Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © PHYCOSPECTRUM
ISSN: 2229 – 6905

Use of Rifampin in the development of microalgal cultures

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Inappropriate use of antibiotics in the development of microalgal cultures can lead to the emergence of antibiotic-resistant bacteria. Antibiotic-resistant bacteria have been shown to be susceptible to Rifampin. This work is thus carried out to determine the minimum concentration of Rifampin that will prevent the growth of bacteria while developing cultures of microalgae. Microalgae were isolated from a fish pond showing green colouration. The microalgal population in the pond water was estimated to be 2.5×10^4 cells.ml^{-1}. Also, the viable heterotrophic bacterial population was estimated to be 1.1×10^5 cfu.ml^{-1}. The pond water supplemented with 20 µg.ml^{-1} Na_2CO_3 was used as the growth medium for culturing microalgal cells. The microalgal cells were cultured in ten conical flasks having different concentrations of Rifampin. The concentrations of Rifampin used are 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, and 5.0 µg.ml^{-1}. The content of the flasks were shaken intermittently. After about 24 hrs of inoculation, the bacterial population in the microalgal cultures reduced to a range of 61 – 157 cfu.ml^{-1}. The microalgal population increased, after 48 hrs, to a range of $2.4 – 7.7 \times 10^5$ cells.ml^{-1}. Blooming occurred after two weeks. At the onset of blooming, 5 ml of each culture flasks were transferred separately to different water-agar plates containing 0.8 µg.ml^{-1} Rifampin. Microalgal growths were observed on the water-agar plates after a week, and there was absence of bacterial colonies. The different microalgal cells from the culture flasks and plates were identified as *Chlorella, Closterium, Ankistrodesmus,* and *Scenedesmus.* The results indicate that Rifampin can be used at a concentration of 0.8 µg.ml^{-1} in the development of microalgal cultures free from bacterial contamination. The use of pond water, which has been supplemented with Rifampin, for the cultivation of native microalgal cells can thus be an effective and inexpensive means of developing microalgal cultures.

Key words: Antibiotic resistance, Bacterial contamination, Microalgal cultures, Rifampin, water-agar

Introduction

Cultures of microalgae are often required for the study of cell physiology, metabolism, genetics, as well as for biotechnological application (Prescott *et al*., 1999; Sadava *et al*., 2009). Microalgae usually occur in the aquatic environment, and share their environment with other microorganisms. Some have been shown to be associated with specific bacteria in their natural environment (Sapp *et al*., 2004; Goecke *et al*., 2013). Thus developing cultures of micro-algae free of bacteria is usually a cumbersome task. Antibiotics are often used in the development of microalgal cultures so as to obtain cultures free of bacteria. The procedures available for the development of cultures of microalgae include the use of agar plating technique, serial dilution of algal bloom samples, spraying, and application of capillary action in picking up single cells (Lim *et al*., 2012; Bacha, 2013). The use of antibiotics in any of these procedures is very crucial since inappropriate application of antibiotics can lead to the emergence of antibiotic-resistant bacteria.

Broad and narrow spectrum antibiotics have been used at differing concentrations in the development of cultures of microalgae. Droop (1967) suggested the use of a mixture of specific amounts of three to four broad spectrum antibiotics, and a twofold dilution procedure whereby the final effective concentration of the antibiotic mixture against the total bacterial population will be determined. Kooistra *et al*., (1991) used Cefotaxime, a narrow spectrum antibiotic. The minimum concentration of the antibiotic against the total bacterial population, which allowed for healthy growth of the alga studied, was determined to be 100 µg.ml^{-1}. Wang *et al*., (2004) used a mixture of Penicillin G and Streptomycin. The minimum concentration of the antibiotic mixture that inhibited the total bacterial population in their algal cultures and allowed for healthy growth of the algal cells was determined to be 100 unit.ml^{-1}. The use of effective antibiotics at sub-lethal concentrations, or the use of antibiotics which are not effective in eliminating the total bacterial population may lead to the emergence of antibiotic-resistant

bacteria. The possible escape of these antibiotic-resistant bacteria into the environment will in the long run pose a threat to human health.

Antibiotic-resistant bacteria have been shown to be susceptible to Rifampin (Peekate and Frank-Peterside, 2015). Rifampin, a broad spectrum antibiotic that kills bacteria by blocking RNA synthesis (Prescott *et al*., 1999), have also been shown to inhibit the growth of most antibiotic-resistant bacteria at concentrations of 0.25 – 1.0 $\mu g.ml^{-1}$ (Suo *et al*., 1988; Bemer-Melchior *et al*., 2000). Rifampin can thus be used in research procedures that require the use of antibiotics, so as to prevent the emergence of antibiotic-resistant bacteria. This study is carried out to investigate the minimum concentration of Rifampin that will prevent the growth of bacteria while developing cultures of microalgae.

Materials and Methods

Rifampin stock solutions

A solution of 2 $mg.ml^{-1}$ of Rifampin was prepared by transferring 0.5 g of Rifampin into 250 ml of sterile distilled water. From this solution, two stock solutions were prepared; 0.04 $mg.ml^{-1}$ and 0.2 $mg.ml^{-1}$. The 0.04 $mg.ml^{-1}$ Rifampin stock solution was prepared by transferring 10 ml of the 2 $mg.ml^{-1}$ Rifampin solution into 490 ml of sterile distilled water, while the 0.2 $mg.ml^{-1}$ stock solution was prepared by transferring 50 ml of the Rifampin solution into 450 ml of sterile distilled water. The stock solutions were stored at 4 ^0C prior to use.

Sample and water collection

Water samples were collected from a fish pond located around the environs of the Rivers State University of Science and Technology, Nigeria. Water from the pond exhibited green colouration which is one of the evidence of algal blooming. The water samples were collected from the surface (0.0 – 0.2 m depth) of the pond with the aid of sterile glass water bottles of 200ml capacity. A large quantity of water was also collected from the pond with the aid of a 10 L jerrycan.

Culture medium

Water from the pond which the microalgal samples were collected was used as the culture medium. The water was filtered through non-absorbent cotton wool, supplemented with 20 $\mu g.ml^{-1}$ Na_2CO_3, sterilized, and used as the culture medium for the microalgal cells. This medium fortified with rifampin was used in developing bacteria-free cultures of the microalgal cells. Also, the water medium was solidified using agar. This solidified medium was used to develop plate cultures of microalgal cells. Rifampin was added to the agar plates at a concentration (0.8 $\mu g.ml^{-1}$) which considerably reduced the bacterial population in the liquid algal cultures. The bacteria population in the water and culture samples were enumerated using nutrient agar.

Enumeration of microalgal and bacterial population

The total microalgal population in the water and culture samples were enumerated using the direct count method. In this method the cells in the sample were stained using Lugol's iodine, and counted directly under the microscope with the aid of a Neubauer haemocytometer.

The total heterotrophic bacterial population in the water and culture samples were enumerated using the viable plate count technique. Plating was done in triplicates for the water and culture samples.

Experimental setup

About 10 ml of the water samples containing microalgal cells were transferred separately to a series of ten conical flasks having sterile water medium. Rifampin stock solution was added to the series of flasks in a stepwise increment so as to obtain predetermined concentrations of Rifampin (Table 1). The culture flasks were cocked with cotton wool. Curved rubber tubing obtained from clinical-drip sets were inserted through the wool, but not into the culture, to allow free exchange of air between the internal and external air space. The culture flasks were kept close to a window in the laboratory where sunlight could reach them, and were agitated manually and intermittently.

The bacterial and microalgal population in the culture flasks were enumerated after 24 and 48 hrs respectively. On observation of blooming, 5 ml of each culture flasks were transferred separately to different water-agar plates having the lowest concentration of Rifampin which considerably reduced the bacterial population and allowed for

microalgal growth. The plates were kept in upright position, close to a window in the laboratory where sunlight could reach them, and wetted daily with sterilized water from the pond.

Table 1: Rifampin concentrations in the microalgal culture setup

Inoculum Volume (ml)	Water medium (ml)	Rifampin stock solution added (ml)		FRC
		(0.04 mg.ml^{-1})	(0.2 mg.ml^{-1})	(µg.ml^{-1})
10	189.5	0.5	-	0.1
10	189.0	1.0	-	0.2
10	188.0	2.0	-	0.4
10	187.0	3.0	-	0.6
10	186.0	4.0	-	0.8
10	189.0	-	1.0	1.0
10	188.0	-	2.0	2.0
10	187.0	-	3.0	3.0
10	186.0	-	4.0	4.0
10	185.0	-	5.0	5.0

FRC – final concentration of rifampin in the culture vessel ($\frac{V_R \times C_R}{200} \times 1000$), V_R – volume of rifampin stock solution added, C_R – concentration of rifampin stock solution, 200 – final volume of the culture broth in ml.

Identification of micro algal cells

The different microalgal cells from the culture flasks and plates were identified based on microscopy and comparison of observed cells with pictures in a published text of Vuuren *et al*. (2006).

Results

The microalgal and bacterial population in the water samples is presented in Table 2 and 3 respectively. The bacterial and microalgal population in the culture flasks containing Rifampin after 24 and 48 hrs are presented in Table 4 and 5 respectively. Blooming was achieved after two weeks. The blooming occurred as greenish sediments (Plate 1).

From Table 4 it can be seen that the lowest concentration of Rifampin that considerably reduced the bacterial population is 0.8 µg.ml^{-1}. Rifampin was thus added to the water-agar plates used to obtain plate cultures of microalgal cells at this concentration (0.8 µg.ml^{-1}). Microalgal growths appeared on the water-agar plates after seven days of inoculation (Plate 2). No bacterial colonies were observed on the plates, even after an extended period of two weeks.

Microscopic observation of microalgal cells from the culture flasks and water-agar plates, and comparison with pictures in a published text of Vuuren *et al*. (2006), revealed that the microalgal cells in the different cultures include *Chlorella, Closterium, Ankistrodesmus,* and *Scenedesmus*. The microscopic description of the microalgal cells is presented in Table 6.

Table 2: Microalgal population in the pond water

Sample	ACC	Population (cells.ml^{-1})
Sample 1	4	4.0×10^4
Saple 2	1	1.0×10^4
Average population		2.5×10^4

ACC – Average cell count per mm^2, Population (cells.ml^{-1}) = average cell count ÷ 10^{-4}ml
= average cell count x 10^4ml [Volume per mm^2 of the Neubauer haemocytometer = 1 mm^2 x 0.1 mm depth = 0.1mm^3, NB: 0.1mm^3 = 0.0001cm^3 = 0.0001ml = 10^{-4}ml]

Table 3: Viable heterotrophic bacterial population in the pond water

Sample	AVC	Population (cfu.ml^{-1})
Sample 1	133	1.33×10^5
Sample 2	87	8.70×10^4
Average population		1.10×10^5

AVC – average viable count, Population (cfu.ml^{-1}) = AVC ÷ (0.1 ml × 10^{-2})
= AVC × 10^3 ml [where 0.1 ml is the volume plated, and 10^{-2} is the dilution of sample plated]

Table 4: Viable heterotrophic bacteria population in the cultures flasks containing rifampin after 24 hours

CF	RFC (µg.ml^{-1})	AVP (cfu.ml^{-1})
1	0.1	157
2	0.2	113
3	0.4	102
4	0.6	151
5	0.8	61
6	1.0	65
7	2.0	67
8	3.0	62
9	4.0	61
10	5.0	63

CF – Culture flask, RFC – Rifampin concentration, AVP – average viable population

Table 5: Microalgal population in the cultures flasks containing rifampin after 48 hours

CF	RFC ($\mu g.ml^{-1}$)	ACC	Population ($cells.ml^{-1}$)
1	0.1	23.5	2.4×10^5
2	0.2	47.0	4.7×10^5
3	0.4	77.0	7.7×10^5
4	0.6	34.5	3.5×10^5
5	0.8	44.5	4.5×10^5
6	1.0	38.5	3.9×10^5
7	2.0	67.0	6.7×10^5
8	3.0	70.5	7.0×10^5
9	4.0	72.0	7.2×10^5
10	5.0	64.0	6.4×10^5

CF – Culture flask, RFC – Rifampin concentration, ACC – Average cell count per mm^2

Plate 1: Greenish sediments of microalgal growth in culture flasks

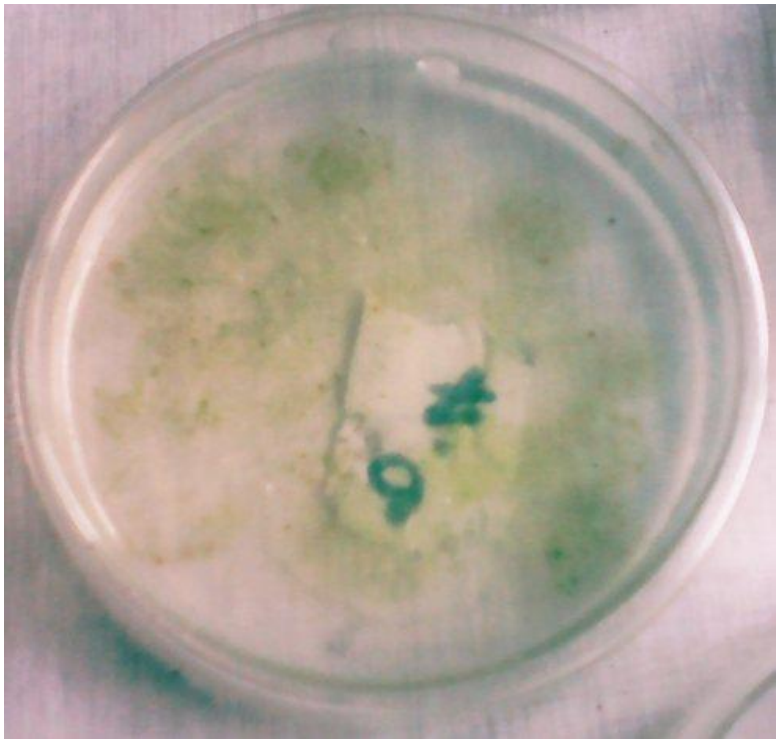

Plate 2: Microalgal growth on water-agar plate

Table 6: Microscopic description of the different microalgal cells

Isolates	Description	Probably identity
BM002	small spherical cells	*Chlorella*
BM003	elongated and bowed shaped single cells, cells are tapered at both ends	*Closterium*
BM005	elongated needle like cells, tapering at both ends	*Ankistrodesmus*
BM006	elongated oval cells in groups of four	*Scenedesmus*

Discussion

In a study carried out on bacterial contaminants isolated from an algal mass-culture unit, it was shown that viable numbers of bacteria increased with increased algal density (Blasco, 1965). Also some of the bacterial isolates exhibited inhibitory effect on algal growth. Thus, various antibiotics have been used in the development of microalgal cultures so as to eliminate the inhibitory effect of bacterial contaminants on algal growth. However, the use of antibiotics not effective against the bacterial population in a microalgal culture can lead to the development of antibiotic-resistance bacteria. These bacteria can become more efficient in exhibiting their inhibitory effect on algal growth. Antibiotic-resistant bacteria have been shown to be susceptible to Rifampin (Peekate and Frank-Peterside, 2015). Rifampin was thus used in this study to develop cultures of microalgae free from bacterial contaminants.

Sterilized water from the sampled pond, supplemented with Na_2CO_3, and fortified with Rifampin, was used successfully in blooming of microalgal cells in the water samples. Various mineral salt media have been used in cultivating microalgal cells. These include BG-II medium, Bolds Basal medium, seawater complemented with F medium, Walne medium, modified Bristol's medium, Proteose medium, etc (Kirrolia *et al*., 2012; Lim *et al*., 2012; Coutteau, 1996; Kamyab *et al*., 2014). The salts used in compounding these media are relatively expensive and sometimes scarce in some countries. Na_2CO_3 is a common salt in Nigeria, and was added to the water media in the hope of providing CO_2 to the microalgal cells. It is worthy to note here that CO_2 is required by microalgal cells for photosynthesis and growth. It was reasoned that gradual release of CO_2 may occur as a result of gradual decomposition of the salt in the water media. With the water media supplemented with Na_2CO_3, blooming was achieved in two weeks. Thus, the use of aquatic media for the cultivation of native microalgae can be a relatively inexpensive means of microalgal cultivation.

The microalgal culture flasks were also fitted with curved rubber tubing (Plate 1) so as to prevent build up of oxygen pressure in the flasks, and allow free exchange of air between the internal and external air space. A build up of oxygen pressure in the flasks as a result of photosynthesis by the microalgae can result in increased dissolve oxygen concentration. High concentration of dissolve oxygen has been cited to be toxic to microalgal cells (Lan, 2013). This is attributed to the production of reactive oxygen species that can damage cellular components. The fitting of curved tubing for the free exchange of air between the internal and external air space is thus advantageous to the system. Also, the tubing been curved will prevent air carrying germs from contaminating the cultures in the flask, just as is the case with Pasteur's swan neck flasks.

Conventional microalgal culture setups are often provided with pressure pumps for provision of CO_2, shaker devices to agitate the culture, and light provision apparatus for 24 hours lighting (Coutteau, 1996; Kirrolia *et al*., 2012). The maintenance of the continuous functioning of such devices is relatively expensive, especially in countries where electrical-power supply is frequently not available. In this work, agitation of the cultures was done manually and intermittently. Blooming was achieved, though as growth sediments. The growth occurred as sediments probably as a result of non-continues agitation, which could have been evaded with the use of a shaker device and continuous power supply. Kirrolia *et al*. (2012) showed that static cultures of a microalga produced biomass only 0.01 $g.L^{-1}$ less of cultures kept under shaking conditions. Also the cultures kept under shaking conditions achieved maximum biomass (blooming) in eleven days. In this work blooming was achieved in two weeks (fourteen days), only three days less than that obtainable in the shaking conditions of Kirrolia *et al*. (2012). Thus in this work, the inherent cost of providing continuous power supply for running a shaker device was evaded and blooming was still achieved within a reasonable time. Also, the inherent cost associated with acquiring a lighting apparatus, and continuous power supply for continuous lighting was evaded by keeping the microalgal cultures near a window in the laboratory where sunlight could reach them. Microalgae in their natural environment are not exposed to continuous lighting, thus the provision of a lighting apparatus and continuous lighting for microalgal cultivation may not be necessary in developing microalgal cultures.

The microalgal population in the different culture flasks after 48 hrs was in the range of $2.4 – 7.7 \times 10^5$ $cells.ml^{-1}$. Since the average microalgal population in the original sample is 2.5×10^4 $cells.ml^{-1}$, then the initial microalgal population in the culture setup as a result of adding 10 ml of the original sample (inoculum) into 190 ml of culture medium (water medium containing the antibiotic) will be 1.25×10^3 $cells.ml^{-1}$ (i.e., 2.5×10^4 $cells.ml^{-1} \times 10$ ml ÷ (10 + 190) ml). It can thus be established that a considerable increase took place. This increase in growth implies that the growth conditions were favourable, and that the concentrations of Rifampin used in this study were not toxic to the microalgal cells. Some other antibiotics which include Amoxicillin, Flumequine, Oxolinic acid, Oxytetracycline hydrochloride, Sarafloxacin hydrochloride, Sulfadiazine, Trimethoprim, Chloramphenicol, Florfenicol, and Thiamphenicol have been shown to be toxic to freshwater green algae and marine algae (Lützhøft *et al*., 1999; Lai *et al*., 2009). However, most of these antibiotics exhibited toxicity at high concentrations.

The bacterial population in the different culture flasks after 24 hrs was in the range of 61 – 157 $cfu.ml^{-1}$. Since the average bacterial population in the original sample is 1.1×10^5 $cfu.ml^{-1}$, then the bacterial population in the microalgal culture setup as a result of adding 10 ml of the original sample (inoculum) into 190 ml of culture medium (water medium containing the antibiotic) will be 5500 $cfu.ml^{-1}$ (i.e., 1.1×10^5 $cfu.ml^{-1} \times 10$ ml ÷ (10 + 190) ml). The reduction of the estimated population from 5500 $cfu.ml^{-1}$ to a range of 61 – 157 $cfu.ml^{-1}$ in the culture flasks reveal that the concentrations of Rifampin used in this study were effective against the bacterial population. On the other hand, the absence of bacterial growth on the agar plates having a concentration of 0.8$\mu g.ml^{-1}$ Rifampin indicate that contamination probably took place during enumeration of the bacterial population in the culture setup.

The microalgae isolated in this study include *Chlorella, Closterium, Ankistrodesmus,* and *Scenedesmus.* The presence of these microalgae has been established in various water bodies including lakes and fish ponds (Ponnuswamy *et al*., 2013). The microalgae were cultured in media having Rifampin concentrations ranging from 0.1 to 5.0 µg.ml^{-1}. Visual and microscopic evaluation of the microalgal population for the period of two weeks showed that these concentrations of Rifampin did not negatively affect the microalgal growth. Thus it could be ascertained that Rifampin is not toxic to microalgae at concentrations of 0.1-5.0 µg.ml^{-1}. At a concentration of 0.8µg.ml^{-1} Rifampin, microalgal colonies grew on agar plates while no bacterial colonies developed on the plates. Rifampin can thus be used at a concentration of 0.8µg.ml^{-1} in the development of microalgal cultures free from bacterial contaminants and in parallel prevent the emergence of antibiotic-resistant bacteria.

Conclusion

The results obtained in this work shows that Rifampin was successfully used in developing microalgal cultures that where free of bacteria contaminants. The concentration of Rifampin that prevented the growth of bacteria was not toxic to the microalgal cells.

The water source from which the microalgae were isolated proved to be a good culture medium for growing the microalgal cells. The water medium probably contained the important nutrients required for growth of the native microalgal cells.

The use of aquatic media, which has been supplemented with Rifampin, for the cultivation of native microalgal cells can thus be an effective and inexpensive means of microalgal cultivation.

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