Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Isolation of Different *Clostridium* Isolates for Acetone-Butanol-Ethanol (ABE) Production from *Sargassum sp.*

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Abstract

Macroalgae with several species are an abundant, carbon-neutral renewable resource and rich in carbohydrates which make it suitable for biobutanol production. Recently, due to advantages of biobutanol as a liquid biofuel, it can be used as a substitute for gasoline and diesel. Many researches mentioned that, different *Clostridium* species are able to use fermentable sugars for production of biobutanol from different biomasses through ABE (Acetone-Butanol- Ethanol) fermentation process. In this study the (*Sargassum* sp.) was used as carbon source for biobutanol production. Thirty-three anaerobic mesophilic isolates were isolated on RCM medium from five soil cultivated with different crops. Only thirteen spore forming, mesophilic, anaerobic Clostridium isolates used for the fermentation process. 100 g/L from *Sargassum sp*. were hydrolyzed by sulfuric acid (6 % (v/v)) followed by thermal pretreatment at 121°C for 20 minutes to produce total reducing sugars (24.151 ± 0.273 g/L), which were fermented by Clostridium isolates. The most promising isolate (HG1) which produce the highest butanol level, acetone and ethanol (3.743, 1.401 and 1.031 g/l) respectively was identified according to Butanol dehydrogenases (bdhA) gene analysis as *Clostridium acetobutylicum.* This paper emphasizes the importance of (*Sargassum sp*.) as a renewable feedstock for the biobutanol production.

Keywords**:** Macroalgae; *Sargassum sp*., Acid hydrolysis; Clostridium isolation; ABE fermentation; Biobutanol.

1. Introduction

One of the most negative impacts of the fossil fuel in our life and environment is steep increasing in global energy consumption. Which lead to fast depletion of conventional energy sources **[1]**. So, many researches tend to produce alternative and clean biofuels from suitable substrates. Actually, the liquid biofuels (biobutanol, bioethanol and biodiesel) seem to be a good substitutional of fossil fuels. Biofuels are also called carbon neutral fuels, because the CO2 produced can be reused for growth of biomass **[2,3]**.

Biobutanol is considered a most suitable and sustainable alternative to gasoline and fossil fuel, this due to its advantages as high energy content, less corrosive nature, low volatility and lower affinity to moisture **[4]**. Also, biobutanol can be used without any modification in the engine **[5]**.

The importance of biobutanol production must be taken into account depending on the use of the appropriate feedstock. Macroalgae are recently considered to be a suitable substrate for biobutanol development and have an important role in solving land use and irrigation water problems **[6]**.

In addition, macroalgae are rapidly growing in contrast to other substrates (4–6 harvesting cycles per year). Moreover, seaweed (macroalgae) can grow in the sea. Seaweed biomass also has a high

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Receive Date: 20 February 2020, Revise Date: 17 March 2020, Accept Date: 29 March 2020
DOI: 10.21608/ejchem.2020.24250.2448

carbohydrate content compared to microalgae **[7-9]**. Sargassum species are the most promising biobutanol macroalgae, as can be found on the coast, on offshore platforms and sea-floating. otherwise, they may be grown in artificial ponds. Sargassum sp. are among the most prevalent brown marine macroalgae **[10, 11].**

It's customary, some Clostridium species have the ability to produce Acetone, butanol and ethanol through the ABE anaerobic fermentation process from different types of sugars such as pentoses and hexoses. Most of Solvent-producing clostridial species have fermentation ability of different sugars from different substrates even the lignocellulosic biomass **[12]**. On the other hand, it is necessary to mention that macroalgae composed of different types of sugars which make it easy to use by microorganisms through the ABE fermentation process **[13].**

This paper was directed to (I) collection of brown macroalgae (*Sargassum* sp.) biomass and its utilization as an unexpansive, potentially available substrate for acid hydrolysis and production of the high concentration of sugars (ii) Isolation of native clostridia from different soil cultivated with different crops. (iii) Evaluating the activity of this native isolates for fermentation of sugars in synthetic medium (T6) and also fermentation of reducing sugars obtained from acid hydrolysis of Sargassum sp. biomass (iv) Phylogenetic identification of the highest ABE producing isolate was performed based on Butanol dehydrogenases (bdhA) specific gene analysis (molecular marker gene).

2. Experimental

2.1. Preparation of macroalgae

The brown macroalgae (*Sargassum sp.)* was collected from Hurghada (Red Sea, Egypt) on September (2017). And then were brought to the laboratory in plastic bags. The seaweed samples were washed thoroughly with distilled water to remove the dirt and other adherent materials, then dried in free air at room temperature. The substrate was chipped at 3-5 cm and grinded to obtain a fine homogeneous powder and sieved to constant size (0.60-0.80 mm). The grinded powder substrate was stored in clean plastic containers at room temperature until further analysis and hydrolysis were performed **[14]**.

2.2. Acid hydrolysis of *Sargassum sp.*

One hundred grams of the algal biomass were put into 2500 ml Erlenmeyer flask with 1000 ml of sulfuric acid (6% v/v), then the flasks were autoclaved at 121°C for 20 min. After autoclaving, the flasks were filtrated to separate the solid materials **[15].** Finally, the filtrate was neutralized and the total reducing sugars (TRS) and hydrolysis percent were determined by the 3, 5-dinitrosalicylic acid (DNS) method.

2.3. Collection of soil samples for bacterial isolation

Samples were collected from deep depths of five agricultural areas cultivated with different crops (bean, cucumber, grass, potato, and eggplant) from five locations in Giza Governorate, Egypt during (2018). The samples were transferred directly into clean and sterilized polyethylene bags and directly used for bacterial isolation.

2.4. Media and anaerobic conditions

The chemical composition of the Reinforced Clostridia Medium (RCM, Oxoid) which used for isolation and cultivation of the native bacteria was : 10 peptone; 10 beef extract;3 yeast extract; 1 soluble starch; 5 glucose; 3 sodium acetate; 5 sodium chloride; 0.5 L-cysteine hydrochloride; 2 sodium thioglycolate, the pH was adjusted to 6.8± 0.2 with NaOH **[16].**

The modified T6 broth (6%glucose) was used for the first fermentation and screening ABE production. The chemical composition of T6 modified medium (g/L): 6 tryptone; 2 yeast extract; 0.3 $MgSO_4.7H_2O$; 10 ml $FeSO_4.7H_2O$; 0.5 KH_2PO_4; 3 ammonium acetate; 2 sodium thioglycolate; 0.5 L-cysteine hydrochloride and 60 glucose **[17].** The medium was sterilized and inoculated with 5% inoculum size (OD = 1.5) at 37 °C under anaerobic conditions for 5 days.

Anaerobic conditions were used to isolate and grow bacterial isolates on the liquid RCM medium: soil samples were inoculated in broth medium, covered with sterilized paraffin oil, then transferred into a water bath (70 °C) for 10 min to inactivate vegetative cells. The cultivated anaerobic bacteria were transferred into glass vials containing sterilized liquid media. Nitrogen gasses were added to establish anaerobic conditions by flushing into the medium for 4 minutes before it was inoculated, the vials were sealed with rubber septa and aluminum crimps, then the medium was sterilized by autoclaving at 121 °C and 15 psi for 20 min. Anaerobic conditions for agar plates were achieved by using gas- producing sachets in an anaerobic jar (MGC, AnaeroPak R Anaero- 3.5 L Japan, code 5233LJ-R).

2.5. Isolation and cultivation of Clostridia sp.

According to **Montoya *et al*. [16],** 1 gram of soil was suspended in 10 ml distilled water. 1ml of suspension was transferred to anaerobic broth RCM

medium as mentioned before. The tubes were then incubated for 5 days at 37°C and checked daily for growth and gas production. Five hundred microliters of growing cultures were placed on T6 agar plate's medium supplemented with (6% glucose). To evaluate acetone production one small drop of 5% w/v sodium nitroprusiate and 25% w/v ammonium hydroxide were added, in the presence of acetone, a red–violet ring was formed **[18].** Acetone-positive colonies were purified on RCM medium at 37°C. The Colonies were checked for appearance of spores using spore stain method by phase-contrast microscopy **[19].**

2.6. ABE fermentation:

- ***ABE fermentation of the of bacterial isolates on T6 medium***

5% (v/v) of fresh RCM growing bacterial culture (at 24 h, the optical density OD =1.5) was inoculated into 50 ml bottles containing 40 ml of T6 medium (6% glucose) under anaerobic conditions. After 120 h at 37°C, the concentration of acetone, butanol and ethanol (ABE) in the fermentation broth was evaluated by a gas chromatograph.

- ***ABE production from the fermentation of Sargassum sp. hydrolysate***

The second fermentation medium was the sulfuric acid algal hydrolysate, the reducing sugars obtained from acid hydrolysis of *Sargassum sp*. were used as the carbon source for the fermentation process. After acid hydrolysis and filtration (described before), the pH of the supernatant hydrolysate was adjusted to 6.5 with 2M NaOH and 2M HCl. The components of T6 medium were added to the hydrolysate except glucose, then the pH was adjusted again. The nitrogen gas was used to create anaerobic conditions as described before. The bottles were sterilized and inoculated with 5% inoculum size (OD = 1.5) at 37 °C for 5 days. Finally, after the incubation period, the concentration of acetone-butanol-ethanol were determined to evaluate the ability of this isolates for fermentation of reducing sugars and select the best isolate for further study.

2.7. Identification of the bacterial isolate using Butanol dehydrogenases (bdhA) gene analysis (molecular marker gene).

- ***DNA extraction***

DNA was extracted for total genomic of sample according to manufacturer protocol of Gene JET Genomic DNA Purification Kit (K0721, Thermo Fisher Scientific.

- ***PCR amplification***

Dream Taq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) was used for specific gene amplification according to manufacturer protocol through Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler.

Butanol dehydrogenases bdhA specific gene was selected as molecular marker gene. *Clostridium acetobutylicum* butanol dehydrogenase isozyme A gene (GenBank: M96945.1) was applied as reference gene sequence. Then, Gene script software (https://www.genscript.com) was applied to design suitable complementary primer for butanol dehydrogenases specific gene to amplified specific amplicon with 300 bp. The reaction consists of The PCR conditions was designed with 95° C for 5 min as initial denaturing step followed by 30 cycles of denaturing at 95°C for 1 min and primer annealed at 54 °C for 1 min and elongation at 72°C for 90 s. Finally, extension step at 72° C for 10 min.

- ***Agarose gel electrophoresis and detection of the amplification products***

1.0 % agarose solution was prepared by adding 0.75 g agarose to 50 ml of 1x TBE electrophoresis buffer in 50ml flask. Heating in a microwave oven then dissolved the agarose. The agarose was cooled in 50°C. A comb was inserted in electrophoresis bed and the agarose was poured in it. Great care should be taken during pouring of the agarose to avoid bubbles formation. The gel solidified within 15 min and became cloudy, the electrophoresis apparatus was filled with the electrophoresis buffer and the comb was removed creating 6 or 10 wells for sample application in the presence of DNA ladder (peq GOLD 1 kb DNA-Ladder, Peqlab, VWR) according to manufacturer protocol. Electrodes were connected to the power supply and the later was turned on. It was adjusted at 80 Volts for 100 min. The gel was removed from its bed and transferred to the gel staining tray for staining with Ethidium bromide for 30 min followed by 20 min distain in distilled water.

- ***Data analysis***

Gel documentation system (Geldocit, UVP, England) was applied for data analysis using Totallab analysis software (www.totallab.com,Ver.1.0.1). Positive amplicons with 300 bp were eluted from agarose gel. Resultant PCR products were purified with Micro spin filters and quantities spectrophotometrically. Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea).

2.8. Analytical Methods

Hemicellulose, cellulose, and lignin concentrations in the collected substrate was determined by National Renewable Energy Laboratory methods NREL, USA **[20].** The elemental analysis was carried out in National Research Center. On the other hand, the total reducing sugars (TRS) ware determined by 3, 5-dinitro salicylic acid DNS method according to **Miller [21]**, and glucose was used as standard. The samples were stored in a refrigerator at -18^{o}C until analysis was performed to prevent spoilage by microbes. The concentration of butanol, ethanol and acetone in the culture broth was evaluated by a gas chromatograph (model 6890, Agilent G1530A, USA) equipped with a flame ionization detector (FID) and a 60m×530 µm×5.00 µm capillary column. The oven temperature was held at 50 °C for 5 min and increased at a rate of 10 °C per min until it reached 230 °C. The final temperature was held for 5 min. The injector and detector temperatures were set at 150 °C and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 2 mL min−1. The bacterial isolates growth was monitored by using a UV–Vis spectrophotometer (JENWAY model 6300) at 600 nm (OD600). All experiments were carried out in triplicate, and the reported results are the average.

3. Results and discussion

3.1. The composition of the collected algal substrate

Cellulose and hemicellulose of any substrate is very important, and can affect biobutanol productivity. On the other hand, studying the lignin content also is very important which determined the method of the pretreatment and hydrolysis. In this study, the total carbohydrates were (40.84%) divided into cellulose and hemicellulose (15.41 and 25.43%, respectively) and the lignin content was 6.95%, also protein content was determined as 6.250 % (**table 1).** These results are correlated with an earlier study **Saravanana *et al*. [22]** who found that, brown seaweed (*Sargassum sp*.) has high carbohydrate content (45%) and low protein content (2.5%). On the other hand, **Solarin *et al.* [23]** found that the carbohydrates content in *Sargassum hystrix* was (58.72%). **Obata *et al.* [24]** studied other examples of the brown macroalgae (*Ascophylum nodosum* and *Laminaria digitate*), they found that carbohydrates content was (57.84 and 64.47%) respectively which supported our results that the brown macroalgae have high carbohydrates content. Also, **Jang *et al.* [25]** reported another study on *Laminaria japonica* which considered an example of brown algae, he found that the carbohydrates content (54.5%). On the other hand, **Rajkumer *et al.* [26]** reported the carbohydrates content in brown macroalgae as (*Laminaria sp., Alaria sp., and Sargassum sp.*) were (33.3, 39.8 and 33.0%) respectively. But, the carbohydrates content in the green macroalgae such as (*Ulva sp., Enteromorpha sp. and Monostroma sp.*) were (42.0, 64.9 and 63.9 %) respectively. Also, carbohydrates content in red macroalgae as (*Porphyra sp., Rhodymenia sp. and Gracilaria sp.*) were (45.1, 44.6 and 61.75%).

The data represented in **table (2)** showed the elemental composition of our *Sargassum sp.* such as Phosphate (P), Sodium (Na), Potassium (K), Magnesium (Mg), Cadmium (Cd), Zinc (Zn), Copper (Cu), Nicale (Ni), Iron (Fe) and Lead (Pb) were determined as 0.020, 2.030, 5.800, 1.320, 0.000, 0.048, 0.001, 0.004, 0.137, and 0.003 ppm, respectively. The order of element composition is K>Na>Mg>Fe>Zn>P>Ni>Pb>Cu>Cd, this data showed that the lowest and the highest concentration of this elements were Cd and K respectively.

As reported by **Murugaiyan and Narasimman [27],** who study the element composition in two brown macroalgae *Sargassum longifolium* and *Turbinaria conoides*. The elemental composition in *Sargassum longifolium* is in the order Mg>Fe> Ni> Cu> Zn>Pb >Cd where as in *Turbinaria conoides* the elemental composition is in the order Mg>Fe >Pb>Zn> Cu>Ni> Cd. In addition, **Indegaard and Minsaas [28]** mentioned that, there are trace elements present in the algal biomass which considered as heavy metals such as (Cd, Cu, Pb, Zn).

Our results significantly proved that, the brown macroalgae (*Sargassum sp*.) have high carbohydrates content compared with lignin content which make it easy for pretreatment, hydrolysis and production of high total reducing sugars. So that, it can be considered as the best biomass for butanol production.

3.2. Acid hydrolysis

Our *Sargassum sp*. biomass was hydrolyzed by sulfuric acid (6% (v/v)) followed by thermal pretreatment at 121°C for 20 minutes, the converted reducing sugars were estimated by the DNS method. The concentration of reducing sugars were 24.151 ± 0.273 g/L (242 mg/g biomass) and the percentage conversion of carbohydrates (cellulose and hemicellulose) sugars represented as (≈ 60%). On the other hand, **Ventura *et al*. [14]** found that the reducing sugars released from Sargassum *fulvellum* by using 0.15 M H_2SO_4 followed by thermal pretreatment at 121°C for 1 h using 100 g/L of the hydrolysate was (8.86). While **Potts *et al.* [29]** found that by using1% sulfuric acid at 70 min of hydrolysis the reducing sugars in *Ulva lactuca* obtained an

average of 15.2 g/L of reducing sugars. Finally, our results proved that sulfuric acid hydrolysis was effective method for releasing high concentration of reducing sugars. These results agreed with **Jang *et al*. [25]** who reported that, the seaweed carbohydrates from all three classes of macroalgae (brown, red, and green) can be effectively hydrolyzed to monosaccharides by dilute H_2SO_4 treatment at high temperature.

3.3. Isolation of Clostridium from agricultural soils cultivated with different crops.

Thirty-three anaerobic mesophilic isolates were isolated on RCM medium from five soil cultivated with different crops. There is a relation between the kind of crops and the number of isolated bacteria as showed in **table (3).** The maximum number of the isolates obtained from grass (30%) followed by potato (27%) while other soils cultivated with cucumber and bean gave low bacterial isolates (18% and 15%) respectively. This result agreed with **Montoya *et al.* [16]** who reported that, 178 solvent producing anaerobic bacteria could be purified from 155 different soil samples of agricultural areas, by referring to **table (3),** the potato crops also showed high bacterial isolates (27%). which also supported by **Berezina *et al.* [30],** who isolated 48 ABE producing clostridial isolates from 52 samples of potato slit and rye grain. Furthermore, similar features have been reported by **Nazari [31]** who found that**,** Potatoes and the roots of nitrogen-fixing legumes have been reported to be excellent material for the isolation of these bacteria. According to the data showed in table (3), 55% (18) of bacterial isolates which produce gas, but only thirteen of these isolates produced acetone on T6 medium (HB1, HB2 HB3, HC2, HC4, HC5, HC6, HG1, HG2 HG9, HP1, HE2 and HE3). These acetone –positive isolates were preliminary identified as Clostridium according to Gram and Endospore stains **[32]**. So that, these thirteen isolates were selected for further studies.

Table 1. The chemical composition of collected brown macroalgae *(Sargassum sp.)* as the percentage for the dray mater.

Chemical composition of *Sargassum sp.*	Unit %
Cellulose	15.41
Hemicellulose	25.43
Lignin	6.95
Protein	6.25

Table 2. Minerals composition of collected brown macroalgae *(Sargassum sp.)*

Minerals composition of *Sargassum sp.*	ppm
P	0.020
In	2.030
K	5.800
Mg	1.320
Cd	0.000
Zn	0.048
Cu	0.001
Or	0.004
Fe	0.137
Pb	0.003

Table 3. Isolation of Clostridium isolates from different soils cultivated with different crops.

Sample number	Crop name	Number of isolates	Name of isolates	Percent%	Gas production	Acetone test
1	Bean	5	HB1	15	+	+
			HB2		+	+
			HB3		+	+
			HB4		+	-
			HB5		-	-
2	Cucumber	6	HC1	18	-	-
			HC2		+	+
			HC3		-	-
			HC4		+	+
			HC5		+	+
			HC6		+	+
3	Grass	10	HG1	30	+	+
			HG2		+	+
			HG3		-	-
			HG4		-	-
			HG5		-	-
			HG6		-	-
			HG7		-	-
			HG8		+	-
			HG9		+	+
			HG10		+	-
4	Potato	9	HP1	27	+	+
			HP2		+	-
			HP3		+	-
			HP4		-	-
			HP5		-	-
			HP6		-	-
			HP7		-	-
			HP8		-	-
			HP9		-	-
5		3	HE1	9	-	-
	Egg plant		HE2		+	+
			HE3		+	+
Total	5	33			18	13

3.4. ABE production from T6 medium by clostridium isolates.

Only thirteen isolates from the thirty-three isolates are able to produce a positive result with acetone test **(table 3)** were selected as spore forming, mesophilic, anaerobic Clostridium isolates for ABE production on T6 medium **(table 4)**, thirteen isolates (HB1,HB2,HB3, HC2,HC4,HC5, HC6, HG1,HG2, HG9, HP1, HE2, HE3) showed the ability to produce ABE with rate ranging between 0.353 to 8.604 g/L on the T6 medium. The maximum Acetone-butanol - ethanol concentration obtained by HG1 were 2.545, 4.471, and 1.588 g/L, respectively. Followed by the two isolates (HC2 and HC6) produced acetone (2.700 and 2.227 g/L), butanol (3.906 and 3.425 g/L) and ethanol (1.206 and 1.1 g/L) respectively. Finally, the three bacterial isolates (HB3, HC5 and HP1) were the lowest total solvent producing bacterial isolates (1.145, 0.392 and 0.353 g/L) respectively**. Salaeh *et al.* [33]** reported that, the maximum biobutanol produced was (2.55 g/L) and the total ABE were (3.65 g/L) on TYA medium which its composition similar to T6 medium by using *Clostridium beijerinckii* TISTR 1461. On the other hand, **Montoya *et al.* [16]** mentioned that, the biobutanol produced on T6 medium from different clostridium isolates from different crops ranged from 1.1 to 11.4 g/L. Similarly, **Abd-Alla *et al*. [32]** tested different spices of clostridium on T6 medium, he found that the maximum biobutanol produced from the isolates *C. roseum* ASU58 (16.77 g/L) followed by *C. beijerinckii* ASU10 (15.66 g/L), then *C. chauvoei* ASU55 (12.92 g/L) after 8 days. While *C. acetobutylicum* ATCC 824 produced 6.463 g/L and used as reference strain. From pervious data we found that different bacterial species produce different biobutanol concentration.

3.5. ABE production from fermentation of algal hydrolysate by clostridium isolates.

The thirteen-spore forming, mesophilic, anaerobic Clostridium isolates were used also for the fermentation of reducing sugars produced by acid hydrolysis of *Sargassum sp*., and we compared the concentration of acetone-butanol- ethanol produced from the fermentation of T6 medium (6% glucose) and algal hydrolysate (2.4% sugar). 10% vigorously growing Clostridium culture was inoculated in 50 ml bottle containing 40 ml of prepared sterilized algal hydrolysate. The bottles were incubated under anaerobic conditions at 37°C for 5 days. The concentration of acetone, ethanol, butanol was evaluated using GC apparatus. The maximum butanol produced from our algal hydrolysate was 3.743 g/L and the total solvents were 6.175 g/L by utilization of HG1 **(table 5)**. This clostridium isolate (HG1) followed by three isolates (HB1, HC2 and HC6) produced butanol (2.435, 2.240, and 2.414 g/L) and total solvents (4.088, 4.641, and 4.729 g/L) respectively. On the other hand, the two isolates (HC5 and HP1) showed no butanol and ethanol production from the fermentation of algal hydrolysate. The result recorded in table **(4 and 5)** agreed with **Ventura *et al.* [14]** who illustrated that, the consumption of the macroalgal biomass as substrate was slower than the more reduced substrate such as glucose **Tashiro *et al*. [34]** which lead to low biobutanol production. Also, **Ventura *et al.* [14]** reported that the *Sargassum. fulvellum* produced 1.76 g/L butanol and 3.14 g/L total solvent by using *Clostridium saccharoperbutylacetonicum* N1-4, this result agreed with our data. On the other hand, **Potts *et al*. [29]** produce biobutanol concentration from *U. lactuca* (green macroalgae) in the fermentation broth reached about 4 g/L. Moreover, our results agreed with **Salaeha *et al.* [33],** who also studied the biobutanol production from *Rhizoclonium* macroalgae (red macroalgae), the maximum biobutanol produced was 2.43 g/L and the total ABE were 4.57 g/L. finally, the results recorded in **tables 4 and 5** illustrated that, the native isolate HG1 produced the maximum acetone- butanol- ethanol from the fermentation of T6 medium and also from algal hydrolysate. So that this isolate was selected as the best isolate for fermentation and production of acetone- butanol- ethanol.

Table 4. ABE production by the bacterial isolates on T6 medium after five days.

Bacterial isolates	Solvent production (g\L)			
	Acetone	Butanol	Ethanol	ABE
HB1	1.26	3.440	0.526	5.226
HB2	1.357	2.014	0.958	4.329
HB3	1.001	0.144	0	1.145
HC2	2.700	3.906	1.206	7.812
HC4	0.347	1.247	0.147	1.741
HC5	0.247	0.145	0	0.392
HC6	2.227	3.425	1.1	6.752
HG1	2.545	4.471	1.588	8.604
HG2	0.057	1.258	0.025	1.340
HG9	1.587	2.358	1.072	5.017
HP1	0.132	0.221	0	0.353
HE2	1.532	1.025	0.147	2.704
HE3	1.725	2.048	1.258	5.031

Table 5. ABE production from the algal hydrolysate after the five days of the fermentation by the bacterial isolates.

Bacterial isolates	Solvent production (g\L)			
	Acetone	Butanol	Ethanol	ABE
HB1	0.642	2.435	1.011	4.088
HB2	0.871	1.908	0.542	3.321
HB3	0.221	0.582	0.105	0.908
HC2	1.8	2.24	0.601	4.641
HC4	0.541	0.874	0.327	1.742
HC5	0.11	0	0	0.11
HC6	1.357	2.414	0.958	4.729
HG1	1.401	3.743	1.031	6.175
HG2	0.432	0.631	0.115	1.178
HG9	1.298	1.74	0.917	3.955
HP1	0.053	0	0	0.053
HE2	0.51	0.891	0.21	1.611
HE3	1.11	1.901	0.895	3.906

3.6. Identification of the bacterial isolate.

The highest ABE producing clostridium isolate (HG1) were characterized based on some morphological and physiological tests. The shape of this isolate on T6 agar medium was opaque, raised, irregular circular margin, brownish center colony at mesophilic temperature. The cells were Gram-positive and endospore former.

This best clostridium isolate (HG1) was identified by Technical Support Department, Delta Scientific Consultancy Center (DSCC). Butanol dehydrogenase (bdhA) specific gene was selected as molecular marker gene for identification. The butanol dehydrogenase (bdhA) is involved in the final step of the butanol formation pathway **[35, 36]** so that it is used as marker gene for identification. The isolate was characterized by bdhA via amplification and sequencing. Suitable complementary primer for butanol dehydrogenases specific gene was design to amplified specific amplicon with 300 bp **(Table 6).** The gene fragment was amplified, the successful PCR amplification of the bdhA fragment was checked by agarose gel electrophoresis **(Fig. 1).** The amplified product was purified and its nucleotide sequence was determined. The obtained sequence was illustrated in **Fig. (2).** this sequence was compared with other related sequences available in GenBank. Alignment in Gen Bank (Blast) indicated that the isolate HG1 was 100% similar with *Clostridium acetobutylicum* butanol dehydrogenase isozyme A gene M96945.1. A phylogenetic tree was constructed from multiple sequence alignment of gene sequences **(Fig. 3).** The nucleotide sequences of bdhA gene of the bacterial isolate HG1 was determined and recorded in GenBank as *Clostridium acetobutylicum* with accession number MN871409.

Table 6. Primer design for gene sequence under study.

Name	Sequence	Position	Tem. °C
SEQ_L1	AGCAATAGGGGGAGGAAGTGC	279	58.68
SEQ_R1	GCTGTTCCCGCTGCTGTTTG	578	59.86

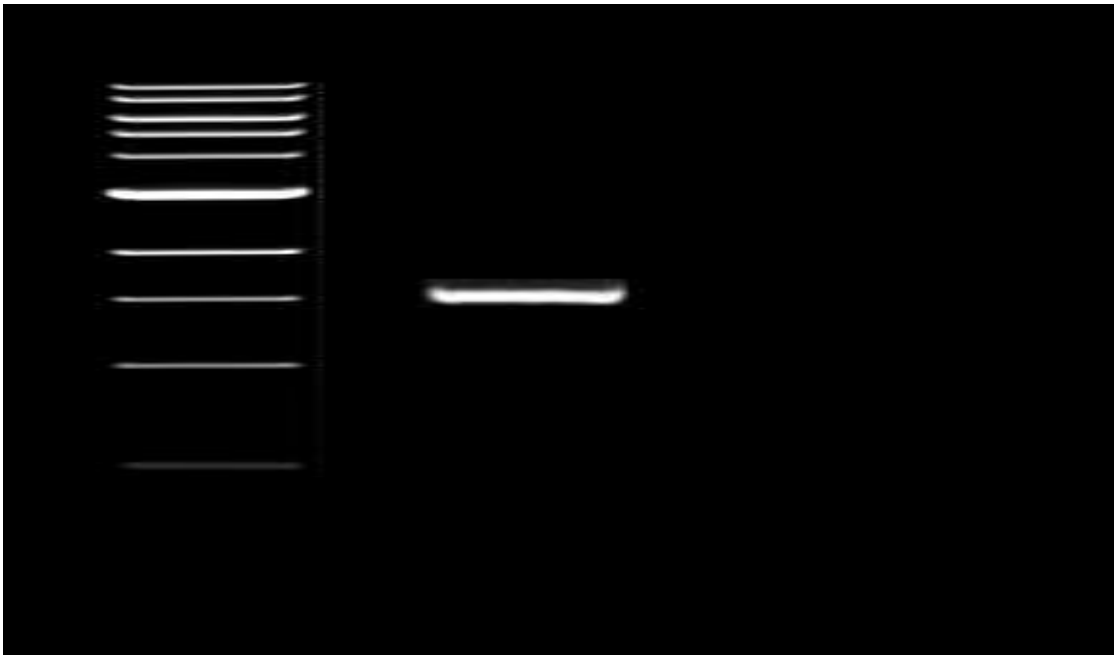

Fig. 1. Specific genomic products for Clostridium acetobutylicum butanol dehydrogenase isozyme A gene (~300 bp).

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>14723_1;
      AATAGGGGGA GGAAGTGCAA TAGACTGTTC TAAGGTAATT GCAGCTGGAG TTTATTATGA      60
      TGGCGATACA TGGGACATGG TTAAAGATCC ATCTAAAATA ACTAAAGTTC TTCCAATTGC     120
      AAGTATACTT ACTCTTTCAG CAACAGGGTC TGAAATGGAT CAAATTGCAG TAATTTCAAA     180
      TATGGAGACT AATGAAAAGC TTGGAGTAGG ACATGATGAT ATGAGACCTA AATTTTCAGT     240
      GTTAGATCCT ACATATACTT TTACAGTACC TAAAAATCAA ACAGCAGCGG                290
```

Fig. 2. Butanol dehydrogenase gene sequence.

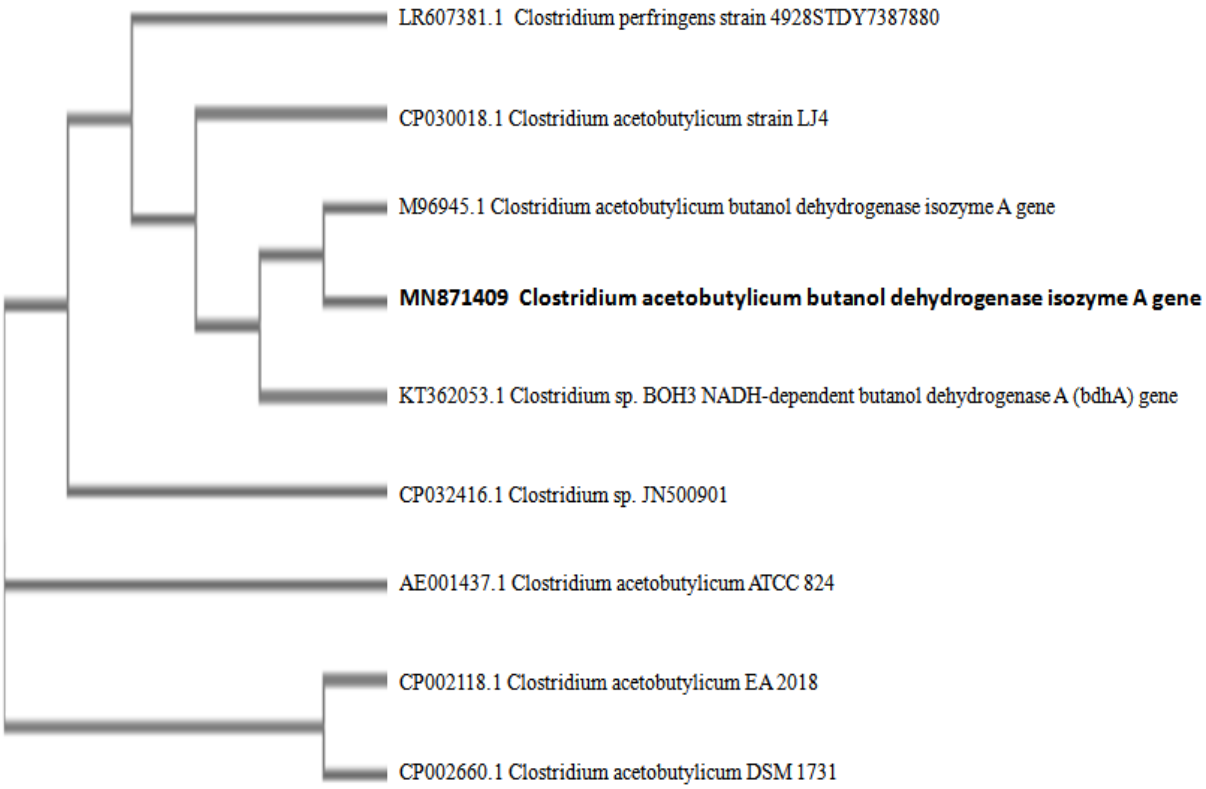

Fig. 3. phylogenetic tree for sample based on butanol dehydrogenase gene sequence.

4. Conclusion.

Thirty-three anaerobic and mesophilic isolates were isolated on RCM medium from five soil cultivated with different crops. The maximum number of the isolates obtained from soil cultivated with grass (30%) followed by potato (27%) while other soils cultivated with cucumber and bean gave low bacterial isolates (18% and 15%) respectively. Only thirteen isolates from the thirty-three isolates are endospore forming, gram positive Clostridium isolates. These isolates have the ability to ferment the glucose in T6 for production of acetone- butanol-ethanol with different range.

The *Sargassum sp*. biomass was hydrolyzed by sulfuric acid (6% (v/v)) followed by thermal pretreatment at 121°C for 20 minutes. The concentration of reducing sugars were 24.151 ± 0.273 g/L (242 mg/g biomass). The thirteen Clostridium isolates were utilized for the fermentation of reducing sugar obtained from sulfuric acid hydrolysis of *Sargassum sp*. biomass. The maximum butanol produced from our algal hydrolysate was ≈4 g/L (166 mg/g sugar) and the total solvents were ≈ 6 g/L (250 mg/g sugar) by utilization of HG1 for fermentation. This isolate was identified by Butanol dehydrogenase (bdhA) specific gene sequencing as *Clostridium acetobutylicum*.

Commercially, the utilization of algal biomass as available and cheap carbon source for biobutanol production by clostridium isolates reduced the cost of fermentation. Although the production of butanol is lower than expected, but the amount of butanol should be increased by improving conditions for the fermentation process.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgements

The authors are appreciative to Egyptian Petroleum Research Institute (EPRI) for providing all the facilities to achieve this work.

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