Complementary Production of Biofuels by the Green Alga *Chlorella vulgaris*

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Abstract- There is increasing growing concerns about global warming and rising of oil prices. The aim of the current work was to evaluate the potential of the green alga *Chlorella vulgaris* as a cheap renewable energy source in term of biofuels. Alga was hetero-trophically grown under both vegetative and induction-conditions. Induction was proceed to enhance cell metabolites mainly oils (for biodiesel) and carbohydrates (for bioethanol-production). BG-II growth medium was used for vegetative growth, while such medium was enriched by ferrous sulfate, sodium acetate and sodium chloride under high light irradiation for induction purposes. De-fatted dried algal cells were subjected to bioethanol production through three different treatments prior yeast fermentation to increase the fermentable sugars content after oils were extracted from algae. This includes direct treatment by *Tricoderma* sp., acid hydrolysis and molasses addition. *Saccharomyces cerevisiae* was used in fermentation action. Results indicated that the oil content of stressed algal cultures was raised to be 14.8% with 26.7% of total carbohydrates versus to 32.14% of crude protein. Fatty acids profile resulted in an increase of saturated fatty acids by about 10.65 %, while unsaturated fatty acids were decreased by 18.57%. Third day of incubation seems to be the optimum for direct fermented or pre acid hydrolyzed-alga in terms of yeast biomass, consumed sugar and alcohol percent By such time 15% of molasses represented the same manner. Also, the maximum alcohol content (38.7%) by acid hydrolyzed; while direct fermentation resulted in 28.7% versus to 36.3% of molasses addition.

Keywords *Chlorella vulgaris*, acid hydrolysis, molasses, yeast, bioalcohol.

1. Introduction

Algae outclass all photosynthetic organisms in both the rate of carbon fixation and conversion potential to energy compounds that could be considered as the most renewable rich energy sources. Biofuels from algae including hydrogen, methane, oil and alcohols open the safe field to produce it and decrease the global warming effect since 40% of the fixed carbon was found to be gained by algae [1].

All algal categories contain the three major cell constituents (i.e.; protein, carbohydrates and lipids). Due to their highly sensitive response to both nutritional and environmental conditions, this constituents are able to modify to produce specific one or more of the above-mentioned compounds. Some algae species contain high levels of lipids which can be extracted through different methods suchas oil press, solvent extraction, supercritical fluid extraction and ultrasound [2,3] that chemically transesterified into biodiesel [4].

Since biochemical composition of the algal biomass can be modulated by varying growth conditions, therefore the oil yield may be significantly enhanced [5] and are capable of photo-biological production of bio-hydrogen to provide carbohydrates and proteins that can be used as carbon sources for fermentation[6]. Theoreticaly, maximum yield is 0.51 kg ethanol and 0.49 kg CO_2 per one kg of carbon sugar glucose [7]. As algal become free lipids or protein it is easily to convert to different types of biofuels such as biomethane; bioethanol and biohydrogen [8, 9, 10].

Commonly, bioethanol from biomass is biochemically produced through fermentation [11] or by thermochemical

process that namely gasification [12]. Renewable, carbon-neutral fuel applications exploiting algal components include transesterification of lipids to biodiesel [13,14], saccharification of carbohydrates to ethanol [15], gasification of biomass to syngas [16], cracking of hydrocarbons and isoprenoids to gasoline [17,18] and the direct synthesis of hydrogen gas [19].

The second generation production of ethanol derived from lignocellulosic materials is now being tested in pilot plants. the most of biomass feedstock which generates bioethanol such as corn, and sugarcane has problems concerning high value for food applications and requires large quantities of land to be produced. The fact that land has many other usages, makes this problem particularly acute, and became a constraint to expand production of biofuel [20].

Microalgae can provide a high-yield source of biofuels without compromising food supplies and the use of rainforest and arable land. *Scenedesmus* sp. is a good source for bioethanol production because it has high sugar content in their cell wall that composed of multilayers, where the interior is cellulose covered by a hemicelluloses matrix and the sugars specified in their rigid wall were glucose (major), mannose and galactose. *Chlorella vulgaris* is consider a good source of ethanol due to the high starch content (ca. 37% dry wt.), and for which up to 65% ethanol conversion efficiency has been recorded methane [21]. *Chlamydomonas perigranulata* was fermented to produce ethanol, butanediol, acetic acid and CO_2. They found that hydrogen recovery from that fermentation was about 139% and carbon recovery was around 105% [22]. Brown seaweed produced higher bioethanol compared to other algae species [23]. Self fermentation of algae to obtain ethanol was simpler with shorter fermentation time compared to conventional fermentation [24,25].

In the first stage, microalgae were fermented in anaerobic and dark environment to produce ethanol. This product can be purified to be used as fuel and the produced CO_2 was recycled to algae cultivation ponds as a nutrient to grow algae. While in the second stage, the residual algae biomass after fermentation was used in anaerobic digestion process and produced methane from this process can further converted to produce electricity. Fermentation process requires less consumption of energy and simplified process compared to biodiesel production system and also, CO_2 produced as a by-product from fermentation can be used as carbon sources for algae cultivation. However, bioethanol production from algae is still under investigation and this technology has not yet been commercialized. Such process was patented by Bush and Hall [26] as they adding yeast (*Saccharomyces cerevisiae*) to algae fermentation broth for ethanol production. Algae are expensive to produce, so there are different systems have been designed for the growth and handling it on a large scale [27-30].

The main purpose in this topic is to minimize the production costs as low possible. In Egypt, reducing of production costs was performed through the selection of promising algae strain with high growth rate under saline and incident conditions [31]; use of low price commercial fertilizers for algae nutrition [32] and increasing production potential by recycling water as well as modifying the growth unit [33, 34]. The current work was achieved to evaluate the potential of Chlorella vulgaris as a cheap renewable source of biofuel through increasing oil content following by oil extraction for biodiesel production and use the remainder biomass for fermentation to produce alcohol.

2. Materials and Methods

2.1. Alga and vegetative growth conditions

The green alga *Chlorella vulgaris* was heterotrophically grown under two growth phases including vegetative and induction-growth to increase carbohydrates and oils. Vegetative growth was performed under the fully recommended nutrition status [33]. During vegetative period, light intensity was adjusted at 120µ.e from one side light bank of 20 lamps x 40 watts. Growth container of 200L vertical sheet photobioreactor was used. Aeration was provided from the lower end of growth container to meet the air-left system by free oil compressed air. Heterotrophic growth was performed through the addition of acetic acid during vegetative growth period ($0.25ml.l^{-1}$)

2.2. Induction technique

Vegetative growth was performed as described above at 200L of growth volume. When alga reached the maximum growth dry weight, turbulence was turned off to allow algal sedimentation. About 90% of growth medium was then discarded and induction was started using tap water 120L with 60L of Red Sea water. Growth media was supplemented by acetate carbon (45mM) and ferrous sulphate ($0.125g.l^{-1}$) with high light irradiation (200µ.e) from two sides. Induction was performed under higher elevated salinity doses of NaCl to reach 5.6%.

2.3. De-fatted algae preparation

By the end of induction period; as the dense culture was obtained aeration was breakdown to allow gravity sedimentation. The upper clear solution was discarded and the remainder slurry was then concentrated by centrifugation at 4000 rpm (RUNNE HEIDBERG model RSV-20) to become containing about 70% of moisture. The de-watered biomass was then refrigerated at 5^oC to allow cell wall cracking. One day later, the obtained biomass was then dried in 45^oC circulated oven, fine grinded and then subjected to oil extraction. Fine algal powder was filled into 100g Cellulose Extraction Thimbles (41x123mm); soaked overnight with solvent mixture of 3:2 (v/v) n-hexane/isopropanol in dim light at room temperature (25^oC). Thimbles were picked up and put into Soxhelt Jacket, covered with the ex-soaking mixture and then subjected to extraction for 24 hrs. Oil fraction was then separated using rotary evaporator. The solvents were evaporated and oil content was then calculated.

2.4. Fatty acid profile

Identification and determination of fatty acids were carried out by Gas Chromatography (GC) Type of GC Perkin Elmer Auto System XL, equipped with flame ionization detector (FID); fused silica capillary column DB-5 (60 mm x 0.32 mm i.d); oven temperature was maintained initially at 150^{o}C and programmed from 150 to 240^{o}C at rate 3.0cm.min^{-1}, injector temp. 230^{o}C, detector temp. 250^{o}C, carrier gas and helium flow rate 1 ml.min^{-1}.

2.5. Bioethanol Production

Three different methods were employed to increase the productivity rate of bio-ethanol from the used alga. First and second methods were employed to determine the most suitable fermentation time, while third method was done to determine the effect of molasses addition at the pre-tested time of fermentation. The first method was done by direct microbial digestion of used algae by *Tricoderma* sp.; following by *Saccharomyces cerevisiae* (ATCC MYA-2034). *Tricoderma* sp. was taken and transferred to 100 ml of liquid Cazpek's medium in 250 ml Erlenmeyer flasks. The flasks incubated in shaker at 200 rpm for 5 days at 30^{o}C, then the medium was centrifuged and supernatant was fermented for ethanol production as glucose source after determination of reducing sugar. Second method was performed by pre-digestion of used algae by sulphuric acid (2.0N). 5.0 g of algal dried biomass were suspended in 25 ml of H_2SO_4 (2.0 N) in three replicates of 250 ml Erlenmeyer flasks. Then the suspensions were autoclaved at 120^{o}C and 15 lb/in^2 for 30 min. The supernatant was taken and the residues were removed by filtration using Whatman 540 (acid resistant). The yielded filtrates were over liming using 0.363 g of calcium hydroxide for adjusting pH to be about 6. Third method includes Five different concentrations (5-10-15-20-25%) of molasses were tested with crude algae. Molasses was added with crude algae to fermentation medium then 1ml of inoculum was added and incubated for 72 hr at 30^{o}C. After fermentation period the media were centrifuged and the yeast cells were dried at 105^{o}C. The ethanol production was spectrophotometrically determined [35] and the consumed sugar was determined by phenol-sulphuric method [36].

2.6. Fungi, yeast and growth media

Tricoderma sp. and *Saccharomyces cerevisiae* were obtained from Microbial Biotechnology Department (NRC). Czapek's medium used for *Tricoderma* sp growing of the following composition (g.l^{-1} of distilled water): sodium nitrate (2.0); potassium dihydrogen phosphate (1.0); magnesium sulphate (0.1) an glucose (20.0) according to Smith and Onions [37]. The pH was adjusted at 5.5 before sterilization. Nutrient agar used as enrichment medium for growth of bacteria and yeasts [38] containing (g.l^{-1}) peptone (5.0); meat extract (3.0) and agar (15.0). Media reaction (pH) was adjusted at 7.0 before sterilization. Ethanol fermentation medium [39] containing (g.l^{-1}) ammonium sulphate (2.0); potassium dihydrogen phosphate (1.0); magnesium sulphate (0.1) and yeast extract (1.0) with 26.3 g.l^{-1} of crude algae. Media reaction was adjusted at pH 5.0. *Saccharomyces cerevisiae* (ATCC MYA-2034) was grown on nutrient agar and incubated at 30^{o}C until measuring of about 1x10^5 CFU.ml^{-1} using haemocytometer technique [40].

2.7. Fermentation

Ten percent of *Saccharomyces cerevisiae* culture was added to all of the flasks that contained *Chlorella vulgaris* sugar extract outcome from aforementioned dried biomass for fermentative production of bioethanol. The process was carried out for different a period (1, 2,3,4,5 days) at 30^{o}C. The flasks of production was used for ethanol

2.8. Ethanol determination

Dichromate solution (1.0ml) and 2.9 ml of the concentrated perchloric acid were added to 100 µL of the sample in a 10 mL Erlenmeyer flask. The solution is homogenized, left to stand for 20 min at 25 ^{o}C and then diluted to the mark with water. The absorbance (A1) is measured at 267 nm against a 3.0M perchlorate aqueous solution. This procedure is applied to a second 100µl volume of the alcohol-free sample as blank (in this case, the solvent is evaporated under vacuum, at room temperature, before adding the reagents). Ao is the new absorbance value measured at 267 nm. Alcohol content of the sample is calculated as:

Volumetric alcohol content (%) = 3(Ao-A1) 11.51D/0.78934v.

where:

D= dilution factor of the sample

V= volume (ml) of the diluted sample withdrawn for the analysis.

(Ao-A1) = amount of chromium(VI) reduced to chromium(III) in the oxidation reaction of ethanol to acetic acid.

2.9. Chemical analysis of alga

Chlorella vulgaris biomass profile was determined by AOAC standard methods [41] in terms of moisture, ash, protein, fat and fiber contents. Total carbohydrate contents were determined according to Dubois *et al*, [36]. Fatty acids profile was determined using gas chromatography (GC Perkin Elmer Auto System XL).

3. Results and Discussion

3.1. Algal growth and Chemical composition

Maximum vegetative growth peak was obtained by the 6th day of incubation as the grown culture was supported by extra nitrogen amount from 0.53 g.l^{-1} urea and 1.44 g.l^{-1} calcium nitrate to reach about 35.2mM N instead of the original that contain 17.6mM N from sodium nitrate. The enhancement of vegetative growth could be ascribed to the presence of both source and type of nitrogen including urea and nitrate [32]. The effect of urea as carbon and nitrogen sources was early recognized, however the specific enzymes including urease and urea amydolyase not detected by the examined alga. Stress factors are expected to reduce dry weight accumulation and increase the rate of chlorophyll de-composition. Here, induction within the same vegetative growth medium safes the

dry weight failure, while chlorophyll de-composition slightly took place. During induction period, protein content tended to decrease against the rise of total carbohydrates and total lipids. The increase of induction potential by full vegetative medium could be ascribed to the presence of $FeSO_4$ plus acetate that enhancing the free radicals generation and This is followed by the huge accumulation of lipids and carotenoids.

Comparing of chemical composition during two different growth periods showed a massive effect of induction on cell metabolites. As shown in Table (1); total carbohydrates were raised from 14 to be 26.7%. The extracted oil content from the green alga *Chlorella vulgaris* was diversified due to the growth conditions (*i.e*. vegetative or stress condition). Cells of vegetative growth represented about 9.7% of crude oil (n-hexane/isopropanol extract). On the other hand, stressed cells were raised on their oil content to be about 14.8%. Increasing of oils and carbohydrates content was found to be on the expense of protein content, where 48% of vegetative cultures was found versus to 32.14 of those stressed cultures of *Chlorella vulgaris*.

Table 1. Major chemical composition (%) of two growth phases grown alga

Analysis	Protein	Fat	Carbohydrate
Vegetative phase	48.0	9.7	14.0
Stress phase	32.14	14.8	26.7

3.2. Oil content and fatty acid profile of vegetative and stressed alga

Fatty acid content and profile of *Chlorella vulgaris* was widely diversified. The variation was found in response to growth media and its effect on metabolic pathway that enhance the accumulation of lipid on the expense of protein and chlorophyll decomposition. Increasing of oil content is accompanied by the rising of total carbohydrate content. Otherwise, the initial fraction of both oil and carbohydrates was also changed regard saturation ratio and polysaccharides content. Oil content was drastically varied under treated and untreated *Chlorella vulgaris* cultures. About 9.7% of oil content was found in *Chlorella vulgaris* grown under control conditions. As conditions became stress (salt stress and nitrogen starvation) oil content was increased and reached 14.8% at the end of cultivation period. Oil content of algae was found to be associated with a huge accumulation of carotenoids under stress conditions such as salinity regime and nitrogen starvation. In this concern, Lardon *et al*, [42] found that the control of nitrogen stress during growth led to maximum oil production from *Chlorella vulgaris*. These results may be explained by the fact that under stress condition, all carbon produced are used for the production of oil and other substances that played an important role in algal tolerance in defense mechanisms. In addition, Sheehan *et al*, [43] suggested that under nutrient stress starvation, the rate of production of all cell compounds is lower and oil production remains higher and this leading to an accumulation of oil in the cells. In the our study, oil content in N- deficient culture of *Chlorella* cells reached 14.8 %. However oil content of 20-30% is easy to induce in several algae species [29] and there are uncommonly an oil content of 86% was reported in *Botryococcus brauna* [44]. The poor growth rate and low microalgal biomass productivity of *B. braunu* was the major hitch in focusing as the industrial organism for biodiesel production [45]. Additionally, Rodolfi *et al*. [46], added that a two phase strategy will require before the cultures is N- starved for oil synthesis, enough biomass (to be used as inoculums) is produced under N- sufficiency followed by a second stage for efficient lipid accumulation under N- deprivation.

Algae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. Unlike other oil crops, algae grow extremely rapidly and among them *Chlorella* species are exceedingly rich in oil. those are desired to maintain the cost efficiency of commercial biodiesel production, the picking of convenient strain according to the site of cultivation is present obligation. The species that are high salt tolerant and are able to grow and reproduce under nutritional deficiency by altering their metabolic pathways efficiently found most promising in this consideration. On the other hand, other stress conditions including high salinity or high temperature and variation in pH are also found to be controlling factors that govern the channeling of metabolism from starch to oil [47]. In conclusion, the data of the present investigation illustrated that under nitrogen starvation and salt stress, oil content of *Chlorella vulgaris* was highly increased compared to control treatment.

The composition of triglyceride Fatty Acid Methyl Esters (FAMEs) of the green alga *Chlorella vulgaris* (Table 2) showed the presence of saturated fatty acids C14:0 (13.47%) and C16:0 (54.3 %). As for unsaturated fatty acids, C14:1 (6.2%); C16:1 (4.8%); C16:2 (1.7%); C18:1 (12.9%); C18:2 (4.1%) and C18:3 (1.4%). This meaning that 68.9% of saturated fatty acids were formed in *Chlorella vulgaris* versus to 31.1% of unsaturated fatty acids making the ratio of 1.7:2.2 in oil fraction. Such structure of the obtained oil represented the promising potential of *Chlorella* oil in biodiesel production in concern the high ratio of saturated fatty acid such as oleic and plamitic acids. Oleic and palmitic acids were considered to be a reasonable balance of fuel properties including ignition quality, combustion heat, cold filter plugging point, oxidative stability, viscosity and lubricity [48].

Table 2. Fatty acid profile of *Chlorella vulgaris*

Fatty acids (%)	Common name	Vegetative	Stress
C 14:0	Myristic	1.85	13.47
C14:1	Myristoleic	4.92	6.2
C16:0	Palmitic	52.98	54.3
C16:1	Palmitolieic	6.12	4.8
C16:2	Hexadecadienoic acid	4.85	1.7
C18:0	Stearic acid	8.83	1.13
C18:1	Oleic	5.02	12.9
C18:2	Linoleic	6.56	4.1
C 18:3	Linolenic	8.87	1.4
Total saturated		63.66	68.9
Total unsaturated		36.34	31.1
TS/TUS		1.75	2.2

The most commonly synthesized fatty acids have chain length that range from C 14 to C18. In *Chlorella vulgaris*, the content of myristic acid methyl ester (C14:0), palmitic acid methyl ester (C16:0) and oleic acid methyl ester (C18:1) are ranged from 13.47, 54.3% and 12.9% respectively. In this study, the composition of FAMEs of *Chlorella vulgaris* was found to be in agreement with the general profile of fatty acids in other microalgae. So, it is established that the FAME composition of the methyl esters has a predominant effect on the biodiesel properties.

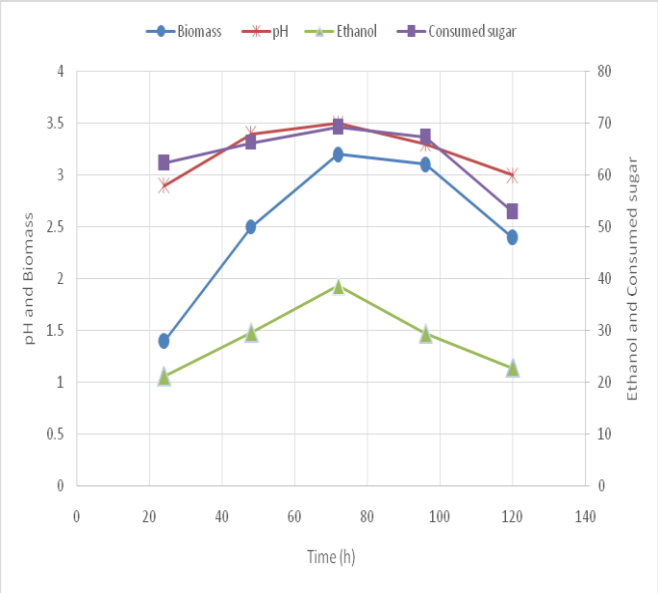
3.3. Direct fermentation

Direct or traditional fermentation method in the current work determined at first the optimum incubation time for maximum alcohol production within the whole incubation time (120 h). Bio-ethanol gained by direct fermentation of the pre fungi hydrolyzed de-fatted alga was varied due to incubation time. Ethanol maximum yield (28.7%) was obtained by the 3rd day of incubation (72h) associated with the formed yeast biomass (2.7 g.100ml^{-1}).

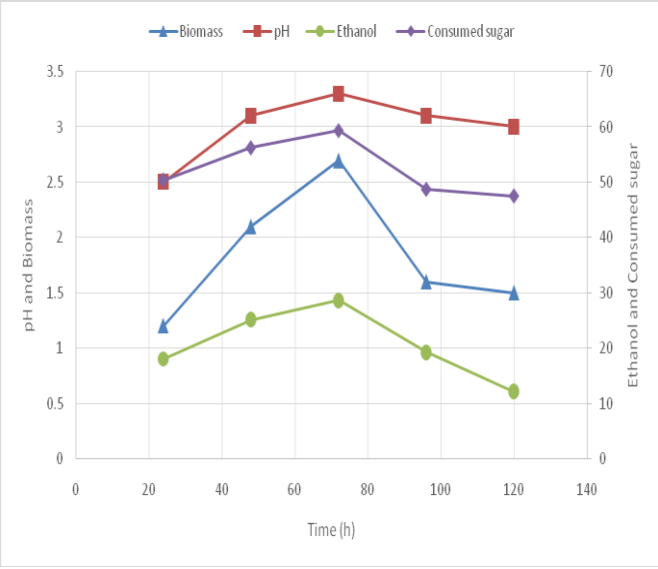
By such time, the rate of sugar consumption was also increased parallel with the formed biomass and bioethanol content. Increasing incubation time up to 72h reduced the above-mentioned result to be lowest after 120 h (fig. 1). This might attribute to the lack of medium component due to yeast growth; however the tested incubation time seems to be commercially in the future use. Concerning media reaction, 3.3 of pH value seems to be the optimum for yeast growth or the result due to the maximum alcohol content, since the obtained pH values were linearly closely related to the obtained biomass, alcohol content and consumed sugars. Acid hydrolysis increases the fermentable sugar content to the maximum depending on hydrolysis efficiency. Regarding both treated samples. By such time of direct fermentation (72h); all of the measured parameters including yeast biomass (3.2g.100ml^{-1}); ethanol content (38.7%); consumed sugar (69.3%) and pH (3.5) were increased.

This finding mainly attributed to the increase of reduced sugar content and sometimes to lower pH value of the initial media at early growth period. Commercially, acid decomposition or hydrolysis at least safe the incubation time for *Tricoderma* sp. Consequently, the obtained biomass and alcohol content could be account by two fold in comparison with the direct fungi incubation within the same defines time.

As the optimum incubation time was assessed by 72h, enrichment of Saccharomyces fermentation media by different concentrations of molasses was performed. Molasses addition at all employed concentrations increased both yeast biomasses and bio-ethanol gained. Comparing with afore-obtained result bio-ethanol gain was raised from 28.7% after 72 hrs of incubation to be 36.3 due to the fertility of growth media by 15% of molasses with the increase of the consumed sugars from 59.3% to 70.1%. Addition of molasses increased both soluble sugars that inlets to increase fermentation potential as well as the extra supplementation of other request for yeast growth mainly nutrients.



(A)



(B)

Fig. 1. Effect of different fermentation time on ethanol production by *saccharomyces cerevisiae* after hydrolyzed de-fatted green alga *Chlorella vulgaris*. (A) = acid hydrolysis. (B) = with fungi hydrolyzed.

Molasses addition at all concentrations increased the obtained biomass of *Saccharomyces cerevisiae* and also increased pH value, since at the maximum bio-alcohol yield resulted in (fig.2). Extra supplementation of molasses (over 15%) decreased the consumption rate of sugars, yeast biomass and the formed bio-alcohol with a slight change of pH value. While, Repeanu [49] reported a maximum ethanol productivity of 2.33 $g.l^{-1}.h^{-1}$ was achieved around 36 hours of yeast fermentation. On the other hand, Roukas [50] reported a maximum productivity value of3.8 $g.l^{-1}.h^{-1}$ for the fedbatch fermentation of beet molasses with initially 250 $g.l^{-1}$ sugars and 3.7 $x10^{8}$ $cells.ml^{-1}$. These conditions are similar to those of Siqueira *et al*. [51] when a productivity of 6.91 $g.l^{-1}.h^{-1}$ was achieved from soybean molasses. However, it is important to remark that the sugar beet molasses is composed mainly of sucrose [52], and soybean molasses contains almost 50% of non fermentable sugars. Consequently, ethanol concentration is lower and production.

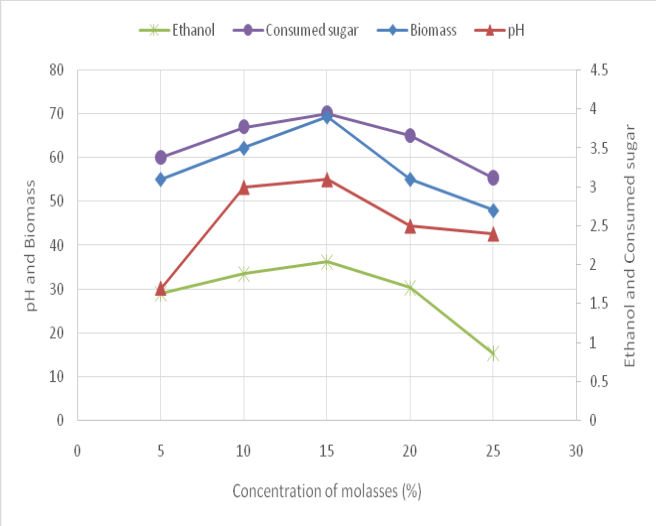


Fig. 2. Effect of addition different concentration of molasses on crude algae 26.3% on the ethanol production by *Saccharomyces cerevisiae* incubated for 72 h.

4. Conclusion

The obtained results during present study indicate that, bio-ethanol production through direct fermentation using fungal prehydrolyzed defatted alga seems to be a promising commercially technology as it required minimum incubation periods. The production process of bio-ethanol increased by addition of molasses, which consider as a cheap source for bio-ethanol production.

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