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Production of Cellulase and bioethanol by ethanol-tolerant coculture of Bacillus cereus and Fusarium solani

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Abstract. Cellulase is an enzyme produced by fungi and bacteria that hydrolyzes cellulose by breaking down the glycosidic bond, β -1,4 that binds sugar glucose units. Bioethanol and cellulase enzyme were produced by ethanol-tolerant of Bacillus cereus co-culture. So the production of filamentous solani were using the wastes of Cynodon dactylon L. The highest activity of enzyme was at 96 hour/30°C of incubation. The optimal pH value was 7.5, while the carbon concentration was 3%. On the other hand, the best inoculum ratio was 0.5/100 mL (52 \times 10⁸ cell/mL bacterial cells and 10⁵ conidia/mL of the fungus). In order to test the efficiency of ethanol production by the co-culture by using different concentrations of pure ethanol was measured in the solid medium. Both *Bacillus* sp. and *F. solani* tolerated incubation with 5% ethanol. The best treatment when using 0.5% sulfuric acid which gave the best concentration of reduced sugars and the results showed a difference in the concentration of sugars produced by the fermentation process, it was 3.9 mg/mL in the first day, whereas it was 2.51 mg/mL after 120 hours of fermentation, the concentration of bioethanol produced after fermentation was 195 g/L. In conclusion Cynodon dactylon L. wastes were treated by sulfuric acid, cellulase digestion, and autoclave treatment to be a good source of reducing sugars.

Keywords. Ethanol, Cellulase enzyme, Fusarium, Bacillus.

1. Introduction

Cellulose is the main component of the plant cell wall, while the massive of cellulosic sources containing raw materials that not exploited or can be used more efficiently [1]. Lignocellulosic biomass in plants consists of about 10-25% lignin, 20- 30% hemicellulose and 40-50% cellulose [2]. Cellulase is an enzyme produced by fungi and bacteria that hydrolyzes cellulose by breaking down the glycosidic bond, β -1,4 that binds sugar glucose units. This enzyme has the ability to hydrolyze cellulose into its essential components, glucose- β or oligosaccharides [3]. Cellulose biomass is an important source of energy. Through this mass, carbon is recycled by microorganisms that secrete the cellulase enzyme, which catalyzes the process of cellulose hydrolysis into cellobiose and then converts it into glucose that can be converted to biofuel such as ethanol [4]. The world faces major economic and environmental problems due to higher fuel prices and increased gas emissions, contributing to global warming. As the sources of these conventional fuels have been depleted, it becomes imperative that alternative energy sources such as bio-ethanol, which has been widely produced in the world by

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fermentation processes be explored [5]. Ethanol is the most important alternative source of fuel. Its production has increased in recent years, and its production reached about 11% of the fuel used due to the rapid depletion of the global extra oil until global production reached 100 billion liters in 2015 [6]. The present study is aimed to produce the cellulase enzyme and ethanol by using of the co-culture of local isolates of *Bacillus cereus* and *F. solani* by culturing of the *Cynodon dactylon* plant as a production medium. The study also identified the optimum conditions for the production of the enzyme and ethanol using the liquid state fermentation.

2. Materials and Methods

2.1. Preparation of Cynodon dactylon wastes

Cynodon dactylon L. was collected from the gardens of the University of Sulaimani and was washed, cut into pieces and then dried using an electric oven at 65 °C for 24 hours. The wastes were then grinded using an electric mill and passed through a 2 mm sieve and stored in sterile polyethylene bags until use.

2.2. Cellulase production

The media for the enzyme production was prepared by dissolving 2 g (NH₄)2NO₃, 2 g (KH₂PO₄), 5 g (MgSO₄.7H₂O), 0.2 g (CaCl₃.H₂O), 0.2 g (MnSO₄.7H₂O), 0.2 g (FeSO₄.7H₂O), 0.2 g (Yeast extract), 0.2 g (Peptone), 40 g (*Cynodon dactylon* wastes) in one liter of distilled water, and adjusting the pH to 7.5. The 1 liter of media was prepared in a 2000 mL flask and sterilized using the autoclave. The media was then allowed to cool, after which it was used to inoculate the selected isolates *F. solani* and *Bacillus* sp. 0.5 ml / 100 ml of media was used to inoculate the bacteria and fungi and incubated in shaking incubator at 30°C with 150 cycle/min for 96 hours [7]. The raw enzyme was extracted by separating the supernatant from the residual media by using the centrifuge at 5000 rpm for 30 min at 4°C and enzyme activity in supernatant estimated according to the protocol by Miller [8], using a microtiter plate reader with wavelength of 450 nanometres.

2.3. Determination of the cellulase activity in the liquid media and the use of carboxymethyl cellulose *(CMC)*

The enzyme activity was estimated in the liquid media based on the standard glucose curve prepared according to the method described in [8].

2.4. Determination of optimal conditions for the production of cellulase enzyme in the liquid cellulose media

Different standards were used to determine the optimal conditions of the production of the enzyme, including the volume of the inoculum, specifically, 0.5, 1.0, 1.5, 2.0, and 2.5 ml / 100 ml media. Each aliquot of the fungi and bacteria isolate was used to inoculate the liquid media in order to detect the effect of amount of inoculum on enzyme activity and production while the pH of the growth media was adjusted to 5.5, 6.5, 7.5, 8.5 to determine the optimal pH for the production of the enzyme in the liquid media. Several concentrations (1%, 2%, 3%, 4%) of the carbon source were used to determine the best concentration of carbon source for the production of enzyme cellulose. The fungal and bacterial isolates tested on liquid media contain ground *Cynodon dactylon* as sole carbon source at different incubation periods (48, 96, 144, and 192 hours). The media was inoculated with both isolates and the activity of enzyme was estimated at each incubation period.

2.5. Pre - treatment for biomass of Cynodon dactylon wastes plant

The process of production of ethanol using the wastes of *Cynodon dactylon*, which was the first pretreatment done for the purpose of increasing the efficiency of both bacteria and fungi for digestion and consumption of cellulose, were done by adding 0.5% diluted sulfuric acid to the crude enzyme and autoclaving.

2.6. Determination of the capacity of isolates to Ethanol Stress Tolerance

F. solani and *B. cereus* were tested for different concentrations of ethanol (2.5%, 5%, 7.5% and 10%) and for different incubation periods (24, 48, 72 hours). After inoculation of the PDA media, pure ethanol, with concentrations suggested by the method of spot assay, was added in order to give an idea of the tolerance of these isolates in the production experiment. The fungal inoculum was prepared and the number of conidia was 10^4 conidia / ml, and the density of bacteria being 52×108 cells / ml. The samples were diluted ten to ten thousand folds after which 5 μ l of each dilution was taken and cultured on the surface of the petri dish containing the culture media with ethanol [7].

2.7. Production of ethanol

The wastes of the *Cynodon dactylon* plant treated with fungus *F. solani* (0.5 ml inoculums), as mentioned above, and containing 104 conidia /ml and 0.5 ml of bacteria (52×108 cells / mL) were placed in a shaking incubator at a speed of 100 rpm at 30 °C and at different incubation periods (24, 48,72, 96 hours). Glucose concentration was measured after each incubation period in the supernatant after removal of liquid media by using centrifuge at 5000 rpm for 15 minutes at 5°C and separating the supernatant from the precipitate. The reduced sugars present in the supernatant were estimated by taking 0.5 ml of supernatant in each test tube followed by the addition of 1 ml 3,5 DNS reagent to each tube. The tubes were then placed in water bath at 100 °C for 5 minutes, after which then the tubes were cooled down to RT. 10 ml of distilled water was added to each tube and mixed well. Absorbance measurements were taken at a wavelength of 540 nm using a Microtiter plate reader. The concentration of the reduced sugars were determined by extrapolating the corresponding absorbance measurements from the standard curve of glucose as described by [8].

2.8. Determination of Ethanol

The process of bioethanol production involved addition of the *Cynodon dactylon* plant waste treated with sulfuric acid into the conical flasks containing fungus and bacteria, and inoculating with the fungus inoculum $(10^4 \text{ conidium /ml})$ and bacteria $(52 \times 10^8 \text{ cell / ml})$. Samples were placed on the shaking incubator, then the amount of glucose were measured by using a microtiter-plate reader, before and during the fermentation process. After the 120 hours fermentation process, the supernatant was separated from the precipitate, distilled at 80° C and the ethanol collected from the distillation process. The ethanol concentration was estimated using gas chromatography (GC).

3. Results and Discussion

The co-culture system has been used in many food biotechnologies, but it is important that the mixed isolates be commensals with each other and not to compete or antagonize with each other [9]. Figure 1 illustrates that there is a synergistic relationship between the two isolates with their growth on the same media and close to each other. This indicates that there is no antagonism between the two

isolates and thus the possibility of using the two isolates together to enhance the production capacity and thereby increase the production of the enzyme and bioethanol



Figure 1. The relationship between *Bacillus* sp. and *F. Solani* (F/), cultured on Luria-Bertani media using the Spot dilution plate assay at 30 ° C for 96 hours; number of fungi / ml (10^5 conidium/ ml), number of bacteria / ml (52×10^8 cell /ml).

3.1. Determination of Ethanol

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The decrease in enzymatic activity when using an inoculum size greater than 0.5 ml / 100 ml was due to competition between bacteria and fungi for nutrient utilization in the culture media [10]. The statistical analysis showed no significant difference between the inoculum sizes used in this study except the size of the inoculum (0.5 and 1 ml) and at a significant level P <0.05. The effect of the change in pH of liquid media on the production of the cellulase enzyme were also studied. Figure 3 shows that the best enzyme activity was at pH 7.5 with mean value of 2.70 unit / ml.



These results were agreement with a previous study [11], which found that the best enzyme activity was at pH 7.5, and to some extent with the findings of [12], which found that pH 7.0 is the best in the production of cellulase enzyme and using *Bacillus* sp. isolate. The presence of more than one or more enzymatic profiles of the enzyme complexes produced by microorganisms may cause fluctuation in pH values, which may lead to reduced enzyme production during fermentation. It has also been reported that the optimal pH for the production of the cellulase enzyme may vary depending on the type of micro-organism used in the production [13]. The statistical analysis showed a significant difference in the values obtain at pH between 6.5 and 7.5 as well as 7.5 and 8.5. There was no significant difference, however, for values obtained at pH between 4.5 and 5.5; 5.5 and 6.5 at P <0.05. Figure 4 shows the effect of the concentration of carbon source on the production of the cellulase enzyme, and from the observation of the results, the best concentration of the carbon source in which the best production of the enzyme was found is 3%, with enzyme activity of 2.75 unit / ml. Next was the concentration of 4%, showing enzyme activity of 2.73 unit / ml while the enzyme activity at 1% and 2%, concentrations were 2.70 and 2.49 respectively.



These results differed from those obtained previously [14], in which the highest enzymatic activity was obtained at a concentration of 1% carbon source by the culture of *Bacillus* on corn husks and using liquid state ferments. The statistical analysis confirmed a significant difference in the enzymatic activity of the cellulase enzyme among all concentrations of carbon source and at the probability level P < 0.05.

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Figure (5) shows the effect of incubation period on the production of the cellulase enzyme using the fungus *F. solani* and *Bacillus*. It was observed that the best enzyme activity was after 4 days of incubation at 30 °C, which yielded an enzyme activity of 2.83 unit / ml. The enzymatic activity decreased to 2.7 unit / ml after a 6-day incubation period and continued to decline to 2.59 unit / ml after 8 days of incubation.



The decrease in enzyme activity with increased incubation duration may be attributed to cell autolysis, release of metabolic substances affecting enzyme productivity, and the possibility of altering the structural composition of the enzyme over time, as well as environmental changes in the production media [15]. [16] reported that most isolates of *Bacillus* sp. begins the production of cellulase enzyme after 3-12 hours of growth. The difference in production depends on the quality of the nutrients in the media and the stage in which the microorganism passes. The results obtained in this study agreed with previous work [17], which showed that the best production of the cellulase enzyme is achieved after 96 hours. The results of the statistical analysis showed a significant difference at P < 0.05 between the incubation period of 2 days and 4 days, while there were no significant differences between the other incubation periods.

3.2. Ethanol Production

Figure (6) shows the ability of the isolates *F. solani* and *Bacillus* sp. to tolerate different concentrations of ethanol (2.5, 5, 7.5, 10%). It is observed that the isolates have a strong ability to tolerate 5% of the ethanol, while there is no growth of the two isolates at the concentrations higher than 5%. These results are consistent with those of [18], who concluded that *B. cereus* is excellent for bioethanol production due to its ability to tolerate ethanol concentration at 6%. [19] reported that *F. oxysporum* had the ability to produce bioethanol from the fermentation of hexoses and pentoses, but there was no growth at 3.5% ethanol level. The increase in concentrations of ethanol and the inability of cells to tolerate high concentrations affect the formation of mycelium and the permeability of the wall of fungi and low net weight of the biomass. The presence of ethanol of about 0.5 - 2% inhibits the secretion of the cellulase enzyme by the fungus *Trichoderma reesei* [20]. Aldehyde Dehydrogenases enzyme is reported to play an important role in the tolerance of ethanol by fungus [21].



3.3. Bioethanol production

The results of the treatments for the *Cynodon dactylon* wastes shown in Figure 7, based on the concentration of sugars produced after treatment, were higher than the 0.5% H_2SO_4 treatment on the rest of the treatments. The concentration of reduced sugars was 3.5 mg / ml while the concentration of sugars of the *Cynodon dactylon* waste treated cellulase enzyme was 2.51 mg / ml. The *Cynodon dactylon* waste treated with distilled water and autoclave recorded the lowest concentration of produced sugars at 2.0 mg / ml. Pre-treatment is essential for obtaining the best degradation of plant wastes by the use of thermal chemical reactions involving the breakdown of solids from the mass of lignocellulose. These factors increase the readiness of the substrate and the redistribution of the lignin so that it can achieve the minimum energy consumption and obtain high percentages of sugars [22, 23]. The results of this study were in agreement with the results obtained by [24] in which H_2SO_4 was used for pre-treatment and high percentage of reducing sugars obtained. Removal of more than 65% of the lignin were observed when *Saccharum* plant was used. The results of the statistical analysis showed a significant difference between all the treatments used in this study at probability level P <0.05.



Figure (8) shows the concentration of glucose produced by the treatment of *Cynodon dactylon* wastes with 0.5% sulfuric acid, on which the *F. solani* and *Bacillus* were cultured. , The daily glucose concentration were then calculated for the 5 days of incubation. The figure clearly suggests that there is a decrease in the concentration of sugars with an increase in fermentation time. Concentration of

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glucose after 24 hours of fermentation time was 3.9 mg / ml and then the concentration decreased after 48 hours to 2.62 mg/ ml with a decrease in pH from 6.8 to 6.3. We noticed increments in sugar concentration after 72 hours of fermentation. The concentration reached 3.2 mg/ ml while the pH dropped to 6.1. The increase in glucose concentration after 72 hours of fermentation time may be due to fungal activity, which increased on the third day. Concentration of glucose started to decrease to 2.89 and 2.51 mg/ ml respectively, after 96 and 120 hours of fermentation time, while glucose concentration for control sample was 3.14 mg / ml, and there was an increase in pH value to 7.7 after 120 hours fermentation.



solani culture and Bacillus.

The growth of isolates in cellulose-containing culture media has been shown to be characterized by the length of the lag phase [25]. Growth depends on the adhesion of microorganisms to the cellulosic fibers found in the culture media and stimulates the secretion of cellulolytic enzymes, in contrast to the culture media containing dissolved substances as the lag phase is short in length. The concentration of reduced sugars in the culture media depends on the enzyme activity in the growth media and on the type of cellulosic material and its capacity of enzymatic degradation, and also depends on the growth phase of the bacteria. The passage of the bacteria in the stationary phase indicates that more sugars are consumed by the bacteria to sustain their activity. This study was conducted to produce bioethanol from the wastes of Cynodon dactylon with Bacillus isolate and F. solani. The important factor in making ethanol production more economical is the use of large quantities of cellulose available in the environment and containing large amounts of cellulose that can be converted into ethanol [26]. Table (1) and Figures (9) and (10) show the concentration of bioethanol produced from the fermentation process after 120 hours of fermentation and saccharification using co-cultures, as well as the results of the analysis of the correlation curve for the produced ethanol and standard ethanol. Table (1) shows that the ethanol concentration was 195 g/liter. This concentration increases with the availability of extra fermented sugars in the media so it is necessary to increase the concentration of sugars in the media to produce ethanol in large quantities and the efficiency of fermentation depends on the speed of sugar consumption by the cell [27]. The concentration of ethanol produced in this study was higher than those obtained by several other researchers who used single isolates. For example, [28], produced bio-ethanol from Saccharum plant and cassava husks using B. cereus bacteria and obtained a concentration of 18.4 g / L of Saccharum wastes sugar and 17.80 g / l of cassava husks Our results also show significant improvement over the works of [29], who reported concentration of bio-ethanol obtained to be 8.3 g / L using B. subtilis and potato wastes. Our work remains supreme when we further compare our results to that of [30] who used rice bran with B. cereus and obtained bio-ethanol concentration of 10.2% at 37 °C and pH 5, after 120 hours of fermentation.

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Table 1. Ethanol concentration (g / L) resulting from the fermentation process Using a co-culture of fungus *F. solani* and *Bacillus* species.

Concentration of Ethanol g/l	Area	R. Time	No. Peak	
195	179108	3.566	6	



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