, *Rhodococcus koreensis* [21–37].

Table 1. Characterization of 2,4-DNP-degrading isolate.

characteristics	isolate 1
colony cell	milky in colour
cell morphology	cocci
gram staining	Gram positive
spore staining	Non-sporulated
motility test	Non-motile
oxidase test	negative
catalase test	positive

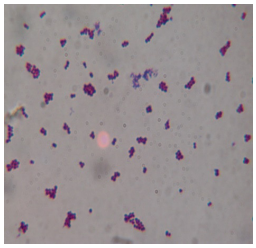

Fig. 1. The cell morphology of isolate 1 under microscope observation. The image was taken in oil magnification of 1000x.

Bacterial growth curve

Fig. 2 represents isolate 1 growth curve over time. It was found that the lag phase occurred for one day where the bacterium was adapting itself by synthesizing the inducible enzymes that are required for the degradation of 2,4-DNP [32,38]. The optimum growth of the bacterium was found to be in the fifth day a rapid decrease in growth was observed, possibly caused by the toxicity of the compound and buildup of metabolic waste [38].

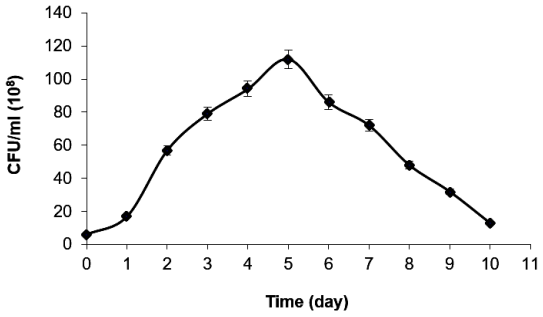

Fig. 2. Growth curves of isolate 1 in 0.25 mM (0.046 g/l) of 2,4-DNP as carbon and nitrogen source over time. The error bars represent mean ± standard deviation of three replicates.

Effect of 2,4-Dinitrophenol concentration on growth

The growth of the bacterium was affected by the concentration of 2,4-DNP. As the concentration of 2,4-DNP was increased, the bacterial growth was decreased (**Fig. 3**). However, the bacterium growth still efficient with 2,4-DNP concentration below 0.5 mM, after which the growth gradually declines to zero growth. At the lower concentration of 2,4-DNP bacteria use 2,4-DDNP as carbon and nitrogen source and at the higher concentration of 2,4-DNP, the 2,4-DNP is known as a toxic compound and inhibit the growth of many sensitive bacteria [21–37]. At these inhibitory concentrations, growth of bacteria on 2,4-DNP as carbon and nitrogen source will be affected.

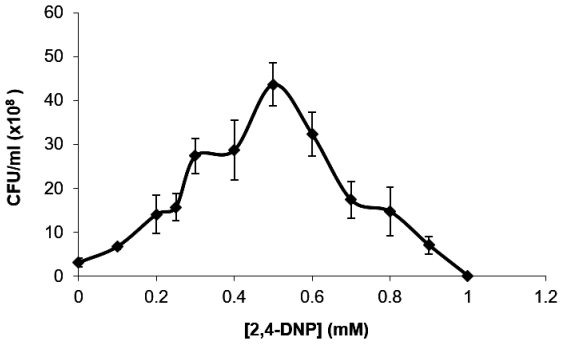

Fig. 3. Effects of 2,4-DNP concentrations on growth of isolate 1. Isolate was grown in medium with different concentration of 2,4-DNP. The error bars represent mean ± standard deviation of three replicates.

Effect of pH

Fig. 4 shows the effects of pH on the growth of the isolate 1. From the results obtained, the optimum growth of isolate 1 was at pH 5 and decrease steadily due to the increasing pH value. According to Blasco and Castillo [1] the uptake of DNP depended on the pH of the medium, showing that a maximal value at pH 7. It is because the pKa of the DNP is 4 at pH 7, and the ratio between the charged an uncharged form of DNP is about 10^3.

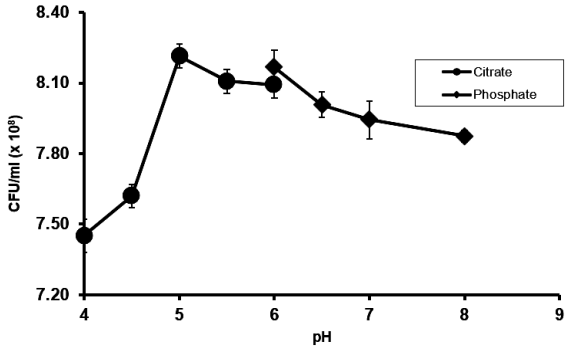

Fig. 4. pH effect on bacterial growth of isolate 1. The experiment wass carried out using an overlapping buffer system. The error bars represent mean ± standard deviation of three replicates.

Effect of Temperature

The temperature of a system is a measure of the kinetic energy of the molecules in the system. An increase in the temperature will enhance the number of collisions of enzyme and substrates per unit time and increase the rate of the decolouration. The effect of temperature on the growth of the isolate was studied at temperature ranging from 20-60°C. The results obtained from the present works are depicted in **Fig. 5**. It is apparent that the isolate showed the highest growth rate at 30 °C. It was also noted moderate growth occur at other temperatures. Growth reached zero at the temperature 60 °C, indicating that it is a mesophilic bacterium. As the temperature of the system is increased (>37 °C), the excess heat may be converted into chemical potential energy beyond which some of the bonds that determine the 3D of the active site of the enzyme may be broken, and this may reduce the growth of the bacteria or adhere the cell wall.

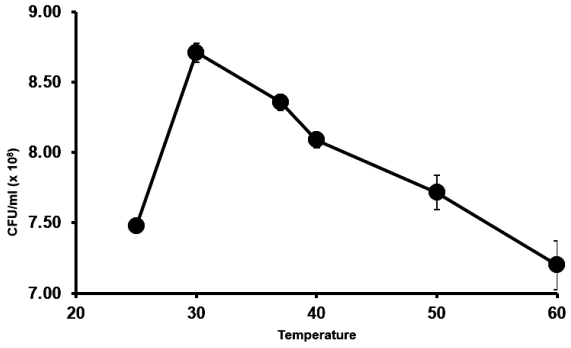

Fig. 5. Effect of temperature. The isolate was grown in 20-60ºC to study the effect of temperature on the bacteria growth. The error bars represent mean ± standard deviation of three replicates.

Effect of nitrogen source

The metabolism of xenobiotic compound usually requires an additional carbon and/or nitrogen source [1]. The effect of nitrogen sources on the growth of isolate was investigated. The result is shown in **Fig. 6**. It was observed that all the nitrogen sources supported the growth; however, urea seemed to enhance growth of isolate 1 while tyrosine and ammonium sulphate showed an inhibitory role the bacterium. This observation agrees with the work of a previous work [1] where DNP reduction is blocked by ammonium, which strongly inhibit DNP uptake and decrease nitroreductase activity. From the previous study, the addition of inorganic nutrient may speed up the process of bioremediation by enhancement of bacteria growth.

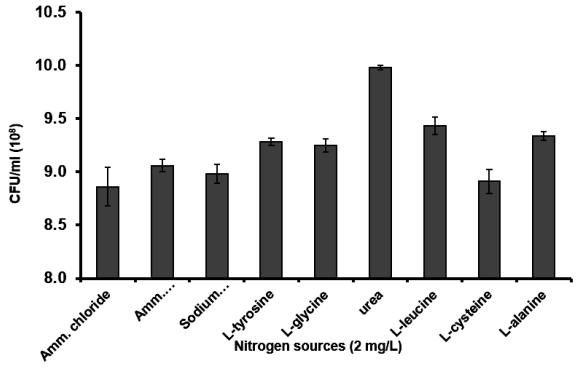

Fig. 6. Effects of various nitrogen sources in bacteria growth. The error bars represent mean ± standard deviation of three replicates.

Effect of yeast

The addition of yeast extract increases bacterial cell number up to 0.4% (w/v) (**Fig. 7**), and after this a decline was noted. Several findings reported in the literature do not report on the use of yeast extract [28,32]. Apparently, the yeast extract functions as an additional supplementary vitamin, especially the B groups to maintain cell ability. The addition of yeast extract will be an important factor for producing mass production of this bacterium for bioremediation purposes. However, since yeat extract is very expensive, other cheaper sources of nitrogen such as molasses and corn-steep liquor can be alternatives to yeast extract.

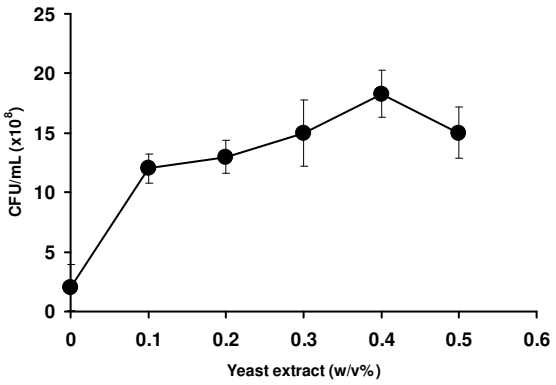

Fig. 7. Optimization of the best yeast extract concentration on the decolorization of 2,4-DNP. The error bars represent mean ± standard deviation of three replicates.

2,4-DNP-degrading activity

Residual 2,4-DNP from the culture medium is monitored through the residual stable yellowish colour that can be monitored spectrophotometrically at 546 nm. The absorbance reading of the 2,4-DNP degradation was taken out every 24 hours. Based on (**Fig. 8**) growth curve of the isolate, decreasing in 2,4-DNP concentration can be seen to be concomitant with bacteria growth showing that phenol is being assimilated for energy and growth. The degradation showed that about 50 % of the 2,4-DNP was degraded within 10 days and the amount of 2,4-DNP degraded was 0.046 mg per day.

The mechanism of reduction in this bacterium needs to be studied, but in the degradation of 2,4-DNP from *Rhodoccocus* sp., the 2-nitro group of 2,4-DNP is first removed in the form of nitrite, concomitant with the formation of the intermediate 3-nitroadipate, of which its nitro group is further metabolized into nitrite [21]. In *Burkholderia* sp. strain KU-46, HPLC and LC-MS analysis indicated that 4-nitrophenol, 1,4-benzoquinone, and nitrite are the intermediates of 2,4-dinitrophenol metabolism [32].

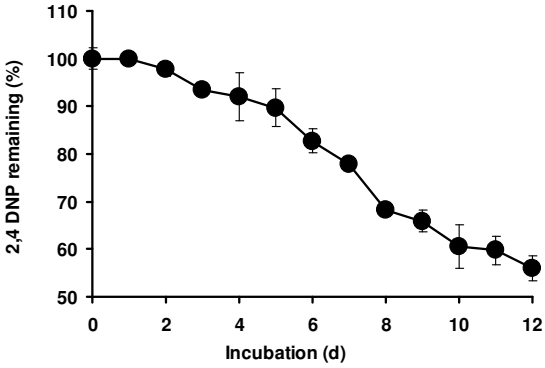

Fig. 8. Degradation of 2,4-DNP by isolate 1. The degradation was observed under spectrophotometer at 546 nm. The error bars represent mean ± standard deviation of three replicates.

CONCLUSION

One isolated bacterium with 2,4-DNP-degrading ability is reported in this study. This isolate was selected to undergo further studies for optimization and characterization. This isolate showed efficient 2,4-DNP removal. The growth was also dependent on temperature, which increase significantly at 30ºC

and at neutral to slight acidic environment. Maximum growth was also observed with 2,4-DNP in presence of various nitrogen sources but optimally in urea. The degradation of 2,4-DNP was about 50 % and 2,4-DNP degraded about 0.076 mg per day. Although, the 2,4-DNP was banned, the compound is still present in the environment due to its persistence and this explaining why many studies are still being carried out to eliminate their presence.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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