GROWTH RATE, MALACHITE GREEN BIODEGRADATION AND CAROTENOID PRODUCTION OF *GORDONIA TERRAE* USTCMS 1066

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ABSTRACT

The *Gordonia* bacteria are known for their exceptional ability to biodegrade a plethora of organic compounds and to produce various carotenoid pigments. *Gordonia terrae* USTCMS 1066, an orange hyperpigmented actinomycete isolated from a contaminated agar plate culture of *Vibrio fischeri* USTCMS 1026, grew rapidly in tryptic soy broth (Merck) supplemented with 0.5% yeast extract and yielded carotenoid content of 207 µg/g dry weight. It exhibited a growth rate of 0.0026 min^{-1} (38 min. doubling time) at 30 ^{o} C with 200 rpm orbital shaking. This strain also grew in nutrient broth (Merck) containing a high concentration of 0.05% (500 mg/L) malachite green and significantly biodegraded the initially bluish triphenylamine dye by 99.8% to colorless after 3 days of incubation at room temperature without shaking.

INTRODUCTION

In bacterial taxonomy, the Gram-positive bacteria which exhibit filamentous growths and spore formation are collectively known as the *Actinomycetes*. Among the member genera of the *Actinomycetes*, the genus *Gordonia* has recently attracted much interest for many interesting observations and valid reasons. Species of *Gordonia* were capable of degrading xenobiotics, pollutants and natural polymers as well as to biosynthesize useful compounds like amino acids, carotenoids and extra-cellular polysaccharides (Arenskötter et al., 2004). The diversity of the metabolic activity of *Gordonia* makes these bacteria potentially useful for environmental and industrial biotechnology. However, because some of the *Gordoniae* are opportunistic pathogens, their application in industry and in environment clean-up may be restricted. *Gordonia* species have been isolated from various habitats such as from soil or mangrove rhizosphere, from oil-producing wells or hydrocarbon contaminated soil, from wastewater treatment bioreactors or biofilters and from diseased human organs. The genus *Gordonia* belongs taxonomically to the suborder: *Corynebacterineae*, the mycolic acid producing group and within the order: *Actinomycetales* (Stackebrandt et. al., 1997), the mold-like bacteria. Recently, its classification has been altered

significantly with numerous species being transferred to other taxonomic groupings and many novel species reported and described. Several metabolically inaccessible organic compounds probably due to their insolubility in water such as the ethers like t-butyl ether, methyl t-butyl ether, t-amyl methyl ether and the hydrocarbons like the cyclic alkanes and polycyclic aromatic hydrocarbons (PAH), can be degraded by species of the genus *Gordonia* (Kästner et al., 1994). Members of this genus have also been known to biodegrade benzothiophenes in diesel fuels by a desulphurisation biochemical process (Kim et al., 2000), catabolize a long aliphatic hydrocarbon: hexadecane (Kummer et al., 1999), degrade the polymeric natural rubbers (Linos et al., 2001), biodegrade pyridine derivatives (Yoon et al., 2000), metabolize ether and alcohol derivatives (Hernandez-Perez et al., 2001), degradation of nitrile chemicals (Brandao et al., 2001), carotenoid production (de Miguel et al., 2000) and production of fine chemicals like amino acids (Kyriacou et al., 1997). These unique catabolic abilities are probably closely related to the production of biosurfactants by species of *Gordonia* (Johnsen and Karlson, 2003). They can also strongly biodegrade natural and synthetic isoprene rubber (cis-1,4-polyisoprene) which is not a common capability among bacteria. Gram-positive bacteria are among the best known producers of non-diffusible pigments rendering their colonies deeply yellow, orange and red in color. These colorful bacteria belong to the genera *Corynebacterium, Flavobacterium, Micrococcus,* and *Mycobacterium* which can synthesize different carotenoids derivatives (David, 1974). *Gordonia* species exhibit pigmented colonies with the reddish color indicative of their capacity to synthesize significant amounts of carotenoids. For instance, *Gordonia jacobaea* MV-1 was isolated and studied because of its ability to produce large amounts of carotenoids specifically the trans-canthaxanthin and trans-astaxanthin. It was also the first *Gordonia* species whose carotenoid biosynthetic pathway was elucidated. Carotenoid compounds are currently produced by Hoffmann-La Roche, Ltd., and are approved by the Food and Drug Administration as food additives in poultry and fish feeds. Carotenoids because of their ability to impart enormous benefits to consumers beyond their known nutritional value can be classified as "functional food component or ingredients".

This study determines the specific growth rate of *Gordonia terrae* USTCMS 1066 (*G. terrae*), an orange actinomycete strain that was isolated from a contaminated agar plate. In addition, its ability to biodegrade the xenobiotic: malachite green (MG) and its ability to produce carotenoids responsible for its dark orange pigmentation were investigated.

MATERIALS AND METHODS

***Determination of specific growth rate*.** The growth rate was calculated by linear regression method from the increasing optical density (O.D.) at 600 nm versus time of incubation during the early exponential

growth phase of *G. terrae*. This strain was inoculated in 300 mL tryptic soy broth supplemented with 0.5% yeast extract contained in 1 liter Erlenmeyer flasks. Incubation was done at 30 ^{o}C with 200 rpm rotary shaking in a Forma Scientifica Orbital Incubator Shaker. Samples were aseptically withdrawn every two hours for 14 hours in the spectrophotometric determination of their optical density.

Cultural Characterization of Gordonia terrae USTCMS (University of Santo Tomas Collection of Microbial Strains) 1066. The color of the colonial growths and their appearance were evaluated 24 hours after streaking in czapek dox-glucose agar (Merck), tryptic soy agar (BD) and nutrient agar (Merck) plates incubated at 30 ^{o}C.

Biodegradation of malachite green in liquid and solid culture media. The triphenylamine dye: malachite green was added to give a final concentration of 500 mg/L (0.05%) in nutrient broth composed of (g/L) tryptone 5.0, yeast extract 1.0 and sodium chloride 1.0. For the solid medium, agar was added to the previously described nutrient broth at concentration of 1.8% (w/v). Sterilization of the culture media was done by autoclaving at 121 ^{o} C for 15 to 20 minutes. Aliquots of 1.0 mL of a 24 hour old nutrient broth culture of *G. terrae* were used to inoculate the nutrient broths containing MG. A 24 hour old slant culture of *G. terrae* was streaked on the plated agar media. The inoculated broths and agar plates were incubated at 30 ^{o} C for 3 days. Decolorization of MG in the broth and clearing zones in the agar plates were observed on the 24 and 48 hours after inoculation at 30 ^{o} C. Un-inoculated broths and plates with the MG dye served as negative control. Six 250 mL Erlenmeyer flasks containing 100 mL of the liquid medium with MG were prepared. Three of the six flasks served as negative control while three that were inoculated served as treated sample. All flasks, control and treated, were incubated at 30 ^{o} C in an incubator without shaking. Aliquots of 2.0 mL samples were aseptically withdrawn daily and centrifuged at 10,000 rpm for 5 min. Dye decolorization in the supernatants of the broth culture was determined spectrophotometrically at 620 nm (Cha et al., 2001, Parshetti et al., 2006). Results were reported as the mean of decolorization for three replicates per reading on the 2nd and 3rd day of the incubation period. The percent decolorization (PD) of the triphenylamine dye was calculated using the formula: PD = 100 x (I - F)/I where I is the mean absorbance of the negative control (un-inoculated broth) and F is the mean absorbance of the dye in the treated samples on the 2nd and 3rd day of the incubation period.

Total carotenoid content analysis. *G. terrae* was cultivated in tryptic soy broth (BD) supplemented with 0.5% yeast extract. Incubation was done for 5 days at 30 ^{o} C with shaking. After 5 days, the cells were harvested by centrifugation at 10,000 rpm for 5 min, washed once with distilled water and dried at 70°C for 5 hours or until constant weight is achieved. The dried biomass pellet was weighed and suspended in 3 mL acetone (AR) by vortexing, after which, 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) and 3

mL of hexane were added to the tube and vortexed again. The two immiscible liquids were then separated by centrifugation at 5000 rpm for 10 min and the pigment-containing upper hexane phase was decanted using a Pasteur pipette and transferred in a clean tube. Absorbance of the hexane portion was measured at 480 nm in glass cuvette and reported as µg Total Carotenoid per g dry weight of the biomass (An et al., 1989). The total carotenoid composition was calculated using the 1% extinction coefficient of 2,100 for the formula:

$$\text{Total Carotenoid (µg/g biomass)} = \frac{(\text{mL of hexane})(A480)(100)}{(21)(\text{dry weight biomass})}$$

RESULTS AND DISCUSSION

G. terrae, grew rapidly in tryptic soy broth supplemented with 0.5% yeast extract. It exhibited a growth rate of 0.0026 min^{-1} or a doubling time of 38 min. at 30 ^{o} C with 200 rpm orbital shaking. Since the microbe is an aerobic actinomycete, the orbital shaking provided more dissolved oxygen to the broth culture that optimized its growth yielding a small value for its growth rate. This observed result is also verified by the emergence of small numerous round colonies after 15 hours of inoculation onto nutrient agar plates incubated at 30 ^{o} C. This rapid emergence of non-fastidious colonies for the wild-type strain: *G. terrae* in agar plates was different compared to the clinical *G. terrae* isolates obtained by Grisold et al., (2007) from catheter-sourced bactereamia which grew on blood and chocolate agar after 48 hours of incubation at 35 ^{o} C. The clinical strains of *Gordonia terrae* have been rendered fastidious due to their pathogenic adaptation in the human body as manifested by the their longer growth rate, higher incubation temperature and growth requirement for special blood-based culture media.

G. terrae achieved the rapid biodegradation of MG as shown by the 99.8% decolorization of the broth culture in 3 days without shaking. Moreover, the biodegradation was achieved at a very high MG concentration of 0.05% w/v (500 mg/L) without inhibiting the growth of this actinomycete. The shifting of the lambda maximum of MG in the broth culture from 620 nm to lower wavelengths was observed in this study as the disappearance of the color of MG in the broth culture indicative of MG's biodegradation (Cha et al., 2001, Parshetti et al., 2006). Fig 1. shows the percent biodegradation of MG on a daily basis showing the disappearance of the color of MG in the broth culture on the 3rd day of incubation.

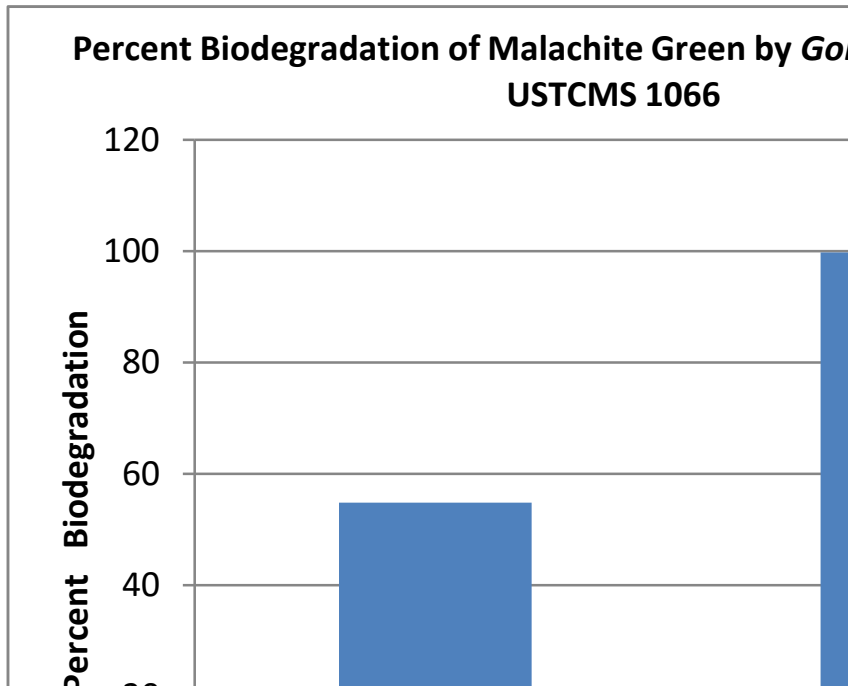

Fig 1.:Percent biodegradation of 500 mg/L malachite green by *Gordonia terrae* USTCMS 1066 in nutrient broth without shaking.

The change in color of MG in the broth culture from blue to green to colorless cannot be attributed to the pH transition of the dye since this can happen only at very high pH of 11.5 to 13.2. Metabolic alkalinity produced by bacteria due to protein breakdown normally reaches a maximum pH of only 9 to10. On the 3rd day of incubation period, the broth culture of *G. t*errae was found to be pH 9 which was still below the pH transition color of pH 11.5 for the triphenylamine dye. Changes in the color of malachite green at acidic pH is also not possible since the metabolic characteristic of the *Gordonia* bacteria is oxidative and so acids are normally not produced in the highly aerobic conditions of the experimental set-up. In addition, there is no sugar present in the nutrient broth used as the culture medium for this biodegradation assay. Simple sugars like glucose are the components of culture media that are usually fermented to produce acids in the broth culture of bacteria. Nutrient broth contains only peptone and beef or yeast extract as ingredients. It is also not a redox effect since the decolorized 3 day old broth culture remained colorless even when it is aerated vigorously by manual swirling. In addition, the decolorization in broth cultures was also observed in agar plate cultures. This was shown by the strong zones of clearing of the bluish triphenylamine dye around the orange colonies of *G. terrae* pointing once again to its biodegradation. The widespread decolorization of MG in the nutrient agar

plates clearly indicated that the biodegradation process was due to the extra-cellular enzymatic action secreted by *G. terrae*. Decolorization attributed to the adsorption of the dye to the biomass of the actinomycete is also not a possible mechanism since the colonies remained as a thick growth on the agar surface. Agar concentration in agar plates is 1.8% to 2.0% (w/v) which yields a firm solid medium that will usually deter access or entry of motile bacteria into its stable matrix. In microbiology, the motility test observed as the movement or migration of bacteria through the agar matrix is done at much lower agar concentration of 0.5% to 0.9% (w/v). The lower agar concentration produces soft agar with a less secure matrix that allows bacteria to penetrate and move readily through it by means of their flagella. In addition, since the *Actinomycetes* in general are not flagellated and thus are non-motile, the cells of *G.terrae* cannot penetrate into the solid nutrient agar substratum where the MG is dissolved and to subsequently absorb the dye in their thick cell walls. Only extra-cellular enzymes secreted by the actinomycete into the solid agar medium can catalyze the break down of MG as manifested by its decolorization effect. Previous study identified the enzyme as triphenylamine reductase which catalyzed an NADH-dependent decolorizing mechanism for the biodegradation of MG (Jang, 2005). Fig. 2 is the photo of an agar plate culture in which the readily visible widespread decolorization of the dye within the solid medium is seen around the thick orange colonies of *G. terrae*. Thus, this microorganism is the one responsible for the metabolic breakdown of MG in its culture medium through the action of its secreted extra-cellular enzymes. Fig. 3 shows the photo of the daily appearance of the broth cultures showing the gradual disappearance of the dye from blue, to green to colorless occurring in a span of just 3 days. The actinomycete was able to grow and tolerate the high concentration of the dye at 0.05% (w/v) and catabolized it rapidly. The high 0.05% (w/v) concentration of MG in the broth culture is already strongly inhibitory to the growth of most bacteria and fungi and it is even 20 times bigger than the concentration found in most selective culture media. Specifically, a highly selective culture medium for the growth of the pathogenic mycobacteria called Middlebrook 7H10 Mycobacteria Agar contains only 0.0025% MG.

In a related study on the microbial biodegradation of MG, an isolated intestinal bacteria that utilized a much lower concentration of the dye at 0.006% due to its toxic effects was observed (Henderson et al., 1997). Recently, *Sphingomonas paucimobilis*, a Gram-negative bacterium isolated from contaminated sites in a textile factory industry in Tunisia, was able to decolorize the MG dye in 4 days but at a much lower concentration of 0.005% (w/v). Decolorization of MG in the broth culture was obtained in 4 days of incubation with shaking at pH 9 and temperature of 25°C (Ayed et al., 2009a). However, *G. terrae* was able to tolerate and decolorize a much higher 0.05% (w/v) concentration of MG with less cultivation energy required since it was achieved without continuous agitation provided by a mechanical shaker. Furthermore, Guo-Ying Lv et al., (2013) used *Deinococcus radiodurans* strain R1, a unique radiation resistant bacterium for the biodegradation of MG

Fig 2. *G. terrae* nutrient agar plate culture exhibiting clearing zones of MG after 3 days of incubation.

Fig 3. Daily decolorization of MG in nutrient broth culture of *G. terrae* from day 1 to day 2 to day 3 (left to right)

observed as the decolorization of the dye in the culture medium. They were able to achieve a decolorization rate of 97.2% for 200 mg/L (0.02% w/v) MG concentration within a period of just 30 minutes. So far this paper reports a very high initial concentration for the biodegradation of MG by a microbial species at 500 mg/L (0.05%) for *Gordonia terrae* USTCMS 1066; 50 mg/L for *Kocuria rosea* MTCC 1532 (Parshetti et al., 2006); 100 mg/L for *Fomes fomentarius* (Jayasinghe et al., 2008); 5 mg/L for Acremonium kiliense (Youssef et al., 2008); 100 mg/L for *Brevibacterium laterosporus* (Gomare et

al., 2008); 100 mg/L for a mixed culture of Pseudomonas sp. and *Escherichia coli* (Tom-Sinoy et al., 2011); 50 mg/L for *Bacillus thuringiensis* (Olukanni et al., 2013) and 100 mg/L for the wild mushrooms (Yogita et al., 2011).

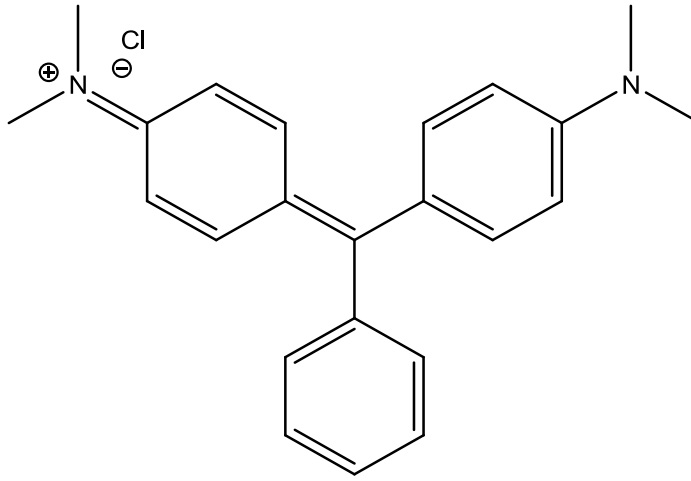

Fig 4. Structural formula of malachite green (Triphenylamine Chloride)

MG is a synthetic colorant used extensively in the textile industries for dyeing cotton, wool, silk, nylon, etc. This dye is generally considered as a xenobiotic and it is naturally recalcitrant to biodegradation by many soil microorganisms due to its stability which is attributed to its aromatic and polycyclic chemical structure. Shown below in Fig. 3, is the chemical structure of MG, a very toxic colorant known by other names like aniline green, basic green 4 or diamond green B with an IUPAC name of 4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline. The dye is also used as an external antiseptic agent for the treatment of parasites, fungal and bacterial infections in fishes, fish larvae and fish eggs. The blue-green colorant is also extensively used in microbiology as a specific stain for the bacterial endospore of the genera: *Bacillus* and *Clostridium*. In 1992 in Canada, it was determined that there is substantial health risk to humans associated with the consumption of fish and fish products treated with MG. The chemical, classified as a Class II Health Hazard, is toxic to mammalian cells and can cause liver tumor. Recent work of Culp et al., (2006) showed that rats fed with malachite green chloride or the leucomalachite green exhibited dose-dependent reductions in their body weight. In addition, they also reported that female rats fed with MG and female and male rats fed with leucomalachite green exhibited a dose-related response of mononuclear cell leukemia. Results that leucomalachite green is an in vivo mutagen in transgenic female mouse liver and that the mutagenicities of MG and leucomalachite green correlate with their tumorigenicities in mice and rats were disclosed by Mittelstaedt et

al., (2004). Mittelstaedt and co-researchers also showed that the lack of increased micronucleus frequencies and lymphocyte *Hprt* mutants in female mice treated with leucomalachite green suggests that its genotoxicity is targeted at specific tissues for tumor induction. There must be a rapid, simple and inexpensive assay for the detection of toxic MG since many processed seafood products from China have been found to be contaminated with the triphenylamine dye which can pose serious public health risks.

In addition to malachite green biodegradation, *G. terrae* exhibited red, orange and yellow colonial growths in various solid culture media. Reddish growth was obtained in czapek dox-glucose agar, orange growth in tryptic soy agar and yellowish growth in nutrient agar. Czapek dox-glucose and tryptic soy agars both contain substantial amount of glucose which seems to favor the production of large amounts of carotenoids. Glucose, a primary metabolite is a good substrate for the synthesis of pigmented secondary metabolites like the microbial carotenoids. The total carotenoid content of *Gt* 1066 in tryptic soy broth supplemented with 0.5% yeast extract was found to be 207 μg/g dry weight. This result is similar to a previous study conducted by de Miguel and co-workers (2000), where they obtained 227 μg/g dry weight total carotenoid from a novel species: *Gordonia jacobaea* MV-1. Antimycin and nitrosoguanidine induced mutation in the yeast: *Phaffia rhodozyma* cultivated on yeast-malt extract agar, yielded a much higher carotenoid content of 1,300 μg/g (An et al., 1989). The mutation prevented feedback inhibition from working metabolically thereby leading to overproduction of carotenoid in the yeast. The observed hyperpigmentation of *G. terrae* is attributed to the presence of carotenoids in its biomass as shown by its deep red-orange pigmentation in Fig 5. Its ability to elaborate various pigments depending on the composition of the culture medium employed for its cultivation indicates that other types of carotenoids are also being metabolically produced by *G. terrae* like trans-canthaxanthin, a mainly dark red pigment and trans-astaxanthin, an orange pigment. Aeration also plays a major role in the production of carotenoids and its production seems to be favored by high dissolved oxygen content in the culture medium (Yamane et al., 1997). In addition, Yamane and co-workers also investigated the influence of glucose concentration and a fixed nitrogen concentration under high level of oxygen supplied by agitation on carotenoid production. Their results showed that the carotenoid production specifically astaxanthin by the yeast: *Phaffia rhodozyma* was enhanced by an initial high carbon/nitrogen concentration ratio (C/N ratio) of the culture medium. *Gt* 1066 an aerobic bacterium achieved high carotenoid production by aeration at 200 rpm and cultivated in tryptic soy broth rich in glucose and protein. Carotenoids are a large group of colorful natural products that are classified as phytochemicals and known from their chemical structure as tetraterpenoids. This group of organic pigments from biological sources are biosynthetically built from five carbon building blocks called isopentenyl diphosphate and dimethylallyl diphosphate both derived from the catabolism of glucose, other sugars and fats. One of the carotenoids known as astaxanthin is classified as a xanthophyll, the yellow

pigment of green leafy vegetables which was the first to be identified in the xanthophyll family of carotenoids. Like many carotenoids, their bright color is due to the long alternating double and single bonds of the compound. This chain system of conjugated double bonds is also responsible for the antioxidant activity of carotenoids. These pigments are abundantly found in fruits and vegetables as well as in microalgae, yeast, salmon, trout, krill, shrimp, prawns, crabs and in the beak, legs and feathers of birds. It provides the bright orange-red color of salmon meat and their eggs as well as the attractive red color of cooked shellfish. Many carotenoids cannot be metabolically converted to vitamin A in the human body. One of the carotenoids known as astaxanthin is poorly absorbed in the intestine and thus exhibits no known toxic effects. Though astaxanthin is a known natural dietary component being produced by many biological sources, it is used as a food supplement for human and animal consumption. Natural astaxanthin as a food ingredient is classified as generally recognized as safe (GRAS) by the US Food and Drug Administration. In addition, it is highly probable that the other pigment that can be produce by *Gordonia* species is canthaxanthin which is a keto-carotenoid that is also widely distributed in nature and first isolated in edible mushrooms. In general, animals cannot metabolically produce carotenoids in their bodies and so must take it preformed from their diets.

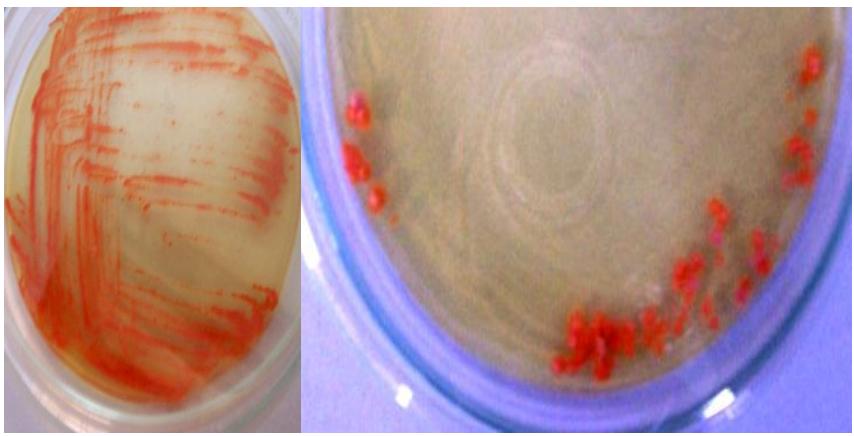

Fig 5. Orange pigmentation due to high carotenoid content (left) and the small colonies at 40X (right).

In conclusion, there is a heightened interest in the isolation of member species of *Gordonia* due to their diverse metabolic activity which could have commercial biotechnological applications and the isolated orange actinomycete: *G. terrae* lived up to its useful and diverse metabolic properties. This strain can biodegrade xenobiotics like MG and produce substantial amounts of the beneficial carotenoid pigments. In the Philippines, this is the first publication of a research work on the isolation of an indigenous species

from the genus *Gordonia* highlighting its physiological characteristics and numerous biotechnological uses.

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