Biobeneficial Spectrum of Halophyte Plant *Avicennia marina* as a Second Generation of Bioethanol Production

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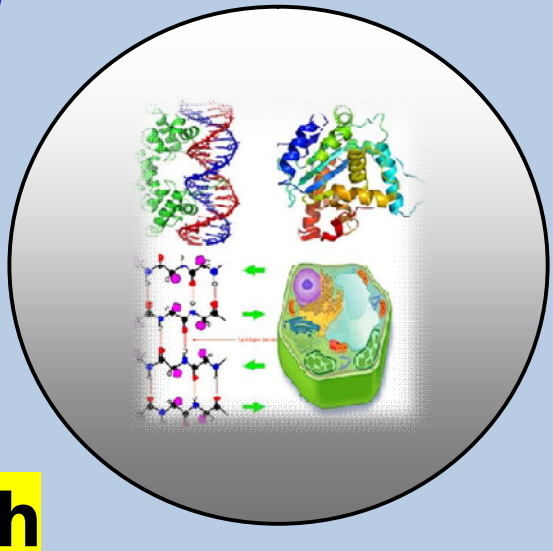

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RESEARCH PAPER

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Biobeneficial Spectrum of Halophyte Plant *Avicennia marina* as a Second Generation of Bioethanol Production

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ABSTRACT

Bioethanol production from cellulosic materials may offer a solution to some of the recent environmental, economic and energy problems facing in the world, therefore the search for renewable sources of fuel is very necessary for the progress of countries. The aim of this study is to optimize the hydrolysis of lignocellulosic materials of Avicennia marina as the basal fermentation medium for bioethanol production with using Saccharomyces cerevisiae. Hydrolysis of lignocellulosic of A. marina with using sulphuric acid, nitric acid and their mixture giving reducing sugars 3.9, 5.2 and 4.9 g/L respectively. Nitric acid was the optimum acid for hydrolysis and their optimum concentration was 5% which giving 9.9 g/L reducing sugar. Reducing sugars yields were 20.1 and 30.6 g/L at heating temperature 90 ℃ and heating time 60 min. respectively. Ethanol production increased with increasing reducing sugar content, the optimum yield obtained was 11.64 g/L at 30 g/L sugar. Finally, the maximum yield of bioethanol obtained was 12.67, 12.52, 13.86 and 15.76 g/L under optimum conditions of an inoculums size (2 ml), incubation period (72 h), pH (6) and temperature (30 ℃) respectively.

Key words: Avicennia marina, Biobeneficial, Bioethanol Production and Second Generation.

INTRUDUCTION

Biofuels represent an alternative to petroleum-based fuel; in particular, bioethanol is the most widely used biofuel for transportation (Balat, 2010). Bio-ethanol is an important chemical product with emerging potential as a bio-fuel as safe alternate to fossil fuels. Bio-ethanol can be produced by fermentation of agricultural products (Rakin *et al*., 2009).

Ethanol can be made of three main, abundant renewable feedstock sources, saccharines, starch materials and cellulosic materials (Kumar *et al*., 2005).

First generation biofuels are often criticised due to their potential adverse impact on food prices (Reuters, 2008). To ease the conflict between "my tank and your plate" second generation biofuels like lingocellulosic bioethanol are promoted (Bmelv, 2008). The second-generation biofuels (*Biomass to Liquid*) are made from organic materials, such as straw, wood residues, agricultural residues, reclaimed wood, sawdust, and low-value timber. The second category is much more inexpensive and because it is a waste material, it is more ethical to use for bioethanol production as compared to the first category. Lignocellulose biomass, including wood waste, agricultural waste, household waste, etc. represents a renewable resource on earth which has stored solar energy in its chemical bonds (McKendry, 2002). It has great potential for bioethanol production, when compared to ethanol produced from grain, tubers and sugar plants, because it is a widely available cheap feedstock which does not compete with human food products (Lynd *et al.*, 1991). Zhang *et al*. (2007) reported that the cellulosic materials are renewable natural biological resources which can be used for the production of bio-fuels.

Mangroves are woody trees and shrubs that grow in the intertidal zones of tropical and sub-tropical regions (Duke, 1992). Mangrove forests have been utilized for many functions including wood production, firewood and charcoal (Tomlinson, 1994). However, wood-related activities or industries based on lingo-cellulose as a manufacturing substrate are very destructive and the rates of mangrove renewal do not match this at all (Kairo *et al*., 2001). Plant cell walls are the most abundant renewable source of fermentable sugars on earth (Saleem *et al*., 2008). The major components of plant cell walls are cellulose, hemi cellulose and lignin, with cellulose being the most abundant component (Han *et al*., 2003). Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemi cellulose by dry weight (Sa-Pereira *et al*., 2003). In recent years, much work has been carried out towards efficient utilization of agricultural residues and forest residues to produce bio-fuels by microorganism (Gnansounou and Dauriat 2010; Wang *et al*., 2011).

According to studies of Lee (1997) and Minhee *et al*. (2011) the biological activities for converting plant-derived lignocellulose to bio-ethanol requires digestion to produce hemicellulose and cellulose, depolymerization of carbohydrate to liberate free monosaccharides and fermentation of mixed pentose and hexose to produce ethanol. Biological and acid treatments of the lignocellulosic waste materials improve the bioethanol production by *Saccharomyces* sp. (Lei *et al*., 2012). In many scientific papers, nitric acid and phosphoric acid at different concentrations and temperature were used to digest bagasse of sugar can (Sun and Cheng, 2004).

The yeast *S. cerevisiae* is the potential yeast for the ethanol production from lignocellulose against toxic chemicals present in the substrate (Tomas *et al*., 2010). *S. cerevisiae* is widely used for the production of alcohol and ethanol fuels through a fermentation process that converting sugar into ethanol.

This microbe can produce a lot of ethanol and has a high tolerance to ethanol and other growth inhibitors compounds (Balat *et al*., 2008; Jasman *et al*., 2013; Saravanakumar *et al*., 2013; Zhenglin *et al*., 2014). Also, Jeffries (2006) stated that, *S. cerevisiae* due to their cell structure, high growth rate under stress conditions of environmental and nutritional factors and greater resistance to contamination are advantageous over other microorganisms for commercial production of bio-ethanol. The aims of this present study were to investigate the use of lignocellulosic materials of *A. marina* in production of bioethanol using *S. cerevisiae* and to optimize the process conditions for enhanced bioethanol production.

MATERIAL AND METHODS

Substrate Employed and acid hydrolysis

Shoot system including stems and leaves of *Avicennia marina* was collected from the muddy coastal area of Jazan, KSA. Collected shoots of *A. marina* were washed with distilled water and air dried for 24 h. then oven dried at 60°C until constant weight. Dry plant parts were grind in an electrical grinder. The dried substrate was then delignified by treating with sulphuric and nitric acids at 3% and mixture of these acids, then the reducing sugar yields were estimated. Effect of different concentrations, heating temperature and heating time of the optimum acid on reducing sugar yields were estimated. NaOH (0.5 M) was then used to neutralize the acidic hemicelluloses hydrolyzate and lignin precipitates were removed by filtration through Whatman filter paper.The pre-treated cellulosic residue was then washed with distilled water to remove residual acid.

Microorganism and their cultural conditions

Saccharomyces cerevisiae (product of France) was colected from market in KSA. One gram commercial lyophilized baker`s yeast (*S. cerevisiae*) was dissolved in100 ml 2% sterile sucrose solution under aseptic conditions then sub-cultured on YEPD agar (1% Yeast extract, 2% Peptone, 2% Glucose, and 2% Agar (Atlas, 2004) plates and incubated at 25°C for 72 h. Single colony was inoculated into 50 ml of YEPD medium in a 250 ml Erlenmeyer flask that were incubated in a shaking incubator at 25°C and 200 rpm for 24 h and then used in fermentation processes. Growth was measured by Optical Density (O.D.) at wavelength 600 nm.

Optimization of fermentation conditions (Osman *et al.,* 2011)

Different optimized conditions were applied in the fermentation processe. Each of optimized condition in the experiment was applied in the nexet experiment as in the following:

Reducing sugars concentration: Different concentrations of mangrove hydrolysate representing different reducing sugars concentrations (5,10, 15, 20, 25, and 30g/l) were prepared as fermentation medium in 250 ml Erlenmeyer conical flasks each contained 100 ml and inoculated with 1 ml yeast culture, and incubated in a shaking incubator at 25°C and 200 rpm for 72 h.

Inoculum size: Fermentation media (pH 7) with 30 g/l reducing sugars were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with different volumes (0.5, 1, 1.5, 2, and 2.5 ml) yeast culture (initial concentration of yeast culture is $7x10^7$ cell ml^{-1}), and incubated in a shaking incubator at 30°C and 200 rpm for 48 h.

Incubation period: Fermentation media contain 30 g/l reducing sugars and pH 7 were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with 1.5 ml yeast culture, and incubated in a shaking incubator at 30°C and 200 rpm for different periods (12, 24, 36, 48, 72, and 84 h.).

Initial pH Value: Fermentation media contain 30 g/l reducing sugars were prepared at different pH values (3,4,5,6,7,8, and 9 pH). Erlenmeyer conical flasks (250 ml) each contained 100 ml media were inoculated with 1.5 ml of yeast culture, and incubated in a shaking incubator at 30°C and 200 rpm for 72 h.

Temperature: Fermentation media contain 30 g/l reducing sugars and pH 6 were prepared. Erlenmeyer conical flasks (250 ml) each contained 100 ml were inoculated with 1.5 ml yeast culture, and incubated in a shaking incubator at 200 rpm and different temperatures (20, 25, 30, 35, and 40°C) for 72 h.

Estimation of reducing sugars: The Dinitro salicylic (DNS) method of Miller (1959) was used to estimate reducing sugars.

Quantitative estimation of ethanol by potassium dichromate method

One ml of the fermented wash was taken in 500ml pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulfuric acid and volume raised to 1 liter). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5°C for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50 ml. Five ml of this was diluted with 5ml of distilled water for measuring the optical density at 600 nm using a Uvikon Xs/60/99-90289 spectrophotometer. Astandard curve was prepared under similar set conditions by using standard solution of ethanol containing 2 to 12% (v/v) ethanol in distilled water. Ethanol content of each sample was estimated and graph was made.

Statistical analysis

The data obtained were subjected to statistical analysis according to the procedure outlined by Sendecor and Cochran (1981) and the means were compared using Duncan`s multiple range test (Duncan, 1988).

RESULTS AND DISCUSSION

In the past few years, the environmental issues, increasing of world oil prices and shortage of fossil fuels are the main reasons which are responsible for search of alternative energy sources, which are renewable cost effective and environmentally safe.

The ethanol production from raw materials of agricultural origin, with lower price, is the aim of most of the studies in the recent years, performed in many laboratories. In our experiments, *A. marina* was used as raw material.

The hydrolysis of lignocellulosic of *A. marina* for maximum extraction of reducing sugars using sulphuric acid, nitric acid and their mixture was investigated (Fig.1), the obtained reducing sugars was 3.9, 5.2 and 4.9 g/L respectively. In the current study, nitric acid was significant highly effective in delignifying of the biomass compared with sulphuric. Moiser *et al*. (2005) reported higher hydrolysis yield from lignocellulose pretreated with diluted H_2SO_4 compared to other acids.

The bioconversion of lignocellulosic materials to bioethanol produces high yield of glucose after hydrolysis (McMillan, 1994, Yasuyuki *et al*., 2011). An experiment was carried out by (Shafizadeh, 1977) revealed that maximum amount of reducing sugars (80-85%) was obtained with using H_2SO_4 from hydrolysis of cellulose. A different concentration of nitric acid was also carried out for detection the optimum concentration of hydrolysis, where reducing sugar increased with increasing acid concentration (Fig. 2). Non significant increase in reducing sugar content (9.9 and 9.7 g/L) at concentration 5 and 6% of nitric acid respectively. According to Rodriguez-Chong *et al*. (2004), 6% HNO_3 was the optimal concentration for hydrolysis of sugar cane bagasse. Various factors influence yields of monomer sugars from lignocellulose. Generally, biological and acid treatments of the lignocellulosic waste materials improve the bioethanol production by Saccharomyces sp. (Saritha *et al.*, 2011).

Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material (Taherzadeh and Karimi 2007). Concerning the effect of heating at different temperature, the obtained results showed that reducing sugars yields increased with increasing temperature up to 90°C (Fig. 3) with the applied optimum concentration 5% of nitric acid compared to other nitrogen sources. This is due to the fact that high temperature increases hemicelluloses degradation and lignin transformation, thus increasing the potential of cellulose hydrolysis (Li *et al*., 2009).

According to Rodriguez-Chong *et al*. (2004), 122 °C was the optimal temperature for hydrolysis of sugar cane bagasse with using 6% HNO_3.

At different heating time the reducing sugars yields are not significantly different especially at 60, 90 and 120 min where it was 30.6, 30.0, and 29.8 g/L respectively (Fig. 4). On the other hand, reducing sugars yields (22.1 g/L) decreased significantly when the biomass hydrolyzed at heating for 30 min. with the applied optimum condition of hydrolysis.

It can be observed that bioethanol yields were significantly increased with increasing the content of reducing sugars and they spend 3.97, 6.01, 8.84 and 11.64 g/L with sugar content 15, 20, 25 and 30g/L. respectively (Fig. 5). Thus, the amount of reducing sugars has a significant role in the bioethanol production. Bioethanol production from the lignocellulosic waste materials depends on their treatment for removal of the hemicelluloses and lignin from the biomass for the production of monosugars such as glucose. This is a challenge to the current researchers on the renewable fuel production (Meinita *et al*., 2012).

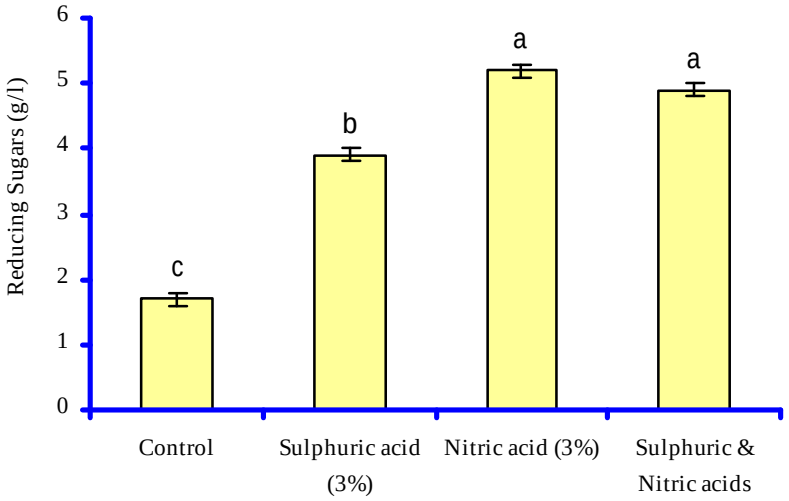

Fig. 1 Effect of different of acids and their mixture on reducing sugars yeilds. Means followed by the same letter are not significantly different.

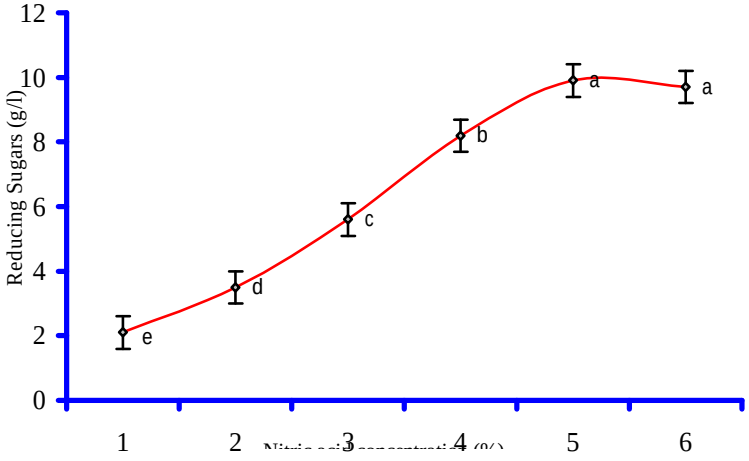

Fig. 2 Effect of different concentration of nitric acid on reducing sugars yields Means followed by the same letter are not significantly different.

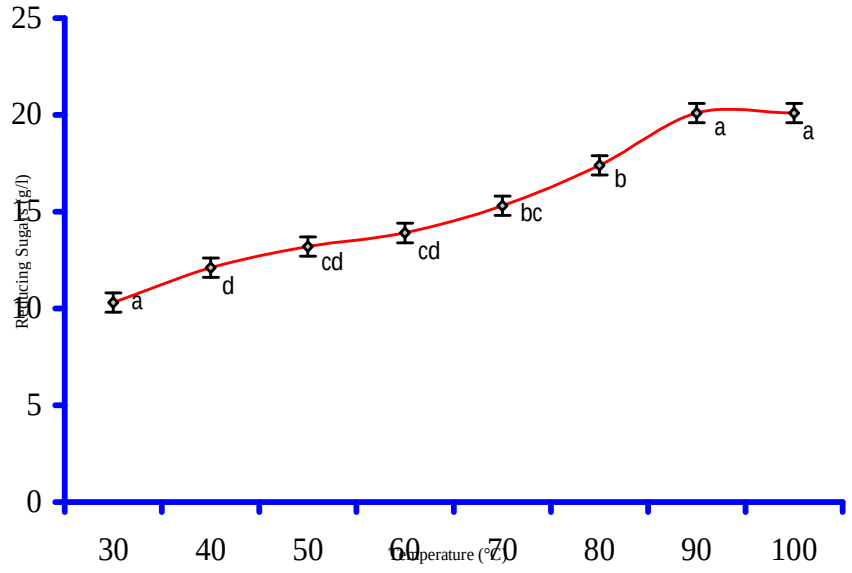

Fig. 3 Effect of different temperature on reducing sugars yields. Means followed by the same letter are not significantly different.

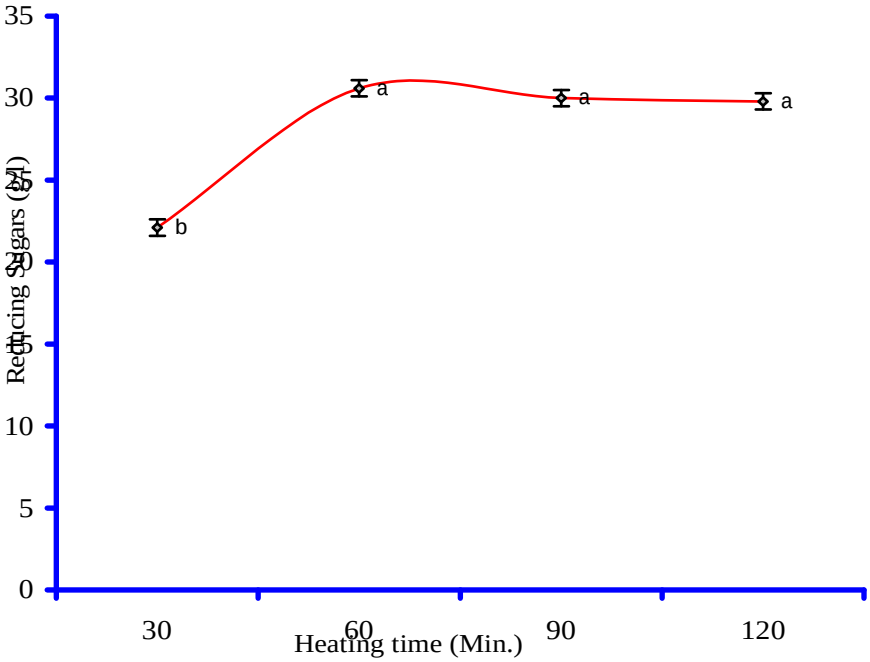

Fig. 4 Effect of different heating time on reducing sugars yields. Means followed by the same letter are not significantly different.

On the other hand, the ethanol content increase gradually as the amount of inoculums used increases to stabilize beyond 2.0 ml. Thus, the ethanol content obtained reached value of 12.67 g/L using inoculums greater than or equal to 2.0 ml. The weakest ethanol content i.e., 9.40 g/L was obtained with an inoculums level of 0.5 ml (Fig. 6). Our finding is an agreement with those obtained by Acourene and Ammouche (2010) with using Baker's yeast. The ethanol content evolve gradually during the fermentation, reaching to 4.75 g/L in 12 h and then 8.60 g/L in 24 h to stabilize at 12.52 g/L beyond 72 h (Fig. 7). The optimum fermentation period for maximum ethanol production is 72 h. Our finding is an agreement with other results (Kongkiattikajorn *et al*., 2007; Acourene and Ammouche, 2010). Recently, Zhenglin *et al*., (2014) reported that under all process conditions, the ethanol concentration became constant after 72 h fermentation. Marine strain of *S. cerevisiae* showed the maximum bioethanol production of 69.58% under the optimal conditions of temperature (30 °C) at 89 h of incubation (Saravanakumar *et al*., 2013). Bioethanol production by *S. cerevisiae* is very sensitive to initial pH of fermentation medium. The maintenance of favourable pH is very essential for the successful fermentation of bioethanol. The obtained result shows that the maximum bioethanol yield (13.68 g/L) was achieved when the initial pH of fermentation medium was kept at 6 (Fig.8). Okeh *et al*. (2014) found that initial pH 7 gave the maximum biogas yield 357 mL/day. The present study attained significant maximum bioethanol production of 15.78 g/L under the optimized conditions of temperature at 30 °C (Fig. 9), the ethanol yield decreased significantly with the increase of temperature.This might be caused by the decrease in the yeast activity. Ethanol yields with using *S. cerevisiae* during fermentation period showed higher values at 30°C compared to that at 33°C (Sulieman *et al*., 2013). Although, Zhenglin *et al*., (2014) stated that the fermentation temperature had no significant effect on the ethanol yield by *S. cerevisiae* and final ethanol concentration, could be set at 35°C to achieve the maximum fermentation rate.

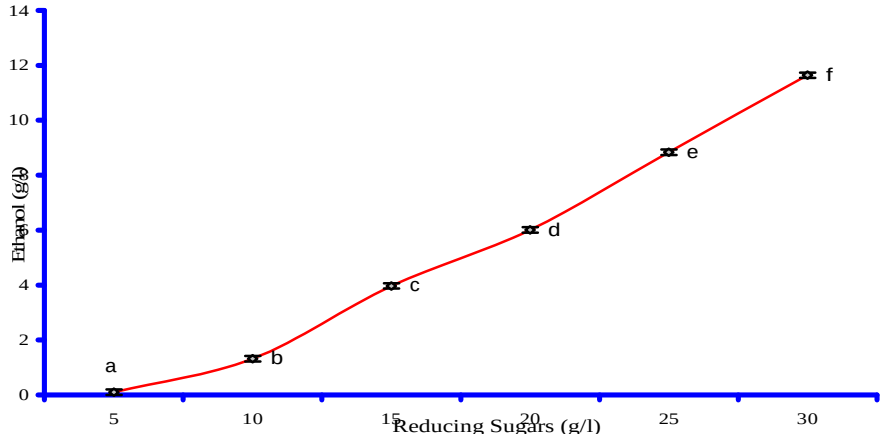

Fig.5 Effect of reducing sugars content on boiethanol production. Means followed by the same letter are not significantly different.

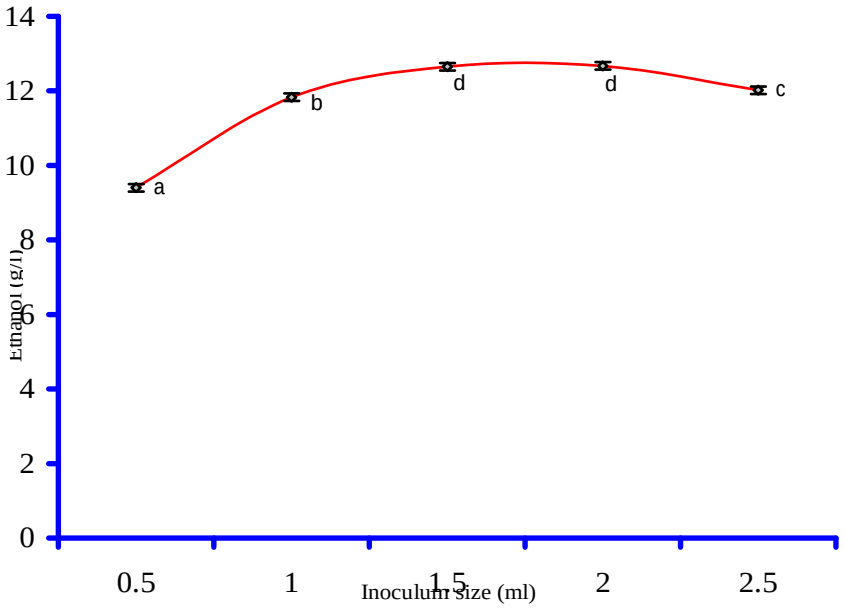

Fig.6 Effect of inoculums size on bioethanol production. Means followed by the same letter are not significantly different.

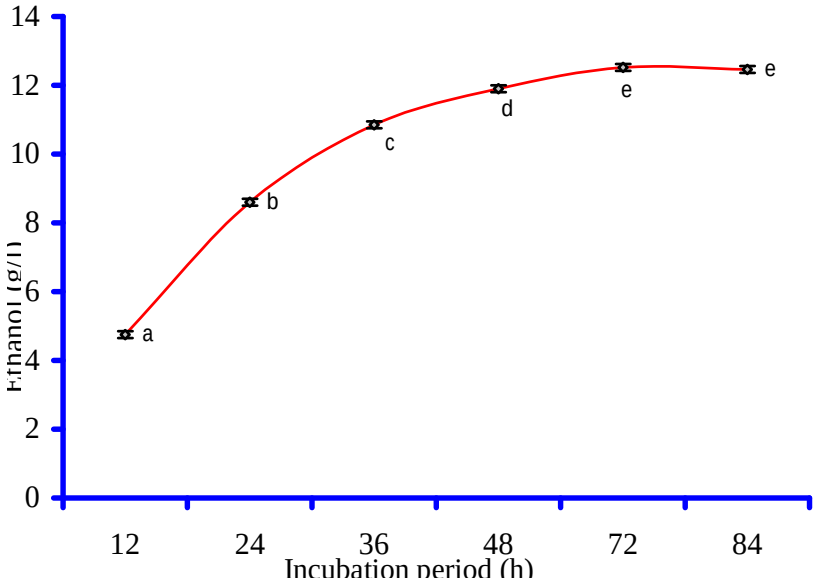

Fig.7 Effect of incubation period on bioethanol production. Means followed by the same letter are not significantly different.

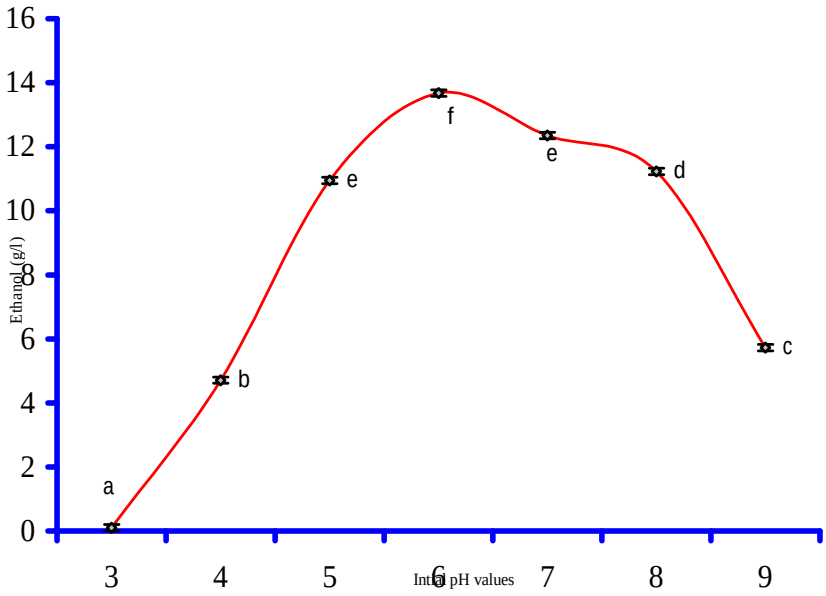

Fig.8 Effect of pH on bioethanol production. Means followed by the same letter are not significantly different.

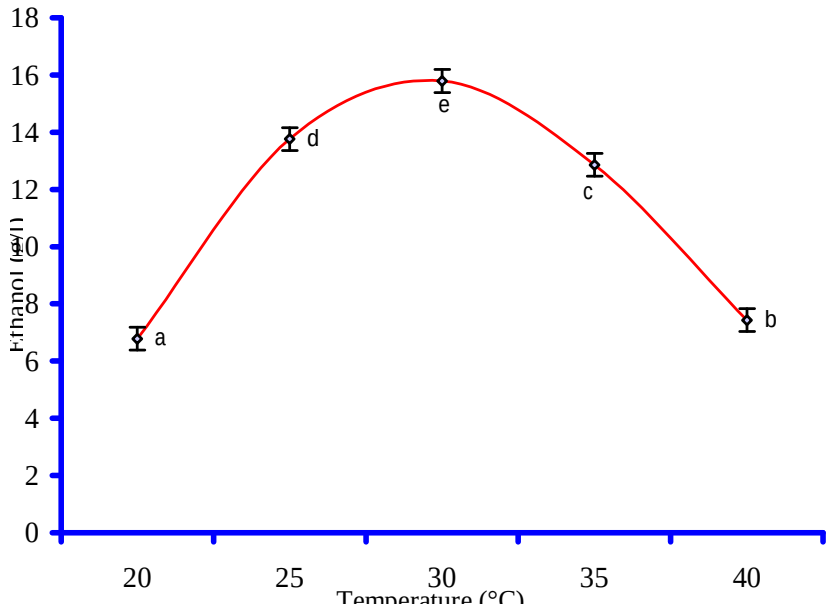

Fig.9 Effect of temperature on bioethanol production. Means followed by the same letter are not significantly different.

CONCLUSION

The production of bio-ethanol from *A. marina* seems very much possible and therefore it has a potential to become a future feedstock for bioethanol production**.** Using the optimal conditions obtained (under hydrolysis and fermentation), in continuous process, substantially increased the productivity of bio-ethanol.

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