Safe technological trend towards the production of bioethanol from algal biomass grown on rice straw

Ahmed Darwish El-Gamal[1], Fawzy Gamal Khedr[2], Eman Yousef Tohamy[2] and Ahmed Mohamed Abouelwafa[3*]

[1]Botany and Microbiology Department, Faculty of science, Al-Azhar University, Cairo, Egypt.
[2] Botany and Microbiology Department, Faculty of science, Zagazig University, Zagazig, Egypt.
[3]Egyptian Ministry of Environment, Environmental Affairs Agency, Cairo, Egypt.

Abstract:

As a result of rapid growth in the population and manufacturing, the demand for ethanol is increasing continuously in worldwide. Because, biofuels produced from first and second-generation became unable to meet the international demand of bioethanol because of their needed value for food and feed. So, algae are among the most important sources of potential biofuels in the future of renewable energy because of accumulating high cellulose and also algae are distributed widely in the natural environment. This paper shows the ability of algae for bioethanol production, by pretreatment, hydrolysis, and fermentation of algal biomass. Two types of algae, *Chlorella vulgaris* and *Arthrospira platensis* were cultured under pre-treated rice straw with advantages as crop residues, a low-cost and carbon-rich source for algal cultivation. The chemical hydrolysates of rice straw (RS) were used for heterotrophic cultivation of *Chlorella vulgaris* and *Arthrospira platensis* for bioethanol production. Algal biomasses of the two microalgae were treated chemically with 4% H_2SO_4 at 121°C in autoclave for 90 min, followed by biological treatment with *Bacillus subtilis* for 72 hours at 30°C and pH 4.5 to increase the reducing sugars production. The fermentation by *Saccharomyces cerevisia*e for 72 hours and distillation of *Chlorella vulgaris* and *Arthrospira platensis* solutions were resulted in ethanol productivity of 8.7% and 2.5 % respectively after 24 hours at 30°C and pH 4.5.

Keywords: Rice straw, Bioethanol, *Chlorella vulgaris*, *Arthrospira platensis*, Saccharification, Fermentation.

Introduction

Microalgae are a large group of microscopic photosynthetic organisms, there are several commercial advantages of algae bioethanol production. These

* Corresponding author: email: aabouelwafa@gmail.com (ISSN: 1110-8649)

advantages include. (i) For algal bioethanol there is no need to compete with food production in either land or water. Additionally, they also need not compete with people for food (**Singh *et al*., 2011a**) (ii) The content of carbohydrate in the algae cell is abundant; the carbohydrates such as starches and sugars can be fermented to produce bioethanol. (iii)Algae are specialized that they have no lignin and very low levels of hemicellulose, which result in an increased hydrolysis efficiency and fermentation yields (**Choi *et al*., 2012; Eshaq *et al*., 2011**); thus, they can reduce the cost of the bioethanol production. (iv) Algae have the ability to take up CO2 from the atmosphere and power plants, and with the use of appropriate technology options, algae bioethanols can yield (greenhouse gas) GHG reductions relative to fossil and other biobased fuels (**Vasudevan *et al*., 2012; Singh *et al*., 2011b**). (v) Algae grow rapidly and can be easily grown in various aquatic environments such as fresh water, saline water, or municipal waste water (**Gouveia and Oliveira, 2009**). (vi) They have a high photosynthetic efficiency; the average photosynthetic efficiency of aquatic biomass is 6–8%, which is much higher than that of terrestrial biomass (1.8–2.2%) (**Ross *et al*., 2008**). Also, the microalgal cells have a very fast productivity and harvesting cycle (1–10 days) compared with other feedstock (harvest once or twice a year) and thus provide high biomasses level for bioethanol production (**Schenk *et al*., 2008**).

At the present time, algae conversion into bioethanol is recaptured attention as future biofuel feedstock in order to replace energy crops and cover any constraint in provide. Many species, especially marine algae are very suitable raw material for the production of different types of biofuels such as biodiesel, bioethanol, biogasoline, etc. and other chemicals (**U.S. Department of Energy Biomass Program,2009**). Also, **Rodolfi *et al*. (2009)** reports that algal biomass can provide about 60 times more alcohol than soybeans per acre of land. According to the study of **Ferrel and Sarisky-Reed (2010)** microalgae can provide amount of ethanol equal ten-fold more than corn per growing area. **Harel** (**2009)** state that microalgae have the ability to consuming high amounts of CO2 during their growth which make them very engaging to use as a friendly environmental feedstock.

Like lignocellulosic ethanol, production of bioethanol from microalgae requires also four major unit operations including pretreatment, hydrolysis, fermentation, and distillation. Schematic flowsheet for the bioconversion of algal biomass to bioethanol is shown in Figure 1 **(Kexun *et al.*, 2014)**. In order to produce sugars from the algae biomass, pretreatment is designed to help separate cellulose, hemicellulose, and lignin so that the complex carbohydrate molecules in microalgae cells can be broken down by enzyme-catalyzed into simple sugars which are easy to be fermented into ethanol. Then the fermentable sugars can be fermented into ethanol by ethanol-producing microorganisms and finally recover and purify the ethanol to meet fuel specifications. Additionally, some separated solids can be recovered and utilized as a fuel to provide process heat and electricity at an alcohol production facility.

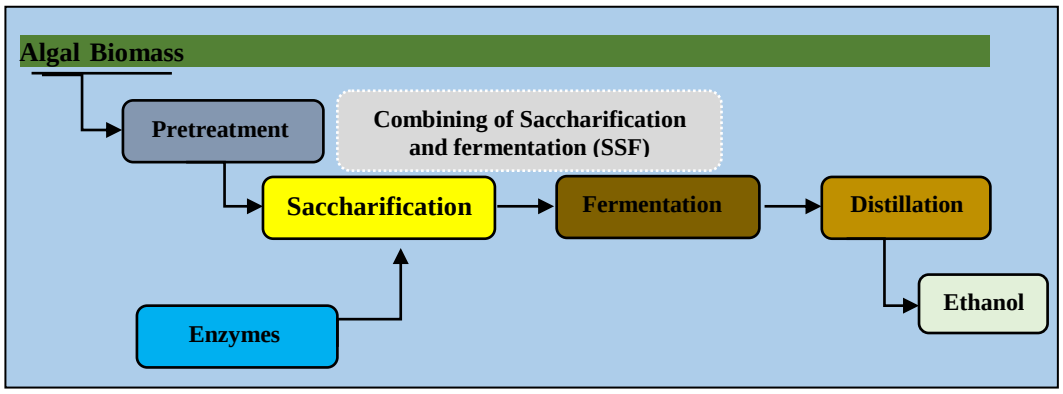


Figure 1. Steps of algae bioethanol production (Kexun *et al.*, 2014).

Microalgal biomass feedstock is the most attractive, alternative renewable feedstock recently studied for bioethanol production (**Al-Lwayzy *et al.*, 2014; Lam and Lee, 2012**). In general microalgae consist about 40% - 70% of carbohydrate, about 10% - 20% of protein and little residues of low molecular weight compounds such as amines, fatty acid and amino acids. Take into consideration microalga feedstock is an enormous source for bioethanol production because of its high content of biomass carbohydrate (**Brennan and Owende, 2010**), fast growth rate, high photosynthetic efficiency, ability to fix

greenhouse gases, non-competitive nature with food production, easily cultivated on non-agriculture site and have no lignin in their cell wall (**Eshaq *et al.*, 2011; Zhu *et al.*, 2014**).

Chlorella vulgaris has 37% - 55% of dry weight carbohydrate content which comes from starch found in the chloroplast and cellulose/hemicellulose found in the cell wall (**Al-Lwayzy *et al.*, 2014**). *Chlorella vulgaris* can be cultivated in different ways heterotrophically, autotrophically or mixotrophically. Mixotrophic cultivation of *Chlorella vulgaris* in presence of organic carbon source produce higher levels of carbohydrate biomass than if it cultivated autotrophically (**Miao *et al.*, 2004; Ceron Garcıa *et al.*, 2000**). When *C. vulgaris* cultivated in limited nitrogen and phosphorus medium, they utilize the carbon substrate easily result in production high quantities of lipid and carbohydrate simultaneously (**Rigano *et al.*, 1998)**. Residual crude glycerol obtained after biodiesel production, agricultural waste converted sugars and cellulosic materials, trapped CO2 which emitted from different industries and molasses production from sugar cane are cheap carbon substrates provide a good way to successful biomass production from microalgae. As a result of processing of plantain, the plantain peels are produced as byproducts. Plantain peels has high mineral contents of phosphorus and potassium, and thus it considered as a major source of carotenoids, polyphenols, dietary fiber and other bioactive compounds (**Wolfe *et al.*, 2005)**. Under Mixotrophic condition, the utilization of *Chlorella* species feedstock using domestic fowl, such as chickens waste as a growth medium resulted in yielding of high biomass production 2.5 g/l (**Agwa *et al.*, 2012)**. Most researchers attributed the ability of algal biomass for biodiesel production rather than bioethanol due to the low yield of sugars after hydrolysis of intracellular starch granules. Due to high cost of carbohydrate hydrolysis by enzyme mixture many researches have been developing simple technologies for such bioconversions such as combination of two chemical reagents; dilute acid or alkaline hydrolysis with enzyme in saccharification process (**Hernandez *et al.*, 2015)**.

Microalgae also are photosynthetic organisms have different polysaccharides. Some of these polysaccharides can be hydrolyzed through

chemical and/or enzymatic processes to yield monosaccharides, which can then be fermented to produce bioethanol **(Darzins *et al*., 2010**). Examples for species that have a high carbohydrate content and are strong candidates for bioethanol production are *Chlorococcum*, *Chlorella*, *Chlamydomonas*, *Scenedesmus*, and *Tetraselmis*. Ethanol production varies depending on the types of hydrolysis strategies and fermentation processes used. Where recently, researchers became able to produce higher levels of sugars through treatment of different types of microalgae: 84–166.1 mg/g for *Clostridium saccharoperbutylacetonicum* **(Castro *et al*., 2015**); 58% (w/w) *Chlamydomonas reinhardtii* of dry cell weight (**Nguyen *et al*., 2009**); release of 57–89.9% of total sugars from *Chlorella* (**Zhou *et al*., 2012**; **Lu *et al*., 2012**), and 41.6% gulcose from *Scenedesmus obliquus* (**Miranda *et al*., 2012a**); and glucose yields greater than 90% from *Dunaliella tertiolecta* (**Geun *et al*.,2013**), 46.92% gulcose from *C. vulgaris* (**Ho *et al*., 2013b**). Ethanol can be excreted directly by microalgae through their cell walls, as shown for several organisms, including *Chlorococcum humicola* and *Chlorococcum infusionum* (**Harun and Danquah,2011**), *Chlamydomonas reinhardtii* (**Choi *et al*., 2010**), *Chlorella vulgaris* (**Ho *et al*., 2013*a***), *Scenedesmus obliquus* (**Ho *et al*., 2013*b***), *Tetraselmis subcordiformis* (**Yao *et al*., 2013**) and *spirulina* (**Shimpei *et al*., 2013**). Due to rigidity of cell wall of microalgae that may be more or less rigid structure therefore, it may be more difficult to break, such as in the case of *Chlorella* sp. that has a harder wall contrary to those of *Scenedesmus* sp. and cyanobacteria *Arthrospira platensis.* Therefore the pretreatments are required.

Carbon sources are usually the most critical and necessary factors for microalgal growth. In general, microalgae can be grown under photoautotrophic, heterotrophic, and Mixotrophic conditions using varied carbon sources, such as carbon dioxide (CO_2), methanol (CH_3OH), acetate ($C_2H_3O_2$), glucose ($C_6H_{12}O_6$), or other organic compounds (**Xu *et al*, 2006)**. For photoautotrophic cultivation, the microalgae utilize inorganic carbon such as carbon dioxide (CO_2) or bicarbonates ($HCO-3$) as a carbon source to obtain the chemical energy through photosynthesis process (**Ren *et al*., 2014**). For heterotrophic cultivation, there are some species of microalgae have the ability to use the organic carbon as a carbon source directly and this is happening in the presence or absence of a light supply (**Chojnacka and Noworyta, 2004**). Although, the most commonly used carbon

source for growth of microalgae and production of biofuels is still CO_2 or $HCO-3$, because using organic carbon sources would be very expensive for producing low-price products such as biofuels, this paper indicates another cheap carbon source for biofuel production, which is pretreated rice straw.

However, when the biofuels are directly converted from using CO_2 as a substrate, CO_2 emissions are reduced from a net-zero CO_2 emission, thus reduces greenhouse effects in the environment. Particularly, photoautotrophic growth of microalgae Contributes to reutilization of CO_2 gases which are coming often from flue gas of most power plants and nearly all industrial activities (**Packer *et al.*, 2009**), as microalgal biomass can be further utilized to produce biofuels or other value-added products (**Hsueh *et al.*, 2007**).

In addition, when compared to terrestrial plants, most microalgae have much higher cell growth and CO_2 fixation (about 10–50 times higher), and this gives proof of another advantage of direct conversion of photoautotrophic growth of microalgae into biofuels. Therefore, it is very important to trend to produce biofuels via photoautotrophic growth of microalgae, as this is important in terms of economy (i.e., cheap) and protection of the environment. However, microalgae grown under heterotrophic conditions have the ability to use organic carbon sources (e.g., sugars) to increase algal biomass content which in turn converted into biofuels. In this way, it is important to focus on biofuel production by heterotrophic cultivation of algae, since heterotrophic growth of microalgae is usually faster than autotrophic growth (**Chen F. *et al.*, 1996**). So because of the high cost of obtaining the organic carbon sources from raw biomass is still a great concern, this paper focused on the production of biofuels from a cheap and available source of carbon; rice straw.

Chlorella vulgaris and *Arthrospira platensis* used for this study was cultivated in rice straw medium which is an excellent carbon source and a low-cost matter. The biomass extracted was exploited as a carbon source for bioethanol production by ordinary Simultaneous saccharification and fermentation process (SSF).

Materials and Methods

1. Rice straw treatment

1.1. Physical Pretreatment of rice straw

Rice straw was sundried for 3 days, then chopped into 2 cm size by using chopper machine and was milled by milling machine to 1-2 mm. As a result of good grinding of rice straw, the homogenized small size particles were obtained and used for further treatments.

1.2. Chemical Pretreatment

There are many chemical pretreatments were used, and the most promising chemical treatment for rice straw include oxidative and Acid-Hydrogen peroxide pretreatments. In chemical treatment; 10 g of rice straw was dissolved in 100 ml of the chemical solution (H_2SO_4 and/or H_2O_2) at ratio of 1:10 (w/v).

1.3. Dilute Acid Pretreatment

Acidic treatment is done at three different concentrations of Sulphuric acid were 2% and 4% **(Anwar *et al.*, 2012; Nikzad *et al*., 2012**).

1.4. Oxidative Pretreatment

Hydrogen peroxide treatment was done at two different concentrations of Hydrogen peroxide were 4% and 6%.

1.5. Acid-Hydrogen Peroxide (AHP) Pretreatment

Acid hydrogen peroxide treatment was done at two different concentrations of Sulphuric acid and Hydrogen peroxide were 2% and 4% respectively.

All the above flasks were the autoclaved at 121˚C, at 15 psi. for 60 and 90 minutes with control flasks. After treatment, the samples were filtered after a

while with cheese cloth. The samples were then washed out gently, first with the tap water and then with the distilled water (**Nikzad *et al*., 2012**). The samples were air dried and then dried at oven at 105°C for one hour, then stored in the refrigerator at 4°C for further use. The filtrate solution was preserved for Dinitrosalicylic acid (DNS) analysis to determine the concentration of reducing sugars present in that samples.

1.6. Microorganisms

The cyanobacterium *Arthrospira platensis* and green algae *C. vulgaris* used in the present study was obtained from the laboratory of Microbiology, faculty of science, University of AL-Azhar, Cairo, Egypt. *Arthrospira platensis* was maintained in 500 mL sterilized Erlenmeyer flasks containing 100 mL Zarrouk's medium at 25 ± 2°C, pH 10.5 (**Zarrouk *et al*.,1966**), while *C. vulgaris* was maintained in 500 mL sterilized Erlenmeyer flasks containing 100 mL Blue-Green 11 medium (BG11) at 25 ± 2°C, pH 9 (**Marrez *et al*., 2013**), with continuous illumination using white fluorescent tubes (2500 Lux) and daily shaking by hand three times.

2. Cultivation of algae on pretreated rice straw

Firstly, 90ml of Zarrouk's medium was poured in two "250 ml conical flasks" and pH was adjusted to 10.5, flasks were autoclaved at 121°C for 30 min, then adding one gram of chemically pretreated rice straw to the first flask and one gram of untreated rice straw to the second flask, then each flask was inoculated with 10 ml freshly prepared *Arthrospir*a *platensis*. Secondly, 90ml of Blue-Green 11 medium was poured in two "250 ml conical flasks" and pH was adjusted to 9, flasks were autoclaved at 121°C for 30 min, then adding one gram of chemically pretreated rice straw to the first flask and one gram of untreated rice straw to the second flask, then each flask was inoculated with 10 ml freshly prepared *C. vulgaris*.

All flasks were kept at room temperature at (25 ± 1°C) under natural day and dark period (approximately, 12:12) for 15 days (**Agwa *et al*.,2012**). Cultures of algae were shaken three times every day. After 15 days, microalgal suspension

was centrifuged, and pellets were washed thoroughly with distilled water and dried at 60°C until constant weight.

3. Filtration of Algal biomass

The growth conditions were observed after two day intervals for the algal cell growth and biomass calculations. Samples were filtered, collected, and subsequently analyzed for optical density (abs) determination using UV spectrophotometer (**Lee *et al*., 2014**). The cell dry weight was measured using a centrifuge at 12,000 rpm for 15 mins. The residue was washed twice with physiological saline (0.85% w/v, NaCl), dried in an oven at 60˚C in a pre-weighed filter paper until constant weight. The amount of dried microalgae was measured as cell dry weight (**El-Sheekh *et al*., 2014**). The biomass productivity was calculated according to **Ho *et al*., (2012)**.

4. Pretreatment of algal biomass

Algal biomass was treated chemicaly with 4% H_2SO_4 then bilogicaly with *Bacillus subtilis*. Biomass pretreatment is one of the most crucial and expensive process steps in the process of converting biomass to fermentable sugars (**Harun *et al*., 2011**), and pretreatment is estimated to reduce about 33% of the total cost (**Choi *et al*., 2010**).

4.1. Acid hydrolysis pretreatment

Algal biomass was subjected to dilute acid pretreatment using 4% (2N H_2SO_4), autoclaved at 121˚C for 120 mins and neutralized to pH 4.5 using citrate buffer.

4.2. Biological treatment of algal biomass

Biological treatment using various types of rot fungi or bacteria, a safe and environmentally friendly method, that does not require high energy for lignin removal from a lignocellulosic biomass **(Kumar *et al*., 2009**). *Bacillus subtilis* has been used for bio-hydrolysis of algal biomass after chemical pretreatment.

Bacterial Inoculum Preparation

Medium was inoculated with 100 µl of *Bacillus subtilis* and incubated at 30°C for 72h then used for inoculation of fermentation yeast.

Yeast Inoculum Preparation

S. cerevisiae was inoculated in 100 ml YPD medium (**Ausubel *et al*., 1994**) under sterilized conditions and was incubated at 30°C for 24h. After incubation the culture was centrifuged for 5 min at 4000 rpm and pellets were washed with sterilized distilled water then were suspended in sterilized distilled water for inoculation immediately.

4.3. Simultaneous Saccharification and Fermentation (SSF)

The strains *Bacillus subtilis* and *Saccharomyces cerevisiae* were grown in Basal Media were added in 500 ml of distilled water and make up the volume to 1000 ml (**Mandels *et al*., 1962; Mukhopadhyay and Nandi , 1998; Karimi *et al*., 2006**) and pH of the media was adjusted to 4.5 using citrate buffer at 25°C. The media was then autoclaved at 121 ˚C and 15 psi for 30 minutes. 5% dextrose was added after the autoclaving of media (**Sharma *et al*., 2007**). 100 ml of this media was then poured in each of 250 ml of flask containing 400 milligrams of pretreated algal biomass. 100 µl of *Bacillus subtilis* was then inoculated in each of these flasks under sterile conditions and then incubated at 30˚C ± 2˚C on the rotary shaker at 120 rpm for 72h. The sampling from these flasks was done for Dinitrosalycilic Acid (DNSA) analysis for estimation of sugar contents, where the solution was centrifuged at 9000 rpm for 15 mins and stored as sugar hydrolysate for the fermentation (**Miranda *et al*., 2012b; Dhull *et al*., 2014**). After 72 hours *S. cerevisiae* was inoculated in the same flasks for the process of fermentation, at an initial yeast cell concentration of approximately 1×10^7 cells/mL and incubated on the rotary shaker at 150 rpm at 30°C for another 72h. Sampling was done every 24 hours for DNSA analysis at 540 nm.

4.4. Filtration and Distillation Process

Samples were then filtered by using cheese cloth to separate the solid substrate from liquid and then distillation was done at 78.37˚C to get the ethanol samples for GC analysis.

4.5. Analytical methods

4.5.1. Estimation of reducing sugars

For reducing sugar estimation: 3,5-dinitosalicyclic acid method was used. By adding 1 ml of DNS reagent to 1 ml of each of the sample then boiled for 5 mins and diluted with 10 ml of distilled water. The absorbance was determined at 540 nm using UV-VIS spectrophotometer. The concentration value was extrapolated from the glucose standard curve (**Itelima *et al*., 2013**).

4.5.2. Estimation of ethanol concentration

Dubios chromate method was used for ethanol estimation (**Dubois *et al*., 1956**). 5 ml of each sample was treated with 2 ml of chromate reagent. The mixture was allowed to stand for an hour and the absorbance measured at 588 nm using UV-VIS spectrophotometer (**Rabah *et al*., 2011**).

4.5.3. Distillation process

Method according to **Oyeleke and Jibrin (2009**); was used for distillation of the fermentation medium to ethanol. the temperature of heating 78˚C was used to heat the bottom of flask until completely distilled. The ethanol percentage resulted from distillation was estimated from ethanol standard curve.

Results

1. Chemical composition of Rice Straw (RS) before and after treatment

Chemical composition of rice straw before and after chemical treatment was shown in table (1). Where cellulose, hemicellulose and lignin for Untreated RS is 38.26%, 25.92% and 11.28% respectively and after Acid-Hydrogen peroxide treatment of RS it became 46.65%, 18.86% and 8.32% respectively, while it was so slightly in untreated specimens where, 38.98%, 25.21% and 10.01% for cellulose, hemicellulose and lignin respectively.

Table 1: Chemical composition of Rice straw before and after chemical treatment

Composition	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated RS (*Native straw*)	**38.26**	**25.92**	**11.28**
After Chemical treatment			
Acid-Hydrogen peroxide treatment of RS	**46.65**	**18.86**	**8.32**
Sulfuric acid treatment of RS	**39.15**	**12.36**	**10.38**
Hydrogen peroxide treatment of RS	**40.55**	**14.47**	**9.05**

2. Chemical treatment of RS

Acid hydrogen peroxide pretreatment at 121°C for 90 mins. was the effective pretreatment in hydrolysis of rice straw than acid or peroxide treatments, where the strength of acid and hydroxyl radicals (•OH) are extremely powerful oxidizing agents that can catalyze highly non-specific reactions leading to the cleavage of covalent bonds in both lignin and cellulose (**Hammel *et al*.,2002**) (**Table 2**).

Figure (2) shows that reducing sugars from rice straw increased after 90 mins. of treatments at 121°C than pretreatments of another chemical either after 60 mins nor 90 mins. So rice straw pretreated with Acid hydrogen peroxide after 90 mins. was used for cultivation of algae.

Table 2: Reducing sugars from rice straw after different chemical treatments

Reducing sugars mg/ml							
	Time of treatment	**2% H_2SO_4**	**4% H_2SO_4**	**4% H_2O_2**	**6% H_2O_2**	**2% H_2SO_4 4% H_2O_2+**	**Untreated RS**
Autoclave At 121 C°	**60 min**	**99.23**	**118.48**	**104.56**	**147.39**	**166.93**	**9.91**
	90 min	**117.60**	**175.85**	**126.56**	**190.33**	**250.56**	**9.93**

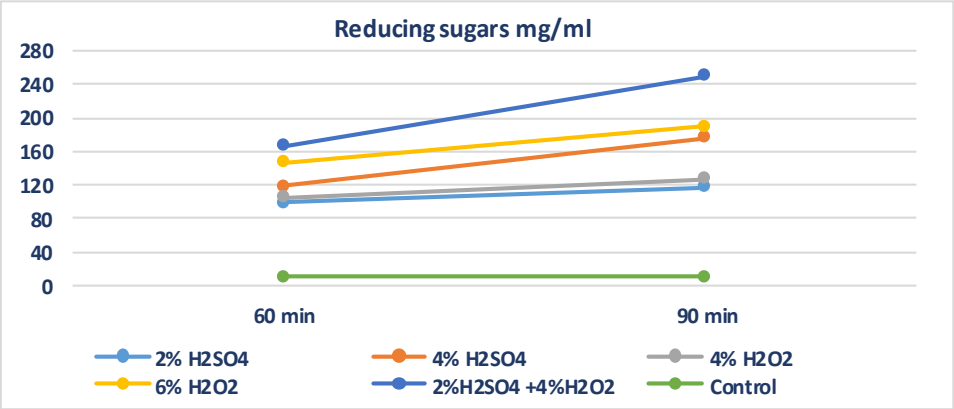


Figure 2. Reducing sugars results from rice straw after different chemical treatments

3. Cultivation of Microalgae on RS as a carbon source

Microalgae *Chlorella vulgaris* and *Arthrospira platensis* were grown under heterotrophic conditions using chemically treated rice straw as a carbon sources (sugars) for bioethanol production.

3.1. Growth rate of Microalgae

The cell growth of microalgae was determined by measuring the Optical Density (OD) at 680 nm using a spectrophotometer. It has been found that growth rate of *Chlorella vulgaris* on chemically pretreated rice straw medium is better than *Arthrospira platensis* (**Figure 3**).

The microalgae *Chlorella vulgaris*, particularly, has been considered as a promising feedstock for bioethanol production because it can accumulate up to 37% (dry weight) of starch. However, higher starch contents can also be obtained for optimized culture conditions (**Nguyen , 2012**).

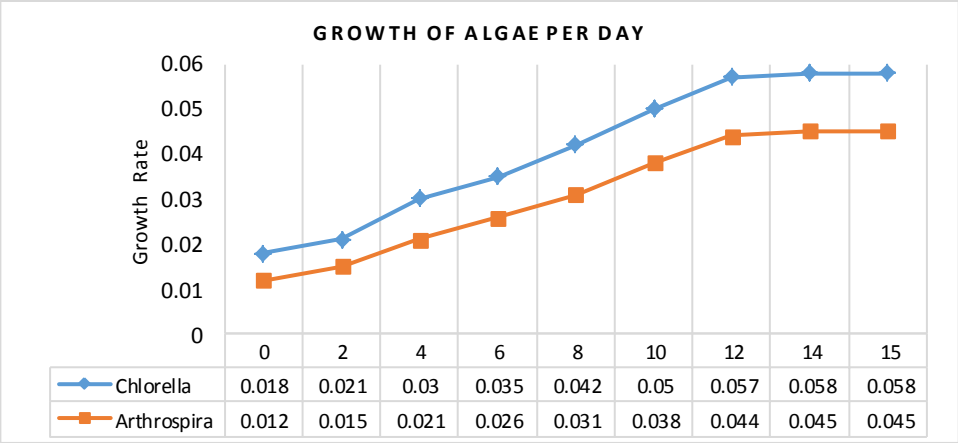


Figure (3). Growth curve of *Chlorella vulgaris* and *Arthrospira platensis* in pretreated rice straw

3.2. Algal biomass

The addition of carbon source (RS) had significant effects on the biomass productivity in *C. vulgaris* and *Arthrospira platensis*. Biomass productivity of *Chlorella vulgaris* was 0.56 g/L was significantly higher ($p < 0.05$) biomass productivity of *Arthrospira platensis* 0.45 g/L (**Table 3**).

Table (3): Biomass productivity of *Chlorella vulgaris* and *Arthrospira platensis*

Microalgae	Biomass productivity (g /L)
C. vulgaris	**0.56 ± 0.06**
Arthrospira platensis	**0.45± 0.02**

3.3. Reducing sugars after cultivation of algal growth

Table (4) shows the quantities of reducing sugars consumed by *Chlorella vulgaris* or *Arthrospira platensis* during the cultivation of each of them on chemically pretreated rice straw. Reducing sugars were measured every 48h.

Table (4): Reducing sugars consumed by *Chlorella vulgaris* and *Arthrospira platensis*

Reducing sugars (mg/ml) Algal cultivation on pretreated RS for 15 days at 25°c				
	Chlorella vulgaris		*Arthrospira platensis*	
Time/day	RS-Treated with 2%H_2SO_4 + 4% H_2O_2	Control	RS-Treated with 2% H_2SO_4 + 4% H_2O_2	Control
0	242.53	11.52	242.78	11.52
2	239.03	11.01	240.78	11.35
4	233.48	10.34	239.04	11.10
6	226.61	9.40	236.18	11.09
8	221.93	9.23	234.97	11.05
10	219.76	9.14	233.38	10.90
12	218.89	8.70	233.27	10.60
14	218.00	8.60	232.45	10.51

It was found that *Chlorella vulgaris* has been consumed 24.53 mg/ml of reducing sugars from the medium as a carbon source after 15 days of its cultivation, while *Arthrospira platensis* has been consumed a significant amount of reducing sugars 10.33 mg/ml after 15 days of its cultivation (**Figure 4**).

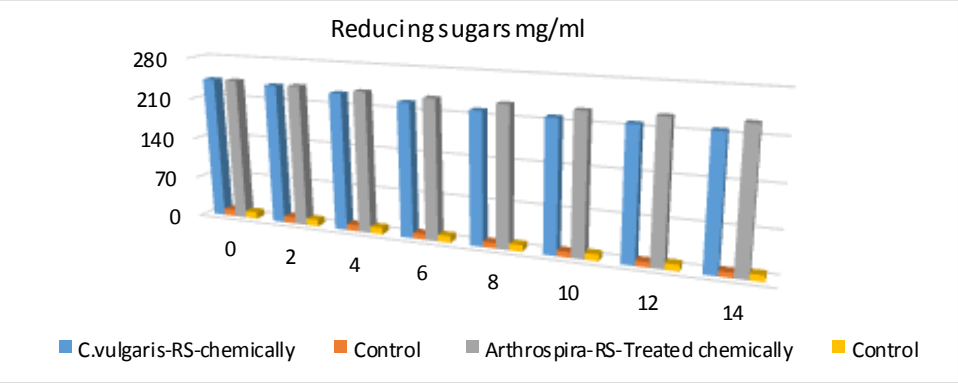


Figure (4). Reducing sugars concentration after cultivation of Microalgae.

3.4. Chemical treatment of Algal biomass

Table (5) shows that carbohydrate extracted biomass had initial sugar composition of 32.68 and 14.78 in mg/ml after acid hydrolysis process for *Chlorella vulgaris* and *Arthrospira platensis* with 4% H_2SO_4 at 121°C in autoclave for 90 min respectively.

Table (5): Reducing sugars from algal biomass treated with 4% H_2SO_4 at 121 °c in autoclave for 90 min

Reducing sugars (mg/ml) Algal biomass treated With 4% H_2SO_4 at 121 °c in autoclave for 90 min				
	Chlorella vulgaris	**control**	***Arthrospira platensis***	**control**
Reducing sugars mg/ml	**32.68**	**3.15**	**14.78**	**1.72**

Acid hydrolysis treatment is more effective with *Chlorella vulgaris* compared to *Arthrospira platensis*, where it gives more reducing sugars **(Figure 5).**

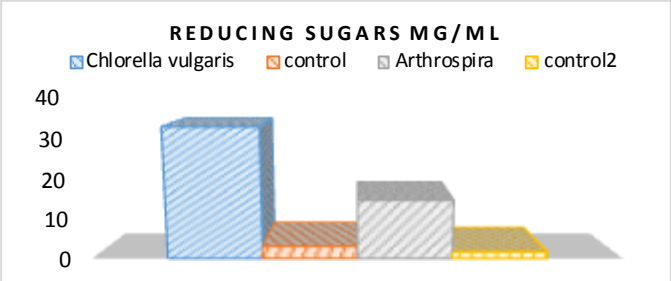


Figure (5). Reducing sugar concentration after acid hydrolysis of algae.

3.5. Biological treatment of Algal biomass

The hydrolysis of algae with *Bacillus subtilis* resulted in carbohydrate extracted biomass had initial sugar composition of 33.54, 35.85 and 36.78 mg/ml

for *Chlorella vulgaris* after 24h, 48h and 72h and respectively while for *Arthrospira platensis* the sugars were 14.90, 16.89 and 17.64 mg/ml after 24h, 48h and 72h and respectively **(Table 6).**

Table (6): Reducing sugars from algal biomass treated with *Bacillus subtilis* for 72h at 30 ˚C and PH 4.5

Reducing sugars (mg/ml) Algal biomass treated with *Bacillus subtilis* for 72h at 30 ˚C PH 4.5				
Time of hydrolysis	***Chlorella vulgaris***	**control**	***Arthrospira***	**control**
24 h	**33.54**	**3.20**	**14.90**	**1.77**
48 h	**35.85**	**3.64**	**16.89**	**1.90**
72 h	**36.78**	**4.11**	**17.64**	**1.96**

Biological treatment is favored for algal biomass after chemical treatment for more reducing sugars production to reach maximum results of ethanol percent after fermentation of sugars by yeast. It has been found that biological treatment results in high reducing sugars for *Chlorella vulgaris* compared to *Arthrospira platensis* **(Figure 6).**

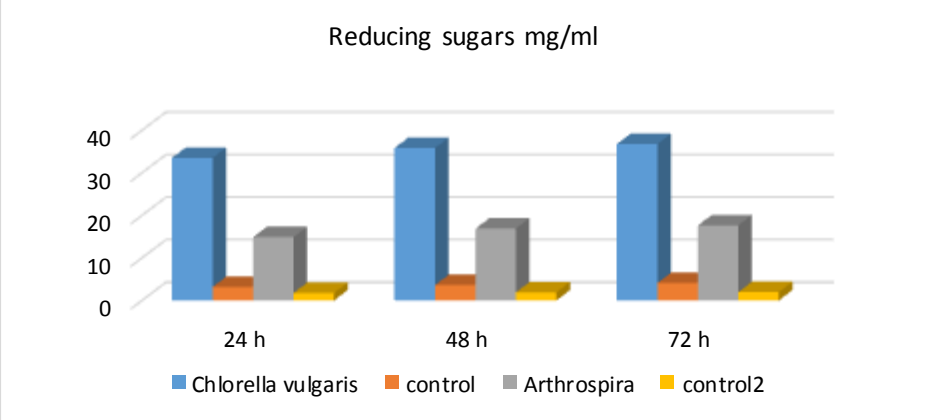


Figure (6). Reducing sugar concentration after biological hydrolysis of algae.

3.6. Fermentation of Algal biomass

After three days fermentation at 30˚C and pH 4.5, the reducing sugar was consumed highly by fermentation broth inoculated with *Bacillus subtilis*, followed by inoculation of *S. cerevisiae*. Most of reducing sugars produced from algal feedstock were fermented by *S. cerevisiae* into ethanol determined by GC.

3.7. Ethanol Determination

3.7.1. Ethanol Yield (Ethanol productivity)

The highest ethanol produced from fermentation of algal biomass was after 24 h of fermentation then it was gradually decreased (**table 7**). Ethanol productivity was 8.7% from *Chlorella vulgaris* after 24h, while for *Arthrospira platensis* it was only 2.5% ethanol productivity after 24h of fermentation by *Saccharomyces cerevisiae* for at 30°C and pH 4.5.

Table (7): Ethanol productivity % from pretreated Algal biomass at different incubation times

Ethanol productivity (%) (ml of ethanol per 100 mL of media) Algal biomass fermented With *Saccharomyces cerevisiae* for 72h at 30 ˚C pH 4.5				
Time of fermentation	***Chlorella vulgaris***	**Control**	***Arthrospira***	**control**
24 h	**8.7**	**-**	**2.5**	**-**
48 h	**7.2**	**-**	**1.8**	**-**
72 h	**7.1**	**-**	**1.3**	**-**

Ethanol productivity results indicates that fermentation of *Chlorella vulgaris* produce ethanol more than *Arthrospira platensis* at the same conditions of fermentation (**Figure 7**).

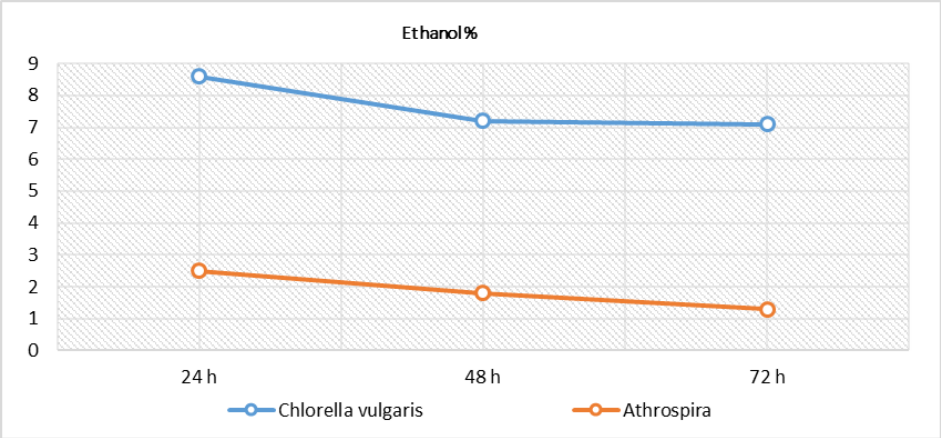
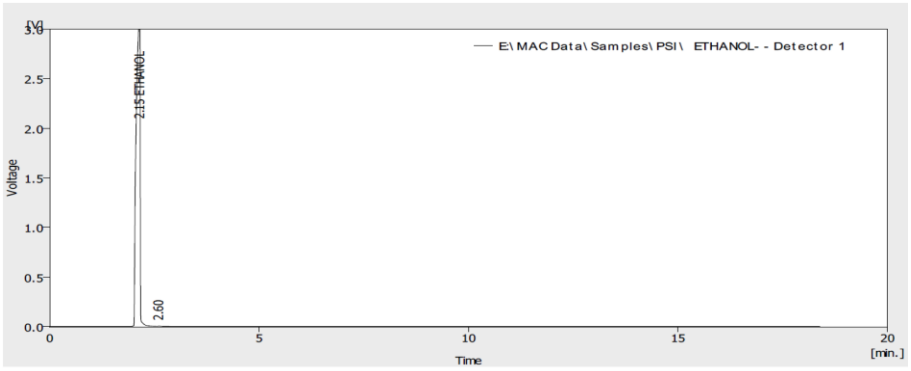


Figure (7). Ethanol productivity of pretreated rice straw during fermentation with *Saccharomyces cerevisiae*

3.7.2. GC Analysis

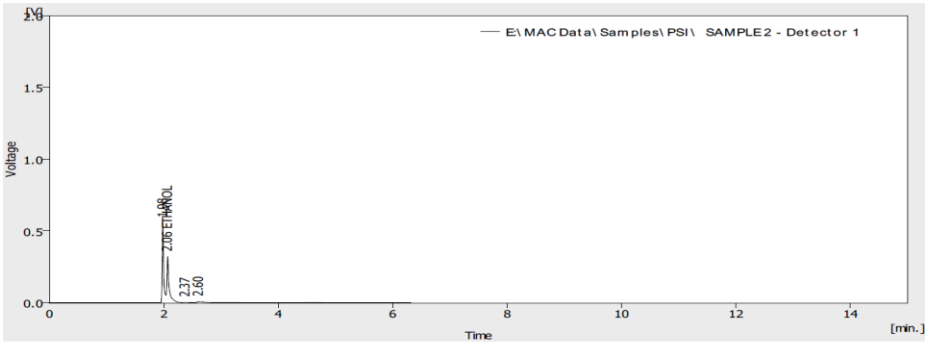
The results of fermentation were obtained using a GC. There was a gradual decrease in the amount of ethanol produced with fermentation time from one day to third days. The results of fermentation are shown in **figure (8a, b and c)** where the ethanol percent produced after fermentation of pretreated algal biomass with *Saccharomyces cerevisia*e was 8.7% and 2.5 % for *Chlorella vulgaris* and *Arthrospira platensis* respectively after 24 hours at 30°C and pH 4.5, then the ethanol percent is gradually decreased at the third day of fermentation.



Result Table (ESTD - E:\MAC Data\Samples\PSI\ ETHANOL- - Detector 1)

	Reten. Time [min]	Response	Amount [%]	Amount [%]	Peak Type	Compound Name
1	2.153	20822.049	99.900	100.0	Ordnr	ETHANOL
2	2.603	40.756	0.000	0.0		
	Total		99.900	100.0		

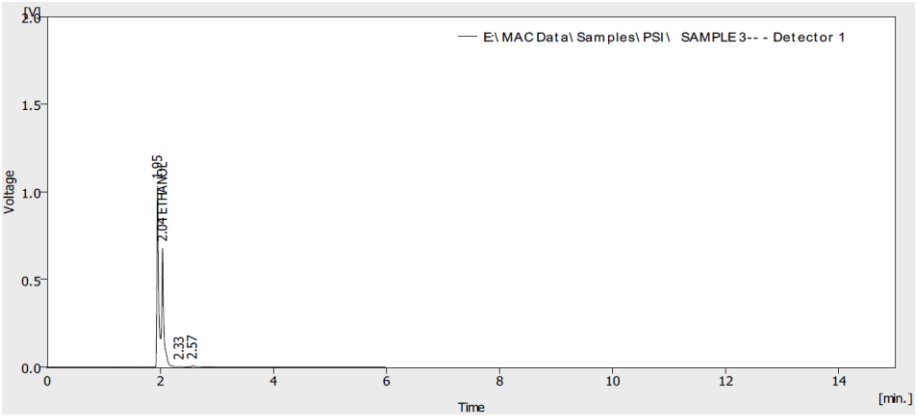
(a)



Result Table (ESTD - E:\MAC Data\Samples\PSI\ SAMPLE 2 - Detector 1)

	Reten. Time [min]	Response	Amount [%]	Amount [%]	Peak Type	Compound Name
1	1.980	873.271	0.000	0.0		
2	2.063	536.906	2.576	100.0	Ordnr	ETHANOL
3	2.370	2.822	0.000	0.0		
4	2.603	47.836	0.000	0.0		
	Total		2.576	100.0		

(b)



Result Table (ESTD - E:\MAC Data\Samples\PSI\ SAMPLE 3-- - Detector 1)

	Reten. Time [min]	Response	Amount [%]	Amount [%]	Peak Type	Compound Name
1	1.950	2209.849	0.000	0.0		
2	2.040	1818.087	8.723	100.0	Ordnr	ETHANOL
3	2.333	15.017	0.000	0.0		
4	2.567	31.749	0.000	0.0		
	Total		8.723	100.0		

(c)

Figure (8). GC Analysis of Ethanol % (a) Pure ethanol, Ethanol from (b) *Arthrospira platensis* and Ethanol from (c) *Chlorella vulgaris*

Discussion

In this study, low cost rice straw which also is available in Egypt in large quantities, was treated as a rich source of carbohydrate for cultivation of two types of microalgae, namely green algae (*Chlorella vulgaris*) and blue green algae (*Arthrospira platensis*), for the production of bioethanol after being chemically treated with diluted sulfuric acid and then biologically using bacterium *Bacillus subtlilis* for breaking down polysaccharides into simple sugars are easily fermented by *Saccharomyces cerevisiae* yeast to produce bioethanol. Rice straw treated with Acid hydrogen peroxide at 121°C for 90 min resulted in high production of reducing sugars than sulfuric acid (4%) or hydrogen peroxide (6%) pretreatments at the same conditions, where it was 250.56 , 175,85 and 190.33

mg/ml for Acid hydrogen peroxide (AHP), sulfuric acid and hydrogen peroxide pretreatments respectively (**Figure 2**), also lignin was reduced to 8.32% by AHP pretreatment as compared to untreated RS (11.28%), sulfuric acid pretreatment (10.38%) or hydrogen peroxide pretreatment (9.05%) and This is evident in **Table (1)** results. This is compatible to the study by **Long *et al.* (2005)**, **Jahan *et al.* (2006)** and **Karin Walter (2009)**. Pretreated rice straw with AHP considered a new chemical treatment added to other chemical treatments of rice straw for bioethanol production. So, it used for cultivation of microalgae *Chlorella vulgaris* and *Arthrospira platensis.*

Results of cultivation of microalgae *Chlorella vulgaris* and *Arthrospira platensis* for 15 days, heterotrophically on rice straw pretreated with AHP as a carbon source showed in **Figure (3)**. it has been found that, maximum biomass of *Chlorella vulgaris* (0.56 g/l) is somewhat higher than of *Arthrospira platensis* (0.45 g/l) at the same conditions (**Table 3**). This is analogous to **Krystian *et al.* (2014)** and is consistent with the results of **Sibi (2015)**. Reducing sugars from pretreated rice straw have been reduced slightly in the mediums of both *Chlorella vulgaris* and *Arthrospira platensis* during the cultivation period, indicating that these microalgae can be cultivated on low cost carbon source lignocellulosic substances such as rice straw (**Figure 4**), and this is analogous to **Penglin *et al.* (2011)** and **Rouf *et al.* (2019)**.

The results of microalgal biomass treatment (*Chlorella vulgaris* and *Arthrospira platensis*) by dilute sulfuric acid showed in **Figure 5**, indicated the effectiveness of chemical treatment in the hydrolysis of microalgal biomass, where it resulted in increasing the amount of reducing sugars from algal biomass, this is agreement with **Lee *et al*. (2011)** and **Laurens *et al.* (2015).** Reducing sugars resulted from *C. vulgaris* biomass (32.68 mg/ml) better than those from *Arthrospira platensis* (14.78 mg/ml) due to the good exploitation of carbon source by *C. vulgaris* than *Arthrospira platensis.*

The results of this study demonstrated that cellulolytic enzymes of *Bacillus subtilis* can be used for microalgae biomass hydrolysis with greater advantages **(Haipeng *et al.*, 2017)**. The yield of the reducing sugars from its hydrolysis is lower than that from dilute acid hydrolysis indicating that saccharification activities of the enzyme are not adequate.

The microalgal biomass hydrolysates in this work were fermented by *Saccharomyces cerevisiae*. Co-culture fermentation by *Bacillus subtilis* and *S. cerevisiae* yielded highest ethanol during the fermentation period after 24h. and then ethanol percent decreased. This may be due to various mixtures of sugars such as hexoses and pentoses released into the hydrolysates which cannot be utilized by *S. cerevisiae* **(Van *et al.*, 2006).** *Bacillus subtilis* is capable of producing carbohydrate hydrolases and certain enzymes like amylases, cellobiases, xylanases which degrade the non-starch polysaccharides resulting to related increase in the amount of soluble sugars available in the fermentation broth (**Huang and Tang, 2007; Panneerselvam and Elavarasi, 2015)** . The susceptibility of some sugars obtained after hydrolysis by *Bacillus subtilis* during the fermentation process to the fermentable activity of *S. cerevisiae* became higher causing corresponding increase in ethanol yield. The result of this study agrees with the study production of ethanol by simultaneous saccharification of *Bacillus subtilis* and *S. cerevisiae* fermentation of potato wastes which stated that most substrates were utilized for ethanol production in co-culture fermentation (**Elsayed *et al.*, 2015**).

High Bioethanol resulted from microalgal biomass fermentation was after 24h at pH 4.5 and 30°C where it was 8.7% from *C. vulgaris* and 2.5% from *Arthrospira platensis*, then bioethanol decreased (**Figures 7&8**). Although the amount of ethanol produced is few (8.7%) compared with the amount of ethanol produced by **Ragaa *et al.* (2017)** which is 40%, But it is a promising and encouraging to complete the study of bioethanol production from microalgal biomass after cultivation on the low-cost carbon source rice straw.

Conclusion

Utilization of crop waste materials for algal biomass is a promising approach to meet the increasing energy needs as a substitute for fossil fuels. This work provides an important strategy using rice straw as a potential carbon source in microalgal cultivation for bioethanol production and showed that pretreated rice straw can be utilized by *C. vulgaris* and *Arthrospira platensis* which provides a

feasible route of reducing production cost of bioethanol from a cheap carbon substrate for biomass and bioenergy production.

Although the slightly quantities of ethanol produced by algae in this study, the results are good and promising for the future production of ethanol from algal biomass, beside another benefits such as,

- Increase the carbohydrate content of microalgae
- Isolation and selection of potential algal strains
- Optimization of their growth in various environments
- proper selection of yeast strain for improved fermentation
- proper knowledge and identification of biochemical triggers, and pretreatment methods and enzymatic methods, they help to improved fermentation process, so that automatically improvement in ethanol yield from algae.

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الاتجاه التكنولوجي الآمن نحو إنتاج الإيثانول الحيوي من الكتلة الحيوية للطحالب التي تزرع على قش الأرز

أحمد درويش الجمل[١]، فوزى جمال خضر[٢]، إيمان يوسف تهامي [٢] و أحمد محمد أبوالوفا [٣]

١- قسم النبات والأحياء الدقيقة ، كلية العلوم ، جامعة الأزهر ، القاهرة ، مصر.

٢- قسم النبات والأحياء الدقيقة ، كلية العلوم ، جامعة الزقازيق ، الزقازيق ، مصر.

٣- وزارة البيئة المصرية ، جهاز شئون البيئة ، القاهرة ، مصر.

نتيجة للنمو السريع في عدد السكان والتطور الصناعي، يزداد الطلب على الإيثانول بشكل مستمر في جميع أنحاء العالم. وذلك لأن الوقود الحيوي الذي تم إنتاجه من الجيل الأول والثاني أصبح غير قادر على تلبية الطلب الدولي على الإيثانول بسبب قيمتهما المهمة كأغذية واعلاف. لذلك، تعد الطحالب من بين أهم مصادر الوقود الحيوي المحتمل في مستقبل الطاقة المتجددة نتيجة لاحتوائها على كميات كبيرة من السليلوز، كما انها منتشرة على نطاق واسع في البيئة الطبيعية. توضح هذه الورقة قدرة الطحالب على إنتاج الإيثانول الحيوي، عن طريق المعالجة الكيميائية، والبيولوجية، وتخمير الكتلة الحيوية الطحلبية. تم استزراع نوعين من الطحالب هما *Chlorella vulgaris* و *Arthrospira platensis* على قش الأرز المعالج والذى يتميز بانة مخلف زراعي ، كما انه مصدر رخيص وغني بالكربوهيدرات اللازمة لزراعة تلك الطحالب. تم استخدام قش الأرز المعالج كيميائيا لزراعة *C. vulgaris* و *A. platensis* لإنتاج الإيثانول الحيوي بالطريقة الغير متجانسة . تم معالجة الكتلة الحيوية للطحلبين كيميائياً بحمض الكبريتيك المخفف تركيز 4٪ عند درجة حرارة 121 درجة مئوية في الأوتوكلاف لمدة 90 دقيقة، ثم تلتها المعالجة البيولوجية باستخدام البكتيريا العصوية الرقيقة لمدة 72 ساعة عند درجة حرارة 30 درجة مئوية ودرجة حموضة 4.5 وذلك لزيادة كمية السكريات المختزلة المنتجة. نتج عن تخمر الكتلة الحيوية الطحلبية لكلا الطحلبين بواسطة خميرة من نوع *Saccharomyces cerevisiae* لمدة 72 ساعة ثم التقطير للمحاليل الناتجة من عملية التخمر ، إنتاج الإيثانول الحيوي بنسبة 8.7 ٪ و 2.5 ٪ لكلا من *C. vulgaris* و *A. platensis* على التوالي بعد 24 ساعة عند درجة حرارة 30 درجة مئوية ودرجة حموضة 4.5.