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DECONTAMINATION OF *Chlorella* sp. CULTURE USING ANTIBIOTICS AND ANTIFUNGAL COCKTAIL TREATMENT

Mokhzanni Mustapa¹, Nor Jannah Sallehudin¹, Mohd Shamzi Mohamed², Normawaty Mohammad Noor³ and Raha Ahmad Raus¹

¹Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, Kuala Lumpur, Malaysia

²Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, UPM Serdang, Selangor, Malaysia

³Department of Marine Science, Kulliyyah of Science, International Islamic University Malaysia, Kuantan, Pahang, Malaysia E-Mail: rahaar@iium.edu.my

ABSTRACT

Direct sampling of microalgae from nature inevitably brings together the problem of massive growth of bacteria and fungi. In this study, bacterial and fungal contamination level was evaluated from the *Chlorella* sp. isolated from two different locations of local freshwater area. Attempts to obtain axenic *Chlorella* sp. culture by combinations of antibiotic and antifungal at different range of concentration treatment were investigated. It is evident that there were three different bacteria and two different fungi present in the culture, but apparently sterility can be achieved when ampicillin, cefotaxime and carbendazim cocktail were employed at concentrations of 700 µg/mL, 200 µg/mL and 0.1µg/mL, respectively. These concentrations are also proven harmless toward *Chlorella* sp. as higher concentrations inhibit the growth of microalgae. It was found that by streaking the contaminated microalgae twice onto the TAP agar containing the previously described cocktail, completely removed the contaminating fungi and bacteria from the culture. In conclusion, this study suggested that axenic *Chlorella* sp. can be attained with this method and cocktail recipe.

Keywords: microalgae, Chlorella sp., bacterial contamination, fungal contamination, ampicillin, cefotaxime, carbendazim, cocktail.

INTRODUCTION

In nature, Chlorella sp. can be found coexist with bacteria and fungus. These microscopic entities may live with *Chlorella* sp. as lichens, facultative or parasitic. Be it symbiotic or not, most researchers find it overwhelming to estimate the association of algae and these microscopic bodies by direct observation. A simple way to find out its association with Chlorella sp. is by observing the colour of algal cell suspension in a culture broth. Chlorella sp. that lives in a symbiotic manner with bacteria and fungus often exhibits dark green colour while contaminated Chlorella sp. culture exhibits yellow green colour with an apparent turbidity of the broth. The discoloration of the broth can be elucidated by the growth of a substantial number of bacteria which might possibly interrupt the photosynthetic activity of Chlorella sp. and as a consequence, decreases the chlorophyll content. In contrast, symbiotic bacteria are naturally diminished under photoheterotrophic and goes into a stationary phase under photoautotrophic condition [1].

Practices of keeping microalgal culture in a steady state for a long period in the fermentation broth and direct sampling from nature inevitably bring together the problem of massive growth of bacteria and fungi. Depending on the industrial application of the culture, algal culture may entail to be axenic for plethora uses in large scale productions especially in pharmaceutical and food grade products [2] or maintained as holoxenic for wastewater treatment [3]. Pure cultural condition of microalga can be attained by various methods comprising

traditional and advanced level to eliminate the contaminants by separating or killing them.

Since 1952, attempts to obtain a clean Chlorella culture with antibiotics, alone or in combination have become a common practice. It is somewhat surprising that some antibiotics bring losses of the chloroplast and damages to the algal cells [4]. Studies on the effects of pesticides on Chlorella sp. to assess the ecological environment truly helped this study in narrowing down the list of suitable antifungal [5, 6]. Since antibiotic and antifungal actions toward microalgae are species dependent, the effect of a cocktail combination of antibiotics and antifungal as one shot solution to treat Chlorella sp. is unknown. Therefore, this study was conducted to obtain a pure culture of Chlorella sp. in a simple, straight forward procedure and recipe by combining ampicillin, cefotaxime and carbendazim into one agar plate.

METHOD

Chlorella sp. culture

Chlorella sp. was isolated from local freshwater area located at Taman Layang-layang, Kepong, Kuala Lumpur and Bentong, Pahang. Both species were then cultured in Bold's Basal Medium (BBM) into 500 mL conical flasks and kept under continuous shaking (Sartorious Stedim, Certomat® IS) with agitation of 120rpm at 24°C, illuminated under 12:12h L:D cycle (T5

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lamp x 2 units). The culture was kept for 10 months in laboratory with routine subculture of every 2 weeks.

Screening of antibiotics and antifungal

Fungal contamination in algal culture was first screened with antifungal; MeiZim 50WP, Thiosin M-70 and BENEX®, each containing active ingredients of carbendazim (50% w/w), thiophanate methyl, TPM (70% w/w) and benomyl (50% w/w). Carbendazim was prepared by adding it to the Tris-Acetate-Phosphate (TAP) agar medium [7] prior to autoclaving while thiophanate methyl and benomyl were dissolved in dimethyl sulfoxide and aseptically added after autoclaving. The sensitivity and susceptibility of alga and fungi were correspondingly evaluated at concentrations of 0.05 μ g/mL to 40 μ g/mL.

To obtain a bacterial-free culture, combinations of antibiotics were attempted initially with broad spectrum antibiotics of ampicillin (Amresco®, US) and cefotaxime (Rekaxime, CCM Pharmaceutical, Malaysia) at a concentration range of 100-1000 μ g/mL and 50-200 μ g/mL, respectively. Aliquots of the antibiotic stock solution for ampicillin (100 mg/mL) and cefotaxime (100 mg/mL), both dissolved in deionized water were filter sterilized with 0.20 μ m sterile nylon membrane (Minisart NY25, Sartorius Stedim) and stored in -20°C. Ampicillin and cefotaxime were added to the warm agar medium prior to pouring the plates.

Algal culture was streaked onto TAP agar and incubated for 1 week at 24°C under illumination of 12:12h L:D cycle, (Philips, TLD 18W). Data was collected and photos were taken.

Cocktail treatment

Contaminated *Chlorella* culture was streaked onto TAP agar, previously mixed with 700 μ g/mL ampicillin, 200 μ g/mL cefotaxime and 0.1 μ g/mL carbendazim and incubated for one week. The grown single colonies were then inoculated into 2 mL TAP broth and incubated for another 4 days. *Chlorella* culture was restreaked on agar with the same cocktail concentration in order to ensure clean condition of the cells. A loopful of single colony was then streaked onto non-treated TAP agar (Figure-1) and further proceeded for mass cultivation.

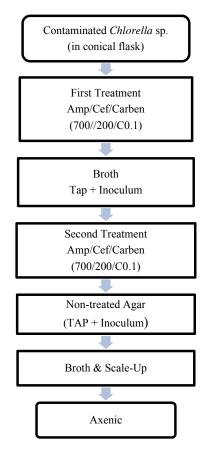


Figure-1. Process flow of cocktail treatment.

RESULTS AND DISCUSSIONS

Culture of Chlorella sp. grown on TAP agar

When grown onto TAP agar, the contaminated cultures demonstrated colonial characteristics similar to bacteria and fungi. Three types of bacteria were noticed, each was classified according to the colour of colony (colourless, white and red) while black and white filamentous fungi were spotted covering the surface of the alga and agar after 7 days of streaking. Colonial identification via colour morphology provides an easy and rapid assessment to the growth of these contaminants. This identification method has been utilized in a previous cocktail study [8]. It was noted that the overgrown contaminants hindered the growth of Chlorella sp. and the formation of single colonies. Given that the growth of alga had worsened by the presence of bacteria and fungi, it can be postulated that these bacteria and fungi are pathogenic to the alga.

Analysis of antifungal and antibacterial screening

Preliminary investigation on three antifungal; carbendazim, TPM and benomyl at different concentrations showed that all antifungal inhibited the fungal growth at concentrations of 10 μ g/mL and above (Table 1 and 2). However, at these concentrations, it also

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inhibited the growth of Chlorella sp. When tested at a lower concentration, 0.1 µg/mL carbendazim showed inhibition of white fungal growth but only partial inhibition of the black fungal growth. While, at the same concentration, TPM and benomyl completely inhibited the growth of both types of fungus. Interestingly, at this concentration, carbendazim allowed vigorous growth of the microalgae, unlike TPM and benomyl. This finding is in agreement with previous studies which demonstrated that the sensitivity and response of antifungal toward microalgae vary by species. For instance, 1 mg/L of each carbendazim, thiram, tebucunazole have been proven safe for Chlorella sp. whereas carbendazim, thiophanate methyl, and benomyl were harmless for Chlamydomonas reinhardtii up to 160mg/L [6] [9]. Although carbendazim at 0.1 µg/mL did not provide strong fungicidal effect, it allows Chlorella sp. to grow unlike the other two antifungal. Based on these, carbendazim at 0.1 µg/mL is used to make up the cocktail for decontamination of Chlorella sp. from fungus.

A closer observation on the black fungal growth on the TAP agar plate containing 0.1 $\mu g/mL$ carbendazim showed that the black fungus demonstrated arrested filament structures on the agar surface whereas at concentrations of 1 $\mu g/mL$ and above, no fungal growth was visibly detected. These observations indicated that various processes such as intracellular transport, maintenance of cell shape and cellular mobility through cilia and flagellar action were disrupted when ^{14}C of carbendazim completely binds to the microtubule of the fungus for complete growth inhibition [10].

Initial screening to obtain bacterial-free culture by antibiotic combination was conducted with broad spectrum antibiotics of ampicillin and cefotaxime at a concentration range of 100-1000 $\mu g/mL$ and 50-200 $\mu g/mL$, respectively. The inhibitions of both antibiotics are dose dependent since reduced and no bacterial colonies were observed as the concentration increased (Table 3). Based on Table 3, ampicillin at 700 $\mu g/mL$ and cefotaxime at 200 $\mu g/mL$ were chosen to be added for the decontamination cocktail.

Ampicillin is broad-spectrum penicillin, which is part of aminopenicillin under the β-lactams group. Ampicillin is known to be effective against both gramnegative and gram-positive organisms. It provides bactericidal effect via inhibition of bacterial cell wall synthesis by binding to one or more of the bacterial penicillin binding proteins (PBPs). Since the cell walls in the growing bacteria are constantly being synthesized, inhibition of synthesis is effective at controlling growth. Ampicillin is known to be effective towards Grampositive: *Streptococcus spp., Enterococcus spp., Listeria monocytogenes*, and Gram-negative: *H. influenzae*, *E. coli, Proteus mirabilis, Salmonella spp., Shigella spp.* [11].

Cefotaxime is a third generation cephalosporin, a group of β -lactams antibiotic and exerts bactericidal activity, like penicillin. However, cefotaxime expresses resistancy to β -lactamase enzymes which are produced by bacteria to inhibit the bactericidal effect [12]. Due to these reasons, the combination of ampicillin and cefotaxime is expected to bring a synergistic effect to the cocktail treatment.

0.05 Carbendazim (µg/mL) 0.1 40 1 20 Chlorella sp. (Bentong) + +++ +++ + Chlorella sp. (Taman + +++ +++ + Layang-layang) +++ White Fungus +++ +++ ++ Black Fungus

Table-1. Chlorella and fungal sensitivity toward Carbendazim.

(-)Not Detected, (+) Limited growth (++) Moderate growth (+++) Abundant growth

Table-2. Chlorella and fungal sensitivity toward Thiophanate Methyl or Benomyl.

Thiophanate Methyl or Benomyl (µg/mL)	0	0.05	0.1	1	10	20	40
Chlorella sp. (Bentong)	+		+	+	1	-	ı
Chlorella sp. (Taman Layang-layang)	+	NC	+	+	-	-	-
White Fungus	+++		-	++	-	-	-
Black Fungus	+++		-	-	-	-	-

(NC) Not Conducted (-) Not Detected, (+) Limited growth (+++) Moderate growth (++++) Abundant growth

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Table-3. Chlorella and bacteria sensitivity toward antibiotic cocktail.

Ampicillin (μg/mL)	0	100	300	500	300	500	700	1000
Cefotaxime (µg/mL)	0	50	100	100	200	200	200	200
Chlorella sp. (Bentong)	+	+++	+++	+++	+++	+++	+++	++
Chlorella sp. (Taman Layang- layang)	+	+++	+++	+++	+++	+++	+++	++
White Bacteria	+++	++	+	+	+	+	-	1
Colourless bacteria	+++	+	+	++	+	1	-	-
Red Bacteria	++	+++	++	+	+	+	-	-

(-)Not Detected, (+) Limited growth (++) Moderate growth (+++) Abundant growth

Analysis of cocktail treatment

As observed in the control plate, *Chlorella* sp. obtained from the freshwater was outgrown by bacteria and white fungi, proving heavy contaminated culture (Figure-2 and Figure-3). These problems resulted from early inoculation and culture procedure such as direct

sampling process, dilution technique and prolonged cultivation time. Apart from that, the cultures displayed distinguished growth behaviour of light green smears (Bentong) and green dotted (Taman Layang-layang) on the agar, suggesting that the two isolated *Chlorella* are of different strains.

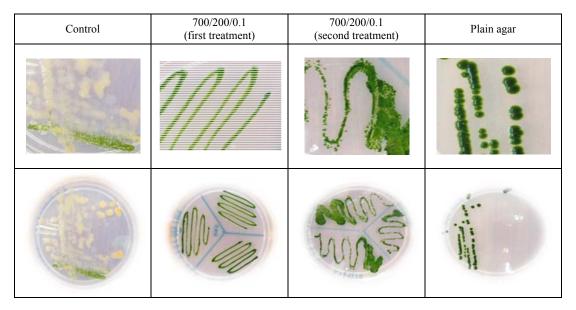


Figure-2. Chlorella sp. from Bentong, Pahang.

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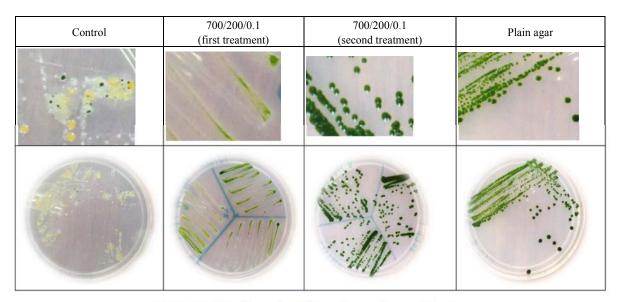


Figure-3. Chlorella sp. from Taman Layang-layang, Kepong.

A clean Chlorella culture was obtained after two serial cocktail treatments with ampicillin, cefotaxime and carbendazim at concentrations of 700 µg/mL, 200 µg/mL and 0.1 µg/mL. However, before the second cocktail treatment was carried out, single colonies from the first cocktail treatment plate had to be cultured individually in TAP broth to ensure clean microalgae. Restreaking on the final plain agar after the second cocktail treatment is essential to ensure no impending bacteria or fungi were formed before mass inoculation. Similar procedure for serial cocktail succession prior to restreaking the cells onto plain agar were reported in the decontamination study of marine microalga, Tetraselmis sp. and green alga Chlamydomonas reinhardtii [8] [13]. There is also evidence that after the decontamination, the colour of the colonies differed between untreated and treated, where the latter was a darker green. The fact that there is variable reaction between microalgae species, higher concentration of carbendazim usage in the cocktail at above 10 µg/mL has a high potential to be an alternative isolation technique in a cross-contaminated culture especially between Chlorella and other microalgae species.

CONCLUSIONS

The cocktail combination of ampicillin (700 μ g/mL), cefotaxime (200 μ g/mL) and carbendazim (0.1 μ g/mL) has proven to be an appropriate cocktail solution to decontaminate infected *Chlorella* sp. culture without affecting the growth. It is possible this similar method can be utilized for other microalgae species as well.

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REFERENCES

- [1] Guo Z. and Tong Y.W. 2014. The interactions between *chlorella vulgaris* and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. Journal of Applied Phycology. 26:1483-1492.
- [2] Wasanasathian A. and Peng C.A. 2007. Algal photobioreactor for production of lutein and zeaxanthin. Bioprocessing for Value-Added Products from Renewable Resources. pp. 491-505.
- [3] He P.J., Mao B., Lu F., Shao L.M., Lee D.J. and Chang J.S. 2013. The combined effect of bacteria and chlorella vulgaris on the treatment of municipal wastewaters. Bioresource Technology. 1(46): 562-568.
- [4] Dube J.F. 1952. Observations on a chlorophyll-deficient strain of *chlorella vulgaris* obtained after treatment with streptomycin. Science. 116: 278-279.
- [5] Ma J., Zheng R., Xu L. and Wang S. 2002. Differential sensitivity of two green algae, scenedesmus obliquus and chlorella pyrenoidosa, to 12 pesticides. Ecotoxicology and Environmental Safety. 52(1):57-61.

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- [6] Cui Y. and Li D. 2014. Effects of six pesticides on the growth of *chlorella vulgaris*. IEEE Workshop on Advanced Research and Technology in Industrial Applications (WARTIA). pp. 106-109.
- [7] Gorman D. S., Levine R. P. 1965. Cytochrome F and plastocyanin: Their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. Processdings of Natural Academy of Sciences of the USA. 54(6): 1665-1669.
- [8] Kan Y. and Pan J. 2010. A one-shot solution to bacterial and fungal contamination in the green alga chlamydomonas reinhardtii culture by using an antibiotic cocktail. Journal of Phycology. 46, 1356-1358.
- [9] Mahan K.M., Odom O.W. and Herrin D.L. 2005. Controlling fungal contamination in *chlamydomonas reinhardtii* cultures. BioTechniques. 39: 457-458.
- [10] Davidse L. C. 1986. Benzimidazole fungicides: mechanism of action and biological impact. Annual Review Phytopathology. pp. 43-65.
- [11] Sharma S.K., Singh L. and Singh S. 2013. Comparative study between penicillin and ampicillin. Scholars Journal of Applied Medical Sciences. pp. 291-294.
- [12] LeFrock J.L., Prince R. A. and Leff R.D. 1982. Mechanism of action, antimicrobial activity, pharmacology, adverse effects, and clinical efficacy of cefotaxime. Pharmacotherapy. 2: 174-84.
- [13] Mohamed M. S., Tan J. S., Kadkhodaei S., Mohamad S., Mokhtar M. N., Ariff A. B. 2014. Kinetics and modelling of microalga tetraselmis sp. FTC 209 growth with respect to its adaptation toward different trophic conditions. Biochemical Engineering Journal. 88, 30-41.