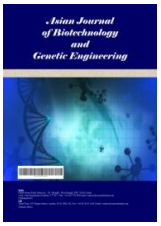

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Mixotrophic Co-Cultivation of *Chlorella lewinii* LC172265 and *Kluyveromyces marxianus* NCYC2791 for Efficient Production of Biomass under Static Condition

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Authors' contributions

This work was carried out in collaboration between both authors. Author CNO conceptualized the research, designed and supervised the experiments, and edited the manuscript. Author OJC carried out the experiments and drafted the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Microalgae and yeast biomass are sources of many useful metabolites such as proteins, lipids, antioxidants, vitamins and a host of pharmaceuticals. However, efficient production of microalgae biomass requires constant supply of carbon dioxide and removal of photosynthetically generated oxygen. On the other hand, production of yeast biomass requires adequate supply of organic carbon and constant supply of oxygen. It is therefore expected that co-culture of the two microorganisms can be achieved without aeration since the culture will be oxygenated by the oxygen released by the microalgae and carbon dioxide will be supplied by yeast fermentation.
Aim: In the present study, the feasibility of co-cultivation of *Chlorella lewinii* and *Kluyveromyces marxianus* for efficient production of biomass without aeration was investigated.
Methods: BG11 medium was used as the basic medium and the effects of nitrogen source on the growth of the cells in monocultures were first investigated. Subsequently, the effects of inoculum ratios and glucose concentrations on the growth of *Chlorella lewinii*, *Kluyveromyces marxianus* and total biomass concentrations in co-cultures were investigated.

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Results: The results showed that urea was the best nitrogen source for the growth of the two strains. In their monocultures, the maximum concentrations of *C. lewinii* and *K. marxianus* were1.62 x 10^9cells/ml and 7.719 x10^8cells/ml, respectively. The optimum inoculum ratio of *K. marxianus* to *C. lewinii* was 1:60 and a total cell concentration of 8.25 x10^9cells /ml was achieved. Although as the initial glucose concentration was increased from 5 g/L to 20 g/L, the total biomass concentration increased, the growth of *K. marxianus* increased while that of *C. levinii* decreased. The highest total biomass yield per gram of glucose was obtained with an initial glucose concentration of 5 g/L.
Conclusion: Co-cultures of *C. lewinii* and *K. marxianus* is an effective method for production of their biomass without external supply of oxygen and carbon dioxide**.**

Keywords: Microalgae; yeast; biomass; monoculture; co-culture.

1. INTRODUCTION

The ever growing human population especially in the developing countries has left many people hungry and mal-nourished in many parts of the world [1]. However, some of these problems of hunger and malnutrition can be tackled with the available bio-resources such as the microbial biodiversity [2]. Many tropical countries like Nigeria are endowed with many species of microorganisms including microalgae [3], yeasts, bacteria and fungi [4,5]. These microorganisms are capable of synthesis and storage of various useful metabolites which can be used in solving human nutrition related and other problems. Microbial biomass have versatile applications due to their rich contents of several useful metabolites such as proteins [6], carbohydrates [7], functional lipids, mineral elements, amino acids and pigments [3,8]. Due to these useful contents, microbial biomass are used as a source of food, food supplements, single cell proteins as well as in animal feed preparation [9]. Among the microorganisms that are used, yeasts [10,11] and microalgae [12] are very popular.

However, the cost of producing microbial biomass for various applications has remained very high. Some of the major limitations of large scale production of yeast biomass for various applications is the high cost of substrates (sugars and nitrogen sources) used in their cultivation [13] and the requirement for continuous aeration [14]. Microalgae on the other hand can grow on inorganic carbon sources such as carbon dioxide [15,16] and cheap nitrogen sources [17]. Nonetheless, the growth rates are low due to light and carbon dioxide limitation. Co-cultivation of yeast and microalgae has the potential of improving the productivity since the carbon dioxide released by the yeast cell is utilized by microalga cells thus overcoming the problem of carbon dioxide limitation. Furthermore, the oxygen generated by microalgae during the process of photosynthesis is utilized by the yeast cells to grow thus overcoming the problem of oxygen limitation in yeast culture. Co-culture of these two microorganisms can also lead to reduced cost of production since only limited quantity of sugar will be added for yeast growth. Furthermore, there is no need for continuous aeration since oxygen is generated by the microalga, carbon dioxide is released by the yeast cells and only mineral salt medium is required to be added. The aim of this research is therefore to explore the potential of co-cultivating yeast (*Kluyveromyces marxianus*) and microalga (*Chlorella lewinii)* for improved microalga and yeast biomass production for various applications.

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture Media

All the culture media components and other chemicals used in this work were procured from Wako Pure Chemicals Industries Ltd, Japan. The microalga (*Chlorella lewinii* LC 172265) Ahamefule et al. [18] and yeast (*Kluyveromyces marxianus* NCYC 2791) Ndubuisi et al. [19] were obtained from the Department of Microbiology, University of Nigeria, Nsukka.

Chlorella lewinii LC 172265 was maintained in BG-11 medium with the following composition (in grams per litre of distilled water): $NaNO_3$, 1.5; $K_2HPO_4 \cdot 3H_2O$, 0.04; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.036; Na_2CO_3, 0.02; citric acid, 0.006; $C_6H_8O_7 \cdot {}_xFe_3^{+} \cdot yNH_3$, 0.006; Na_2EDTA, 0.001; and 1.0 ml of A_5 + Co stock solution. The A_5 + Co stock solution was prepared by dissolving 2.86 g of H_3BO_3, 0.222 g of $ZnSO_4.7H_2O$, 1.81 g of $MnCl_2.4H_2O$, 0.079 g of $CuSO_4.5H_2O$, 0.39 g of $Na_2MoO_4.2H_2O$ and 0.0494 g of $Co(NO_3)_2.6H_2O$ in 1.0 L of distilled

water [20]. The initial pH of the BG 11 medium was adjusted to 6.0 using 0.1N HCl before sterilization in each case. The microalga was activated by sub-culturing in freshly prepared BG-11 medium and subsequently sub-cultured every 4 weeks by incubating at 27 ± 2 ^{O}C. The culture was illuminated continuously [21] at a light intensity of 150 $\mu molm^{-2}s^{-1}$ using a 32 W white fluorescent tube (ASTRA NU-PARK, CHINA).

Kluyveromyces marxianus NCYC 2791 was maintained in yeast peptone glucose agar (YPGA) test tube slants. The YPGA was composed of the following (in g/l of distilled water): glucose, 10; yeast extract, 5; peptone, 5 and agar, 20 [21]. The yeast cells were activated by sub-culturing in freshly prepared yeast peptone glucose broth. The cells were transferred into yeast peptone glucose agar (YPGA) in test tube slants and stored in the refrigerator at 8^{O}C ± 2^{O}C for subsequent experiments.

2.2 Microalga Seed Culture

The BG-11 medium was used for seed culture of the microalga under photoautotrophic condition for 14 days at 27 ± 2 ^{O}C, at a light intensity of150 $\mu molm^{-2}s^{-1}$. The resulting culture broth was used as an inoculum for mono- and co-culture experiments.

2.3 Yeast Seed Culture

The yeast seed culture was prepared by picking a single yeast colony from the test tube slant to inoculate 50 ml of yeast peptone glucose broth (YPGB) in 250 ml Erlenmeyer flask. The flask was incubated at 27 ± 2 ^{O} C with intermittent manual shaking for 24 hrs. Then one milliliter of the seed culture broth was aseptically transferred into 50 ml BG-11 medium containing 5g/L glucose and incubated under the same culture conditions described before.

2.4 Effect of Nitrogen Sources on the Growth of Microalga and Yeast

The effects of five nitrogen sources: NH_4NO_3 + Peptone, urea, NH_4NO_3, $NaNO_3$, and KNO_3 on the growth of *Chlorella lewinii* LC 172265 and *Kluyveromyces marxianus* NCYC 2791 were investigated by culturing them separately in 250 ml Erlenmeyer flasks with 100 ml of the BG-11 medium containing 5 g/l of glucose. In each case, the $NaNO_3$ in the BG 11 was replaced with each of the above nitrogen sources. They were illuminated at a light intensity of 150 $\mu molm^{-2}s^{-1}$. The cultures were not aerated but occasionally shaken to disperse the cells.

2.5 Effects of Initial Cell Ratio on the Growth of *Chlorella lewinii* LC 172265 and *Kluyveromyces marxianus* NCYC 2791 in a Co-Culture System

A modified method of Shikai et al. [22] was used to determine the optimum initial yeast: microalga cell ratio for biomass production in a co-culture system. The BG-11 medium containing 5 g/L glucose with urea as the nitrogen source pH6.0 was used for the co-cultivation of *K. marxianus* and *C. lewinii*. The ratios of the inoculum cell numbers of *C. lewinii*: *K. marxianus* were examined (30:1, 40:1, 60:1, 80:1 and 100:1). The culture conditions were the same as described for the mono-cultures. The cell numbers were determined by counting the yeast and microalga cells separately using a Thoma Haemocytometer under a Light Microscope.

2.6 Effect of Initial Glucose Concentration on Biomass Production In The Co-Culture of *C. lewinii* and *K. marxianus*

The effects of glucose concentration in BG-11 medium on the growth of *C. lewinii* and *K. marxianus under* co-culture system were investigated. One hundred milliliters (100 ml) of BG 11 were dispensed into 250mL Erlenmeyer flasks and 5, 10, 15 or 20 g/L glucose was added. They were sterilized by autoclaving at 121^{O}C for 15min at 15 psi and inoculated with 5% inoculum comprising *C. lewinii* and *K. marxianus* in a ratio of 60:1. Each flask was prepared in triplicates and they were incubated at 27 ± 2 ^{o}C under a light intensity of 150 $\mu molm^{-2}s^{-1}$ with continuous illumination for 7 days. The cultures were not aerated but shaken manually twice daily to prevent cell sedimentation and improve mass transfer. The numbers of the individual cells and the total cell concentrations were determined as described above.

2.7 Statistical Analysis

All the experiments were done at least three times and the results were expressed as Mean ± Standard Error of the Mean (SE). They were subjected to one way Analysis of Variance (ANOVA) and the Means were separated using the Least Significance Difference (LSD).

3. RESULTS AND DISCUSSION

3.1 Effect of Nitrogen Source on the Growth of *K. marxianus*

Nitrogen source is a very crucial nutrient for the growth of yeasts as they require it for DNA replication and protein synthesis. Five nitrogen sources were tested for biomass production by *K. marxinus* namely: sodium nitrate, urea, ammonium nitrate, potassium nitrate and a mixture of ammonium nitrate and peptone. Inorganic nitrogen sources were chosen since organic nitrogen sources are coloured and thus limit light penetration into the culture during co-cultivation with microalga. Since peptone is a good nitrogen source for yeast growth, low concentration was mixed with ammonium nitrate to reduce the colour intensity. With sodium nitrate, urea, and ammonium nitrate, the maximum cell concentrations were obtained after 96 hours and prolonging the culture to 120 hours resulted in decreases in the cell concentrations. However, in the case of potassium nitrate and a mixture of ammonium nitrate and peptone, the maximum cell concentrations were obtained after 72 hours and thereafter decreased as the culture period was prolonged. The cell concentration obtained with a mixture of ammonium nitrate and peptone (9.359 x 10^9) was the highest (P = 0.05) but the colour of the medium was brownish due to the colour of peptone. This would reduce light penetration and thus the growth of *Chlorella* during the co-culture. There was no significant difference between the cell concentrations obtained with urea (7.719 x 10^9 cells/ml) and ammonium nitrate (7.703 x 10^9 cell per/ml) after 96 h of cultivation. These were significantly higher than the value obtained with Potassium nitrate (4.016 x10 ^{9}) (P = 0.01). Zohri et al. [23] also reported that urea was the best nitrogen source for the growth and fermentative ability of Baker's yeast.

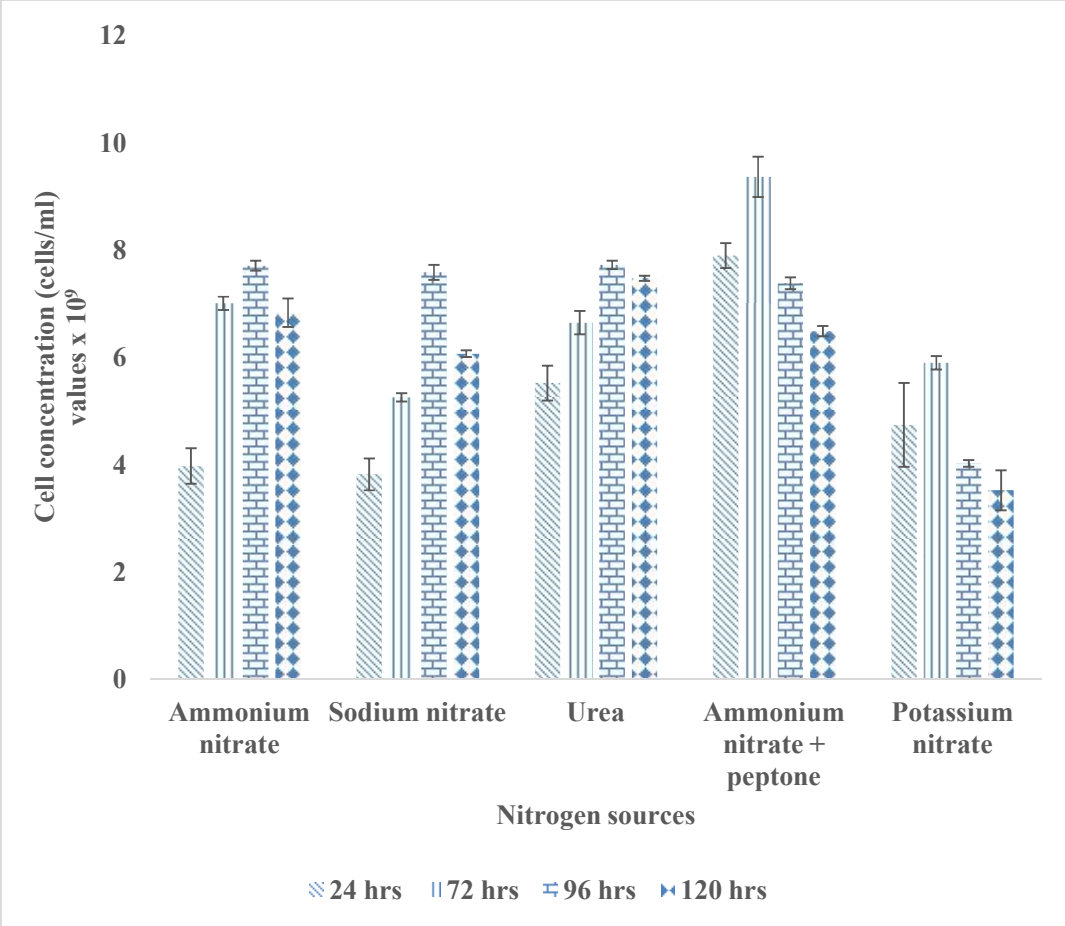

Fig. 1. Effect of nitrogen source on the growth of *K. marxianus*

3.2 Effects of Nitrogen Sources on the Growth of *Chlorella lewinii*

The same five nitrogen sources used to cultivate *K. marxianus* were used to cultivate *C. lewinii* in monoculture. With all the nitrogen sources investigated, the cell concentrations increased with cultivation time up to 96 hours after which there were slight decreases in the cell concentrations. Urea gave the highest concentration of *C. lewinii* after 96hrs of cultivation at 27±2^{o}C (P = 0.01). The cell concentration obtained with urea (1.62 x 10^9 cells/mL) was more than 70% higher than the value obtained with ammonium nitrate (9.5 x 10^8 cells/ml) and this was the second to the highest. This result agrees with the work of Sharma et al. [24] who reported that urea was the most favourable nitrogen source for the growth of their microalgae consortia. The least *C. lewinii* cell concentration was obtained from sodium nitrate with a value of 5.969 x 10^8 cells/mL.

Since urea is relatively cheaper than all the other nitrogen sources tested and gave the highest concentrations of *C. lewinii* and supported good growth of *K. marxianus*, it was used as the nitrogen source for the co-culture of the two microorganisms.

3.3 Effects of Inoculum Ratio of *C. lewinii* and *K. marxianus* on Biomass Production in a Co-Culture

Generally, microalgae grow slower than yeasts and preliminary experiments showed that at inoculum ratio of 1:1, *K. marxianus* completely overgrew *C. levinii*. The initial number of *C. lewinii* cells to combine with *K. marxianus* cells in a co-culture system for stable growth was therefore investigated by varying the ratio of *C. lewinii* to *K. marxianus* from 30:1, 40:1, 60:1, 80:1, to 100:1. Among the five cell ratios tested, a ratio of 60:1 gave the highest cell biomass of 8.25 x 10^9/cells per ml. This was followed by a ratio of 40:1 with cell biomass concentration of 8.016 x10^9. The least cell concentration was obtained with a ratio of 30:1 where the cell concentration was 4.206 x 10^9 cells per ml.

It is interesting to note that the total cell concentrations obtained in the co-culture (8.25 x 10^9/cells/ml) was more than 61% higher than the value obtained for *K. marxianus* in a monoculture (5.12 x 10^9 cell/ml), and more than 93% higher than the value obtained for *C. levinii* in a monoculture (4.27 x 10^9 cells/ml).This therefore demonstrates the effectiveness of co-culture in biomass production of the two strains.

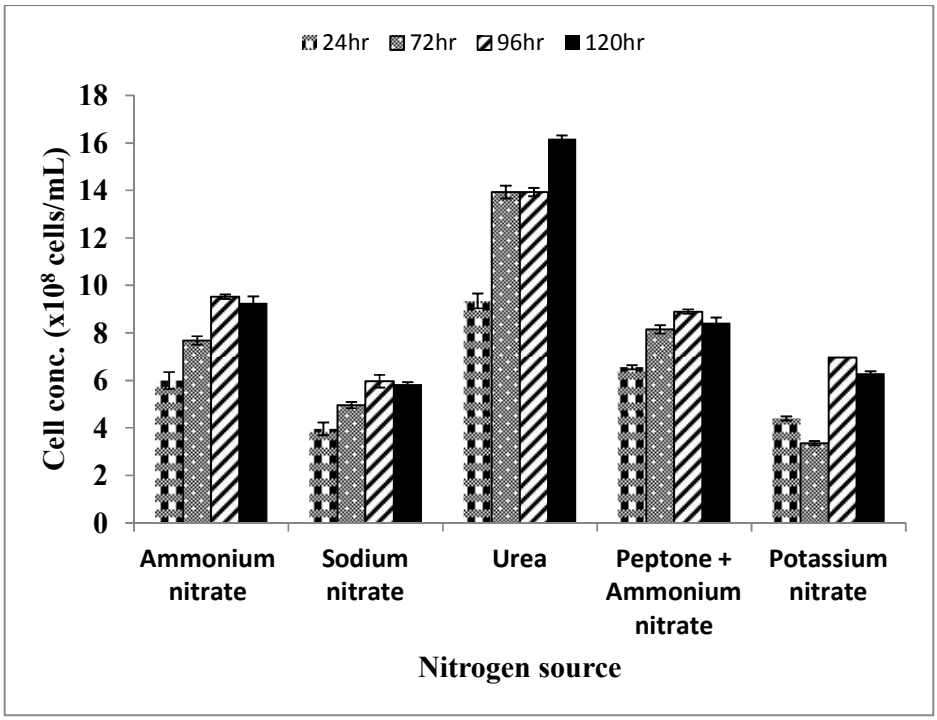

Fig. 2. Effects of Nitrogen sources on the growth of *C. lewinii*

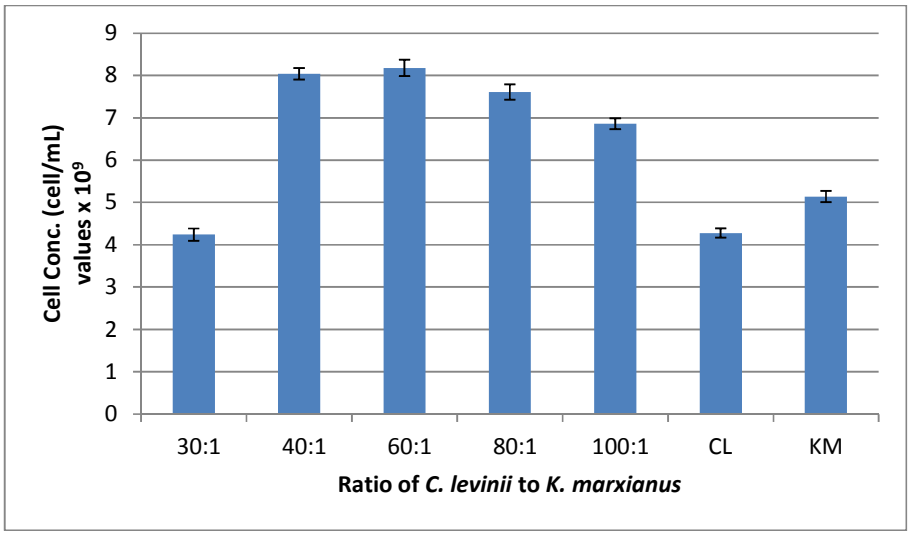

Fig. 3. Effects of the inoculum ratios of *C. lewinii* and *K. marxianus* on their growth in co-cultures. CL= *C. lewinii* monoculture, KM = *K.marxianus* monoculture

3.4 Effects of Initial Glucose Concentration on Biomass Production by *C. lewinii* and *K. marxianus*

As shown in Fig 4, in the monocultures, the final *K. marxianus* cell concentrations increased with increase in the glucose concentration but the concentration of *C. lewinii* increased only up to the glucose concentration of 15 g/L. There was no significant difference in the *C. lewinii* cell concentration obtained in the media containing 15 g/L and that of 20 g/L. It is interesting to note that with low glucose concentrations (up to 15 g/L), the concentrations of *C. lewinii* were higher than those of *K. marxianus*.

In the co-cultures, the concentrations of *C. lewinii* decreased with increase in glucose concentrations while the concentrations of *K. marxianus* increased as the glucose concentration was increased. In other words, *K. marxianus* has competitive advantage over *C. lewinii* at high glucose concentrations. This is consistent with previous reports that heterotrophic metabolism in mixotrophic cultures increased with increase in the concentration of the organic carbon source [25,26]. It is also known that at high carbon dioxide concentrations (which is expected at high glucose concentrations), glucose metabolism by microalgae is suppressed [27,28]. In the co-cultures, especially at glucose concentrations higher than 5 g/L, *K. marxianus* grew much better than *C. lewinii*.

Comparison of the monocultures and the co-cultures shows that although with all the concentrations of glucose investigated, the concentration of *C. lewinii* in monocultures were significantly higher than those in the co-culture ($P = 0.01$), there were no significance differences in the concentrations of *K. marxianu* between the monoculture and co-cultures in the medium containing 15 g/L and 20 g/L glucose. However, on the whole, the total cell concentration (*K. marxianus* + *C. lewinii*) in the co-cultures is higher than the concentration of either *K. marxianus* or *C. lewinii* in their monocultures, showing more efficient utilization of the carbon sources.

Although in the co-culture, the total cell increased with increase in glucose, the yield per gram of glucose decreased. The cell yields per gram of glucose were 1.313×10^{11}, 6.813×10^{10}, 5.813×10^{10}, and 4.45×10^{10} (cells/gram) for the glucose concentrations of 5, 10, 15 and 20, respectively. Thus, from economic point of view, the optimum glucose concentration is 5 g/L.

It is interesting to note that co-culture of *K. marxianus* and *C. lewinii* resulted in significantly higher biomass than their individual monocultures regardless of the glucose concentration. This can be attributed to synergetic relationship between the two microorganisms in the co-culture. *K. marxianus* supplies carbon dioxide to *C. lewinii* and removes the photosynthetically generated oxygen which under monoculture could lead to

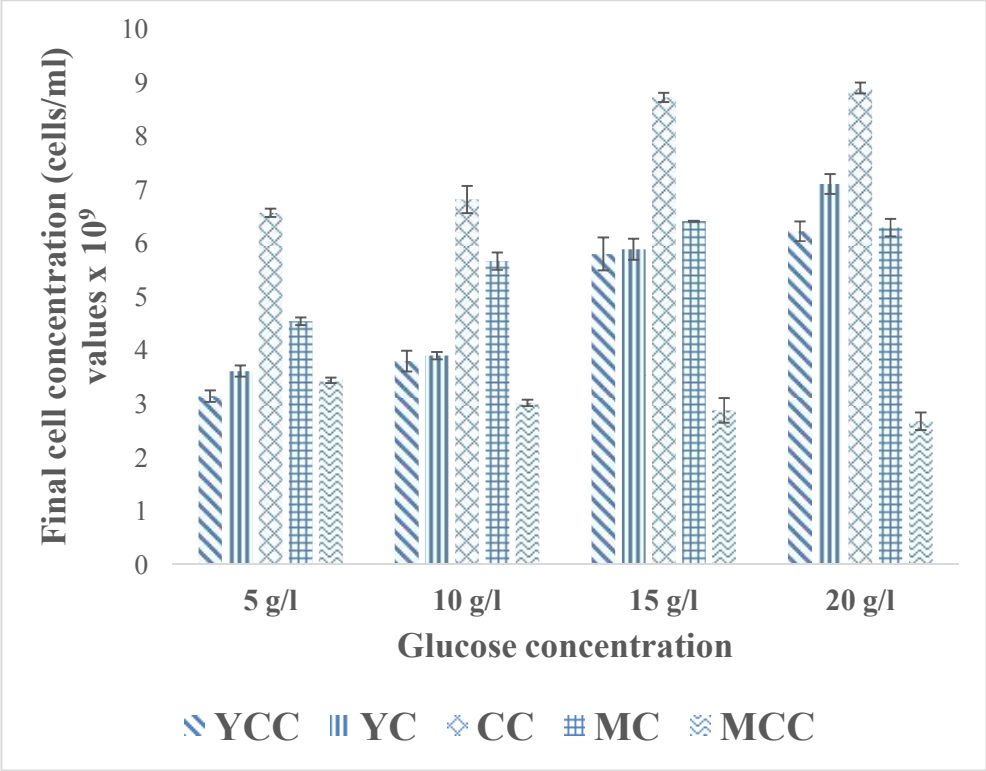

Fig. 4. Effects of initial glucose concentration on the growth of *K. marxianus* and *C. lewinii* in a co-culture system.YCC =Yeast in co-culture; YC = Yeast in monoculture; CC = Yeast + algae in co-culture; MC = Microalga in monoculture; MCC = microalga in co-culture.

oxygen inhibition. On the other hand, *C. lewinii* supplies oxygen to *K. marxianus* for glucose metabolism.

4. CONCLUSION

The major advantages of the co-culture of yeast and microalgae include: (i) It is environmentally friendly since the carbon dioxide generated by the yeast is absorbed by the microalgae (carbon neutral). (ii) The cost of carbon source is low since only small amount of organic carbon is required, and there is no need for external supply of carbon dioxide. (iii) The energy cost is low since there is no need for continuous agitation and aeration because the supply of oxygen and carbon dioxide are *in situ*. Only occasional mixing may be required to avoid cell sedimentation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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