GREEN Gold

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Abstract

Recently, renewable energy sources and new methods for wastewater treatment have become the main interest of scientists and leading countries worldwide. In terms of energy, I have chosen biodiesel production as an alternative energy source. The biodiesel was produced from moringa oleifera (MO) seeds and co-culture of the genetically modified Rhodotorula glutinis "yeast" with Chlorella vulgaris "microalgae". Directed evolution methodology was used by adding sethoxydim, which is an acetyl coA carboxylase "ACCase" inhibitor, to obtain highly lipid productive co-culture via natural selection. Furthermore, the effect of nitrogen depletion on the co-culture lipid productivity was assessed for enhanced biodiesel production. In addition, the yeast exhibits high amounts of carotenoids and riboflavin (Vitamin B2) while the algae tend to contain high amounts of cobalamin acid (Vitamin B12). These valuable nutrients could be extracted from the cells after the lipid extraction, which is used in the biodiesel production. The MO seeds exhibited 32.5% of lipid content. The residual MO biomass was mixed with macroalgae Sargassum fusiforme to obtain Moringa oleifra - Sargassum fusiforme (MO-SF) powder to test its wastewater treatment ability (96% turbidity; 99% bacterial load; and Cu, Cd, Fe, Hg, Pb heavy metals removal). The sludge produced can be reused to be a biofertilizer after removing the heavy metals using bioleaching technique or dilute acid treatment. The obtained wastewater was further processed to enhance its water-quality parameters. The treatment was done by culturing co-immobilized Chlorella vulgaris and Azospirillum brasilense, and immobilized Bacillus subtilis and Bacillus licheniformis co-culture. The previous treatment enhanced the water-quality parameters by 61% EC, 59% TDS, 95% TSS, 95% COD, 94% BOD, 98% phosphorus, and 90% TN removal respectively. The microalgae showed significant growth with the bacteria, ensuring the symbiotic relationship between Chlorella vulgaris and Azospirillum brasilense.

Introduction

The world confronts several problems that impede its development like increasing the use of alternative energies, water pollution, and recycling. In my project, I focused on the production of biodiesel as a renewable energy source and developed an efficient bioremediation process for wastewater with relatively significant enhancement algal biomass production. Biodiesel (fatty acid methyl esters "FAMEs") is considered a clean and renewable energy source, cleaner than petroleum diesel with approximately 74% lower emissions. The MO oil was extracted using cold pressing technique then converted to biodiesel. The biodiesel production from MO oil can be considered as a long-term biodiesel production source since MO normally takes 8 months to grow. So, a short-term biodiesel production source must be developed for faster supply. Co-culturing oleaginous yeast R. glutinis and microalgae C. vulgaris proved its efficiency for lipid (oil) production thus biodiesel, exploiting their symbiotic relationship. Firstly, the yeast cells were genetically modified by transforming the ACCase operon from E. Coli to the R. glutinis cells. This transformation would increase the enzyme concentration in R. glutinis cells thus the lipid content. Secondly, the co-culture was exposed to ACCase inhibitor to naturally select the cells, which have high lipid content and yield. These superior cells were tested under nitrogen starvation condition and excess glucose (carbon source) to see the effect on lipid content. The lipids (oil) were extracted and converted to biodiesel. The oil and lipids from MO seeds and algal-yeast co-culture was converted to biodiesel via transesterification reaction, using a novel acid catalysis of trifluoroacetic acid. The glycerol, commercially known as glycerin, is a by-product from the transesterification reaction that could be used in cosmetics industry. The biodiesel quality from the MO and algal-yeast co-culture was evaluated by measuring the percent of the three propitious fatty acids for biodiesel production in the cells' fatty acid profile using gas chromatography test (GC); they are oleic (C18:1), linoleic (C18:2), and Linolenic fatty (C18:3) fatty acids (unsaturated fatty acids). The residual MO biomass was used to produce MO-SF powder, used in wastewater treatment from heavy metals, bacterial load, and turbidity. The water can be further treated with culturing chlorella vulgaris along with the plant growth promoting bacteria (PGPB) A. brasilense alginate beads for better algal biomass productivity during the treatment. I also used B. subtilis - B. licheniformis co-culture alginate beads for enhanced bioremediation. After the treatment, the algae could be used as a food supplement, pharmaceutical industry, or in the biodiesel production process along with the yeast. The bacterial alginate beads could be reused with the resulted sludge (after treatment from the heavy metals) in the MO-SF powder treatment to produce high quality fertilizers.

Methods

1MO oil for biodiesel production:
20 grams of seeds were dried in sun for 3 days then shell-removed to get the kernels. The kernels were further dried for a week in room temperature. The dried-kernels were poured in the receiving funnel of the cold presser, and 6.5 grams of oil were obtained.

Presser

2. MO-SF powder for wastewater treatment:
The SF was oven dried at 105 Celsius for 6 hours. The dried SF and MO were grinded to obtain the MO-SF powder. 1 gram of the powder was added to 1 liter of the wastewater before entering the shaker for 5 minutes. The mixture is, then, let to settle for 2 hours before taking the measurements. Side note: the heavy metals concentrations were determined before and after the treatment to calculate the precent removal.

3. Algae-immobilized bacteria wastewater treatment 96 hours:

- *Immobilized bioremediation bacteria:*
  - B. subtilis and B. licheniformis were cultured in nutrient broth (NB) medium (pH 7) in a shaker incubator (120 rpm) at 30°C for 24 h. An immobilized mixture of the two species at equal ratio was transferred to 100 ml of a 5% aqueous solution of alginate. The alginate beads were obtained by dripping the mixture into a solution of 0.2% CaCl2.
- *Co-immobilization of A. brasilense and chlorella vulgaris:*
  - 20 ml of A. brasilense (cultured in nutrient broth medium at 37°C) and 20 ml of Chlorella vulgaris (cultured in mineral growth media (C30) – 5 x 10^6 cells/ml) was mixed with alginate solution to obtain a final 2% of alginate solution. The alginate beads were obtained by dripping the mixture into a solution of 0.2% CaCl2.

Algae Alginate beads

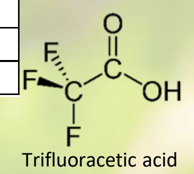
  Because the immobilization normally reduces the number of cells in the beads, we needed to incubate it at synthetic growth medium (SGM) (40 grams of beads / 500 ml of SGM for 6 days at 28°C).
- The immobilized beads were placed with the wastewater for 4 days, and the water quality parameters were assessed before the treatment and after the treatment. After the treatment, 5 beads of the A. brasilense – C. vulgaris beads were dissolved in 4 % sodium bicarbonate solution at room temperature for 30 minutes, then the C. vulgaris was counted with Neubauer hemocytometer to investigate the algae biomass enhancement due to the co-culture.
  - Note: all the beads formed were stabilized for 1hour at 28°C and washed in sterile saline solution, and the treatment took 4 days before assessing the water parameters.

4. Algae-yeast for biodiesel production :

- The plasmid carrying the acetyl coA carboxylase operon was transferred from E. coli to the R. glutinis yeast via chitosan nano particles technique. The expression of the enzyme was measured before and after the transformation.
- The R. glutinis was co-cultured with C. vulgaris in the presence of sethoxydim, which is an ACCase inhibitor, to obtain highly lipid productive co-culture via directed evolution.
- After the genetic engineering and directed evolution work, an experimental design was prepared to test the nitrogen starvation effect and symbiotic relationship between the algae and the yeast in two steps. Firstly, two 1Liter flasks of N-rich yeast and N-rich yeast and algae co-culture was prepared and let in a batch system for 3 days. Secondly, each flask is then divided into a N-rich and a N-poor treatment and were incubated for 5 days before assessing the total biomass, lipid yield, and calculating the lipid content for each treatment.
- The lipids were extracted from the cells using Soxhlet method for extraction, using chloroform and methanol as solvents (2:1 ratio by volume).

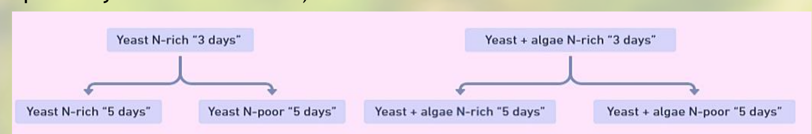
Component	Nitrogen rich medium (g/L)	Nitrogen poor medium (g/L)
Glucose	70	100
Urea	5	3
Yeast extract	10	7

Culturing media



Trifluoracetic acid

5. The MO oil and microbial lipids were converted into biodiesel and glycerol (by-product) via transesterification reaction with methanol (1:16 respectively for the molar ratio) at 120°C for 5 hours with 2 molar trifluoroacetic acid.



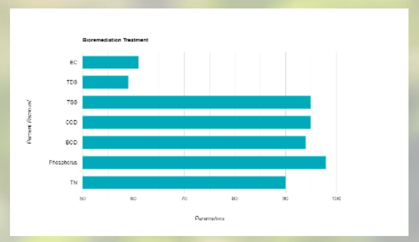
Experimental setup for microbial biodiesel production

Results

1. The oil yield of moringa seeds was 6.5 grams out of 20 grams MO seeds biomass, corresponding to 32.5%.
2. **Table 1:** MO-SF powder

Parameter	Percent removal %	parameter	Percent removal %
Bacterial load	99	Fe	98
Turbidity	95	Hg	79.3
Cd	95	Pb	85.1
Cu	93		

3. The algae biomass increased to be 6 x 10^6 cells/ml
The results of the co-immobilized C. vulgaris and A. brasilense and the immobilized co-culture of B. subtilis and B. licheniformis on the wastewater quality parameters are represented in the following graph.



4. The acetyl coA carboxylase (ACCase) enzyme expression was 6.9 nanograms before the transformation and turned to 11.6 nanograms after the transformation; the results are obtained by qRT-PCR analysis test for ACCase.

	Lipid yield (g/L)	Total biomass (g/L)	Lipid content %
N-rich yeast	1.57	6.23	25.16
N-poor yeast	1.35	4.45	30.25
N-rich co-culture	4.24	9.76	43.49
N-poor co-culture	5.33	8.90	59.84

5. **Table3:** GC test for fatty acids profile (N-poor co-culture).

Fatty acid Methyl Ester	Moringa oleifera percent %	Algae-yeast co-culture percent %
Pentadecanoic fatty acid (C15 :0)	0.4	0.9
Palmitic fatty acid (C16 :0)	6.73	35.6
Palmitoleic (C16 :1)	3.17	2.9
Stearic fatty acid (C18 :0)	4.81	8.7
Oleic fatty acid (C18 :1)	75.54	29.3
Linoleic fatty acid (C18 :2)	1.59	12.8
Linolenic fatty acid (C18 :3)	3.2	3.5
Others	4.56	6.3
Total desired fatty acids	80.33	45.60

Analysis & Discussion

Egypt suffers from alternative energy scarcity and pollution. So, in my project, I applied alternative energy generation and innovative way for wastewater treatment to overcome grand challenges like pollution and climate change. These grand challenges have always hindered the world's development. So, in efforts to reduce pollution, climate change, and improve alternative energy sources, I have proposed biofuel synthesis -biodiesel- from two sources: moringa oleifera (MO) seeds oil and the lipids produced from C. vulgaris and R. glutinis co-culture. In terms of the wastewater treatment, the residual biomass from the extraction of the MO seeds was used with Sargassum fusiform (SF) macroalgae to treat water turbidity, bacterial load, and heavy metals, and the water quality parameters were enhanced by co-immobilized C. vulgaris and A. brasilense and the immobilized co-culture of B. subtilis and B. licheniformis.

1 MO oil for biodiesel production:
The MO trees were one of the most abundant crops in ancient Egypt. The seeds of this tree like many other seeds contain oil; this makes it a possible source for biodiesel production.
To assess whether the MO seeds oil is a potential source for biodiesel or not, the lipid content of the seeds and the quality of the produced biodiesel were determined. The quality of the biodiesel is dependent on the total percent of the three propitious fatty acids for biodiesel production; they are oleic (C18:1), linoleic (C18:2), and Linolenic fatty (C18:3) fatty acids (unsaturated fatty acids).

The results indicated that biodiesel production from MO seeds oil is very efficient with a lipid content of 32.5% after extraction by cold pressing technique. In addition, the quality of the biodiesel was perfect with total desired fatty acids of 80.33%.

2. MO-SF powder for wastewater treatment:

- Turbidity removal "95%": It is caused by the sludge suspended in the wastewater itself, causing the change in the wastewater color. The MO-SF powder contains fine-grinded MO seeds, which has high coagulant activity. This coagulant activity arises from its ability of adsorption and neutralization of the colloidal charges, causing the sludge particles to settle down after the treatment.
- Bacterial load removal "99%": The antimicrobial activity of the powder arises from the antimicrobial proteins in the MO seeds not the macroalgae because the macroalgae treatment alone doesn't result in bacterial load removal. The previous results suggest that the MO-SF powder can be used instead of the chlorin in the chlorination step of the wastewater treatment plants.
- Heavy metals removal: The results indicate high efficiency of the MO-SF powder in treating total hardness in the water, particularly Cd, Cu, Fe, Hg, and Pb with 95.0%, 93.0%, 98.0%, 79.3%, and 85.1 percent removal respectively. The principle behind this is that the cell wall of the macroalgae is filled with polysaccharides and the MO powder is filled with negative radicals. The negative polysaccharides and radicals can achieve successful biosorption with the ions (heavy metals) using electrostatic attraction, thus decreasing the free heavy metals in the water.

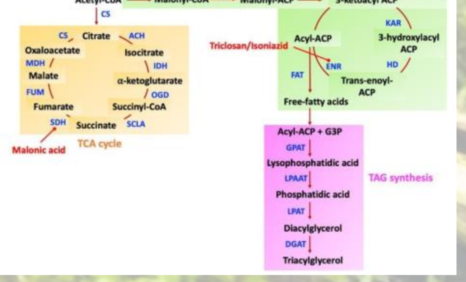
Note: The MO-SF powder was fine-grinded to increase the surface area thus maximizing the treatment efficiency.

3. Algae-immobilized bacteria wastewater treatment:
The immobilization technique used in the treatment has many advantages like biocompatibility and effective bio-catalysis. It also could be recycled again for several times in the same process and serves a great function in cell separation if the process involves the usage of many species.

- Immobilized bioremediation bacteria: Bacillus subtilis and Bacillus licheniformis were used as the main bioremediation bacteria in the wastewater. Theses bacteria can express a wide array of catabolic and hydrolytic enzymes like amylase, xylanase, cellulase, and lipase. These enzymes are capable of degrading a wide range of chemical pollutants in the wastewater thus achieving a very successful bioremediation process.
- Immobilized C. vulgaris with A. brasiliense: These beads were chosen specifically to enhance the total nitrogen and total phosphorus specifically in the wastewater due to the algal demand for these essential nutrients.
- The presence of the plant growth promoting bacteria A. brasiliense, which is found normally in the rhizosphere of the plants, is to solubilize the phosphorus and nitrogen presented in the wastewater from complex forms into much simple and bioavailable forms to nourish the algae growth. Furthermore, A. brasiliense is a nitrogen fixing bacteria: it can fix the atmospheric nitrogen for the algae (essential nutrient for algae growth. In addition, A. brasiliense produces indole-3-acetic acid (IAA), which is a form of auxin (a plant growth regulating hormone). IAA has the ability to promote chlorella vulgaris growth thus the total biomass as it does in plants by signaling cell elongation and division. A. brasiliense has the ability to produce this hormone thus signaling the growth of C. vulgaris, resulting in 6 x 10^6 cells/bead.
- In summary, the previous cultures enhanced the water-quality parameters by 61% EC, 59% TDS, 95% TSS, 95% COD, 94% BOD, 98% phosphorus, and 90% TN removal respectively and produced 6 x 10^6 cells/ml (biomass).

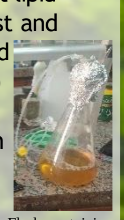
4. Algae-yeast for biodiesel production :

- ACCase transformation: the acetyl coA carboxylase enzyme is the enzyme responsible for the catalysis of the rate determining step in the lipid biogenesis, catalyzing the irreversible carboxylation reaction of acetyl coA to malonyl coA. Thus, by transforming the operon that is responsible for the expression of that enzyme, we could increase the enzyme concentration inside the cells thus promoting the lipid biogenesis. The operon was transferred from E. coli to our oleaginous yeast R. glutinis using chitosan nano particles technique. The concentration ACCase enzyme went from 6.9 nanograms before the transformation to 11.6 nanograms after the transformation.
- Directed "guided" evolution: this methodology is simply a robust technique to design proteins with desirable functions via applying a certain stress along with the natural selection mechanism. The aim of my directed evolution was to produce high lipid productive co-culture of yeast and algae using stress condition. The co-culture was exposed to sethoxydim, which is an acetyl coA carboxylase "ACCase" inhibitor. This stress would result in two different possibilities.



Lipid Biogenesis cycle

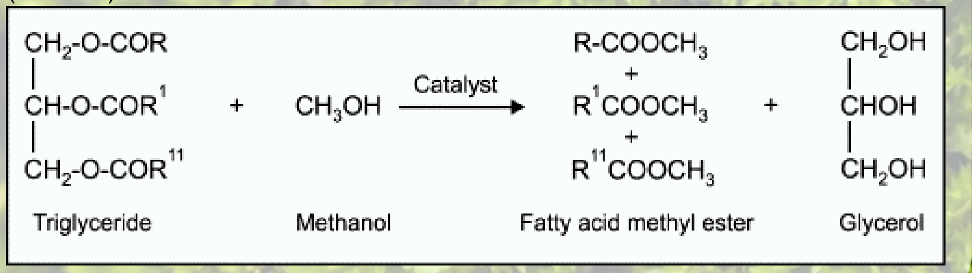
- The first one is to induce a genetic mutation to elevate the amounts of lipids inside the cells via up regulating any enzyme involved in the lipid biogenesis pathway. Then, the high lipid productive cells would be naturally selected, and the other cells that could not develop a useful mutation for the lipid biogenesis would die. The second possibility is that the population itself of the co-culture would normally have high lipid productive mutants before adding the ACCase inhibitor. Thus, by adding the inhibitor, those high productive cells would have been selected naturally by natural selection mechanism. Those two possibilities would result in the production of hyper lipids accumulating cells.
- Co-culturing the yeast and algae: the first step of the co-culture was done in N-rich medium to ensure a high biomass before the N-poor treatment in the second step that would result in a decrease in the biomass. The second step was done to test the nitrogen starvation effect on the lipid content. From the results section, we can infer that the C. vulgaris and R, glutinis under N-poor conditions exhibited the highest lipid content. This suggests the mutualistic symbiotic relationship between the yeast and the algae. It obviously depends on the O_2/CO_2 balance, substance exchange, and dissolved oxygen. Furthermore, the algae growth with carbon source (glucose) changed the algae growth mood from autotrophic to mixotrophic, which is theoretically better in lipid production. In addition, it is clear that the starvation treatment positively affects the lipid content for better biodiesel production but negatively affects the biomass. This makes sense because, at nitrogen stress conditions, the cells respond by accumulating lipids as high carbon and energy-storing molecules to tolerate the unfavorable conditions "nitrogen depletion" at the expense of its total biomass. The quality of the produced biodiesel is relatively high because it has 45.6% of the three propitious fatty acids out of the whole fatty acids profile.



Flask containing the growth medium with aeration and air filter

Note: the control group of the co-culture was culturing the yeast alone (dominant species in the co-culture).

5. The MO oil and microbial lipids were converted into biodiesel and glycerol (by-product) via transesterification reaction with methanol (1:16 respectively for the molar ratio) at 120°C for 5 hours with 2 molar trifluoroacetic acid. This reaction with these conditions resulted in 95.6% biodiesel yield, measured by the gas chromatography mass spectrometry test. The molar ration of oil :methanol was intentionally high because the transesterification reaction is a reversible reaction, and based on Le Chatelier's principle as the concentration of the reactants increases the equilibrium point of the reaction shifts towards the product side (biodiesel).



Transesterification reaction

Conclusions

In conclusion, the MO seeds contains high amounts of oil corresponding to 32.5% for the lipid content on dry mass bases with an extraordinary high-quality biodiesel while the oil from the algae-yeast (N-starvation) co-culture exhibited 59.84% in terms of the lipid content with 45.6% of the three main fatty acids for biodiesel production. The biodiesel was obtained using transesterification reaction of methanol with oil (16:1 respectively for the molar ratio) at 120°C for 5 hours with 2 molar trifluoroacetic acid. The biodiesel yield of this reaction was 95.6% "novel catalysis". The MO-SF powder was very successful since it achieved 95% turbidity; 99% bacterial load; and 95% Cd, 93% Cu, 98% Fe, 79.3% Hg, 85.1% Pb removal. Furthermore, the treatment of the water quality parameters was perfect showing 61% EC, 59% TDS, 95% TSS, 95% COD, 94% BOD, 98% phosphorus, and 90% TN removal respectively. In addition, the algae biomass was improved after the co-immobilization with the plant growth promoting bacteria A. brasiliense in alginate beads.

The by-products of these processes can be utilized turning problems into solutions. The sludge from the MO-SF powder could be used with the immobilized A. brasiliense as a high-quality fertilizer. The glycerol (transesterification reaction by-product) could be used in cosmetics industry. The residual biomass of the algae-yeast co-culture could be used in pigments industries, pharmaceutical industries, and biofertilizers.

Recommendations

In the end, I would like to give some valuable recommendations for further researches:

1. Search for more effective oil extraction methods for MO seeds and the algae-yeast co-culture.
2. Optimizing the conditions for heavy metals removal using the MO-SF powder.
3. Optimizing the growth and lipid productivity conditions of the algae-yeast co-culture, for example, the pH, air volume flow rate, carbon source (glucose, glycerol, or molasses).
4. We can use mutagenesis to: 1.knock down the ADP-glucose pyrophosphorylase gene. This is the rate-limiting step enzyme of starch synthesis, a competing cycle for lipid biogenesis in chlorella vulgaris. 2. Another mutation could be introduced to knock down the beta-oxidation pathway in the algae and yeast cells, which is responsible for lipids catabolism.
5. Use directed evolution to produce powerful oleaginous strains.

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