



ISSN: 0067-2904

## Improvement Efficacy of *Bacillus Subtilis* Cellulose Hydrolyzing by Using Cold Plasma Technique

Isam Shaker Hamza<sup>1</sup>, Labeeb Ahmed Alzubaidi<sup>2</sup>, Aziz Latif Jarallah<sup>1</sup>, Rehab S. Ramadhan<sup>1\*</sup>

<sup>1</sup>AL-Esraa University College

<sup>2</sup>Food contamination research Center, Directorate of Environment & Water, Ministry of Science & Technology

Received: 16/4/2021

Accepted: 7/6/2021

Published: 30\4\2022

### Abstract

*Bacillus subtilis*, an isolate of *bacillus* genus, was obtained from the laboratories of Ministry of Science and Technology. The best efficient *Bacillus subtilis* isolate in cellulose and semi-cellulose hydrolysis was treated with Dielectric-barrier Discharge (DBD). Atmospheric cold plasma technique (non-thermal) was used by exposing them at different times (2, 3, 4 and 5 mins) separately as a first stage, and then 60 seconds after any treatment separately as a second stage. After 48 hours, the difference between the plasma source and the sample was fixed at 0.5 cm. The results showed a variation in the growth of the isolate according to the exposure time by the appearance of culture turbidity and the estimation of optical density. Positive results appeared between exposure times, the amount of optical density, and the cellulose and semi-cellulose decomposition into glucose. *Bacillus subtilis* increased its efficacy in cellulosic hydrolysis and semi-cellulosic materials. *Bacillus subtilis* showed malleability and the ability to increase the efficiency in cellulose and semi-cellulose materials hydrolysis. We conducted a new and extensive study by using cold plasma technique to increase the hydrolysis efficacy of food microorganisms.

**Keywords:** Cellulose, Hydrolyzed, *Bacillus subtilis*, Cold plasma.

### تحسين كفاءة عزلة *Bacillus subtilis* المحللة للسليولوز باستخدام تقنية البلازما الباردة

عصام شاكر حمزة<sup>1</sup>, لبيب احمد الزبيدي<sup>2</sup>, عزيز لطيف جار الله<sup>1</sup>, رحاب صبحي رمضان<sup>1\*</sup>

<sup>1</sup>كلية الاسراء الجامعة

<sup>2</sup>مركز بحوث تلوث الغذاء، دائرة البيئة والمياه، وزارة العلوم والتكنولوجيا

### الخلاصة:

تم الحصول على عزلة بكتيرية عصوية الشكل *Bacillus subtilis* من مختبرات وزارة العلوم والتكنولوجيا. تمت معاملة العزلة البكتيرية *Bacillus subtilis* الكفوة في تحليل السليولوز واشباه السليولوز بتقنية البلازما الباردة باستخدام الهواء الجوي بتعريضها بأوقات مختلفة (2، 3، 4 و 5) دقيقة كلا على انفراد كمرحلة اولى و 60 ثانية كل على انفراد كمرحلة ثانية (بعد 48 ساعة) وتم تثبيت المسافة بين مصدر البلازما والعينة عند 0.5 سم. واستخدم النمو البكتيري بدون معاملة) مجموعة سيطرة). أظهرت النتائج تباين في نمو العزلة وحسب زمن التعرض من خلال ظهور العكورة في الوسط الزرع وتقدير الكثافة الضوئية حيث ظهرت نتائج طردية بين زمن التعرض ومقدار الكثافة الضوئية والتحلل المائي للسليولوز واشباه السليولوز الى الكلوكون. يمكن

\*Email: dr.rehab2018@esraa.edu.iq

ان نستنتج بإمكانية استخدام تقنية البلازما الباردة في تغيير التوازن الايضي للأحياء المجهرية ومن ضمنها بكتريا *Bacillus subtilis* لزيادة كفاءتها في تحليل المواد السليلوزية واشباه السليلوزية. اظهرت بكتريا *Bacillus subtilis* قابليتها في زيادة التحلل المائي للمواد السليلوزية واشباه السليلوزية. نوصي بالاستمرار باجراء دراسات جديدة ومستفيضة في استخدام تقنية البلازما الباردة في رفع كفاءة الاحياء المجهرية في معالجة الاحياء المجهرية الأخرى الملوثة للأغذية.

### Introduction:

Enormous amounts of agricultural and industrial cellulosic wastes have been accumulating in the environment. Celluloses are considered as an important renewable resource for bioconversion. Many Cellulosic substances are hydrolyzed to mono or di sugars to make Single Cell Protein sweeteners. It has become an economic interest to develop an effective method to hydrolyze the cellulosic biomass [1]. Huge amount of plant waste can be utilized to produce many value-added products such as biofuels, animal foods, chemicals, and enzymes. Cellulose is the main component of agricultural waste. Cellulosic waste is hydrolyzed into glucose and other soluble sugars using the hydrolysis enzymes of cellulose and quasi-cellulose. Bacteria isolated from thermophilic environment, can produce cellulases and also utilize agro-waste biomass. It has a high potential for developing thermostable cellulase required in the biofuel industry. The cost for cellulase represents a significant challenge in converting lignocellulose to fermentable sugars for biofuel production [2]. Cellulase enzyme complex consists of three units of soluble extracellular enzymes: 1, 4- $\beta$ -endoglucanase, 1, 4 - $\beta$ -exoglucanase, and  $\beta$ -glucosidase ( $\beta$ -D-glucoside glucohydrolase or cellobiose [3].

Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters. With the increasing knowledge of the mode of action of Cellulase, they have been used in enzymatic hydrolysis of cellulosic substances [4].

The most important agents of cellulase enzymes are bacteria and fungi. These microorganisms are commonly found in soil and agricultural waste. The potential cellulases-produced bacteria are *Cellulomonas*, *Pseudomonas*, *Thermoactinomyces* and *Bacillus spp.* However, enzymes of microbial origin are more widespread due to their broad biochemical diversity, feasibility of mass culture, and ease of genetic manipulation. Moreover, they possess high degree of stability under extreme conditions [5]. Plasma has been used in sensitive materials such as human tissues, food products, medical devices, and the packaging industry, but the results are impractical. However, over the past few years, a remarkable technique has been developed that can produce low-temperature plasma under atmospheric conditions and offers several advantages. Low temperature plasmas are initially composed of a background gas medium subjected to an electric or electromagnetic stress that allows initial seed electrons (naturally present in the gas medium) to store energy high enough to undergo many inelastic processes with atoms or molecules of the background gas. The inelastic ionization or dissociation or excitation collisions, due to the energetic electrons, leads to the creation of new plasma species as radicals, ions, long-lived excited species, neutral byproducts, photons and even electric field self-induced by the space charges present in the plasma. The physico-chemical properties of these active plasma species are exploited in many fields, for instance biomedical and plant biology [6]. The plasma-chemical technologies using non-equilibrium and low-temperature plasmas could be a promising alternative to the hydrolysis methods. The bacterial species that dominate the thermophilic phase of compost processes, belong to the genus *Bacillus*. They play an important role in the degradation of complex substrates, such as cellulose [7].

Therefore, the aim of the research is to study the application of cold plasma technique in improving the efficiency of one of the bacterial isolates hydrolyzed to cellulose and semi-cellulose.

### **Materials and Methods:**

#### **Bacterial isolates culturing and confirmation:**

Salts-cellulose medium modified form [8], was used for cellulose hydrolyze bacteria growth consisting of (KH<sub>2</sub>PO<sub>4</sub> 0.5, NaHPO<sub>4</sub>.12H<sub>2</sub>O 4.5, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.25, MgSO<sub>4</sub>. 0.25, Yeast extract 2, carboxy. methyl cellulose-CMC 10, cellulose 0.25, gelatin 2 (for the enhancement of the bacterial activity)) g/l, adjusted medium pH at 7.2. The medium was sterilized with an autoclave at 121 °C for 20 mins. 0.1 ml of *Bacillus subtilis* isolate, obtained from the culture collection of the ministry of science and technology, was added to the broth medium and incubated at 55 °C for (24±2) hours. After its growth, 0.1 ml of the bacterial growth was spread on the culture media and incubated anaerobically at 55 °C for (24±2) hours. The culturing process was repeated to purify the bacterial isolates using the planning method. The most efficient bacterial isolation was identified in the cellulose hydrolysis by the phenotypic characteristics. Characteristics of bacterial colonies were recorded according to shape, size and texture while growing on nutrient agar. Furthermore, diagnosis was further extended using the gram stain method for further confirmation.

#### **Identification of *Bacillus subtilis*:**

*Bacillus subtilis* isolates were primarily identified on the basis of taxonomic properties. The characteristic morphological, cultural and biochemical properties were observed [9].

#### **Cultural characterization:**

Isolates on nutrient agar plates were examined for size, pigmentation, form, margin and elevation of the colonies.

#### **Morphological characterization**

Morphological characteristics such as the cell shape, cell arrangement as well as the Gram's reaction of the organism were determined by Gram staining technique. Endospore staining technique was also carried out to morphologically characterize the isolates.

#### **Biochemical characterization:**

Biochemical tests such as catalase, motility, citrate, urease, indole, Methyl red, Voges Proskauer, Nitrate reduction, starch hydrolysis and sugar fermentation were carried out according to the standard procedures.

#### **Treatment the bacterial isolate with cold plasma technique:**

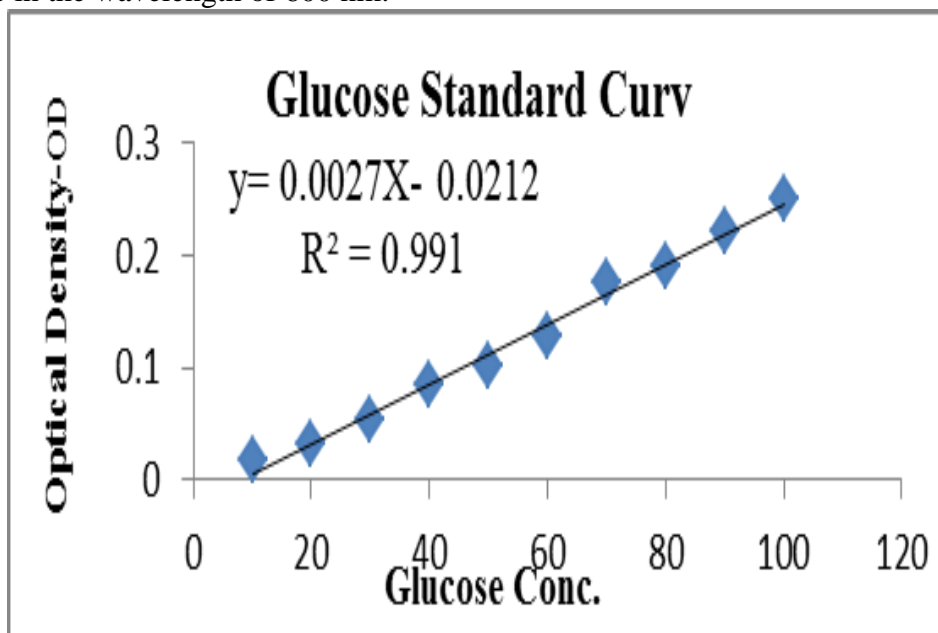
The most efficient bacterial isolation was treated with atmospheric cold plasma technique (non-thermal) at different times (1, 2, 3 and 4 minutes) separately as a first stage and then (60 seconds) as second stage with 48 hours separately. The distance between the plasma source and the sample is at 1 cm, and a variable DC voltage (7.5-8.5) kV, at a frequency of 28 kilohertz, according to [10]. The laboratories of the College of Science for Women and the University of Baghdad compared the bacterial growth without treatment (control group).

#### **Enzymatic assay**

Cellulase activity was assayed using Dinitrosalicylic acid (DNS) reagent by estimation. The reducing sugars resulted from carboxymethylcellulose (CMC) as a substrate. The bacterial growth centrifugation precipitate, treated with formic acid 6.6 % concentration 10 minutes, was added to 0.5 ml of 1% CMC 0.05M Phosphate Buffer and incubated at 50°C for 30 min. The reaction was then ended by adding 1.5 ml of DNS reagent and boiled at 100 °C in a water bath for 10 mins. The quantity of reducing sugar, resulting in the hydrolysis of CMC, was measured at 600 nm using UV-Vis spectrophotometer. Cellulases fabrication was estimated using Glucose standard curve. The unit of enzyme activation was defined as the amount of enzyme that is necessary to release 1µmol of glucose per min under standard assaying circumstances [11].

**Standard curve for glucose:**

Serial dilution was prepared from the stock glucose solutions 1mg/ ml in decimal series 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 mgs/mls by adding the 3ml of Dinitrosalicylic acid-DNS reagent for colorant for sugar, reducing it to 1 ml of each concentration of glucose. The solution was heated for 10 minutes until it reached the boiling point and then cooled down to the room temperature. In order to obtain the standard curve of glucose, we measured optical density (OD) using a UV-VIS spectrophotometer (Optima) with a wavelength of 600 nm and drew the standard curve. The sample glucose concentration was also determined. Concentration of glucose was measured in the suspension of bacterial growth on broth of salts - cellulose media from centrifugation process by using DNS for the purpose of measuring OD of glucose in the wavelength of 600 nm.



**Figure 1**-Glucose standard curve measured by reducing sugar in the presence of DNS.

**Estimating the efficacy of cellulose degradation:**

Bacterial isolates were grown on component salts medium. Incubation time was  $(24 \pm 2)$  hours, at a temperature of  $55^{\circ}\text{C}$  to select the most efficient cellulose hydrolyzing isolate by measuring the optical density of bacterial growth using a spectrophotometer at a wavelength of 540 nm and comparing it to the culture medium without bacteria (control sample). The growth media was covered with Congo red stain 0.1% after the emergence of bacterial growth for 15 minutes and washed with a sodium chloride solution at 1M concentration for many times. The positive result was observed when the transparent yellow halo appeared around the bacterial growth medium, and the degradation diameter was measured to determine the isolation efficiency in cellulose degradation [8].

**Results and discussion:****Examine the bacterial isolates purity.**

The bacterial colonies appeared mucous, circular, cream-colored, having the ability to grow at  $55^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  temperatures. As shown in Figure 2.



**Figure 2** - *Bacillus subtilis* growth on nutrient agar medium bacterial appeared after 1 day of incubation at 37°C.

Upon microscopic examination, it was observed that the bacterial was bacilli Gram-positive, forming Endospore.

#### **Estimating the effectiveness of cellulose degradation:**

The results in Table 1 showed the activity of bacterial isolate in hydrolyzing the cellulose through estimating the OD of bacterial growth as compared to the control sample. Results showed that the bacterial isolates have effectiveness in utilizing and analyzing cellulose by using it as an energy source by analyzing it into simpler units in order to carry out biological activities, necessary for growth and reproduction.

**Table 1-** Activity of *Bacillus subtilis* in cellulose hydrolysis compared with control sample

Samples	1	Control
OD	1.028±0.004	0.703±0.009

The results showed that the whole isolation showed a clear growth on CMC medium through the appearance of a transparent yellow halo around the bacterial growth with different diameters as shown in Table 2.

**Table 2-** Estimate of bacterial isolates activity into cellulose degradation.

Samples	1	Control
Diameter of clear zone in CMC medium	1.3	0.5

The appearance of yellow transparent index indicateds the exo-production of cellulose degrading enzyme, and that the degree of cellulose degradation varies between microorganisms depending on the composition and amount of cellulases enzymes in addition to the nature of the substrate material [12].

#### **Standard curve for glucose:**

Result of glucose consumption standard is illustrated in Figure 1. Serial dilution of 1 mg/ml glucose stock was reduced by dinitrosalicylic acid - DNS as a reducing agent and was detected by spectrophotometer. Readings were recorded to draw the standard curve for the glucose concentration.

#### **Measurement of glucose concentration:**

The glucose consumption by bacterial growth was measured by spectrophotometer at 600 nm wavelength and applied in the straight-line equation of the standard curve of glucose.

The results show the emergence of bacterial growth in all cellulose concentrations in the culture medium. Upon estimating the glucose concentration, the presence of sugar in different concentrations was observed, as well as when calculating the percentage of cellulose

consumption and its presence in varying percentages of consumption., It was noticed that a significant utilization rate was obtained using the bacterium under study. Cellulose concentration was measured to be 1.5% while high O.D absorption rate was also recorded. These results reflect the efficacy of local *B. subtilis* in utilizing cellulose (0.3-2) %, while the researchers concluded [13] that the optimal cellulose concentration for the bacterial effectiveness in producing cellulose-degrading enzymes is 1% and 0.75%. The cellulose-degrading enzymes ability was not inhibited due to low cellulose concentrations, since the cellulose assimilation was still same even in low concentration, suggesting active operon expression cellulose in the medium.

#### Activity of bacterial isolate after treatment with cold plasma technique:

*Bacillus subtilis* isolate was tested depending on the results of cellulose hydrolysis and because it gave the highest OD. The appearance of yellow transparent halo is evidence of the effectiveness of bacterial isolation in secreting the enzyme that hydrolyzes cellulose to the outside and its effectiveness in analyzing cellulose into simpler units.

The results of Tables 3 & 4 show the bacterial isolate growth treated with DBD cold plasma technique, different doses, two stages for stabilizing the genes mutation, encoding the hydrolysis cellulose enzymes through the appearance of turbidity in the culture media and the estimation of OD as compared to the control sample.

**Table 3-**Optical density of the efficient bacterial isolate growth after treated with DBD cold plasma technique with different exposure times (first stage)

Exposure time (min)	1	2	3	4	control
OD	0.804±0.03	0.85±0.03	0.91±0.04	1.02±0.03	0.708±0.04

**Table 4-**Optical density of the efficient *Bacillus subtilis* growth after treated with cold plasma technique with different exposure times (second stage)

Sample with exposure time (1min)	1	2	3	4	Control
OD	0.818±0.01	0.916±0.05	1.21±0.02	1.07±0.02	0.711±0.02

Through these results, it appeared that the bacterial isolates were different in activity and benefit from cellulose hydrolysis by using sugar as a food carbohydrate sourced by hydrolyzation into simpler units in order to carry out the necessary biological activities for growth and reproduction., All isolates showed an uneven growth on CMC medium as well as in the effectiveness of clear degrade of CMC, through the emergence of a transparent yellow halo around the bacterial growth with varying diameters as shown in Table 5.

**Table 5-**Assessment of the efficient bacterial isolate in cellulose hydrolyzed after treated with cold plasma technique and with different exposure times (second stage).

Samples	1	2	3	4	Control
Diameter of CMC hydrolyzed	0.7	0.8	1.4	1.1	0.5

The percentage of cellulose degradation varies between the micro-organisms analyzing cellulose, depending on the composition and quantity of the enzymes that degrade cellulases as well as the nature of the cellulose material, as it is mainly composed of long polymers of glucose units. Cellulose is a producer of glucose and other materials [12].

#### Glucose concentration measurement:

Results show the differences in bacterial growth in cold plasma treatments. Depending on the glucose concentration, presence of sugar in varying concentrations was observed, as well as when calculating the percentage of cellulose consumption and the presence of varying percentages of consumption. The results obtained by the percentage of consumption of cellulose, reflect that the activity of the local bacterial isolate *B. subtilis* in all values of cellulose concentrations under study (0.3- 2) % is not directly proportional to the increase in

exposure time. The researchers concluded [13] that the optimal cellulose concentration, when the producing enzymes, bacterial cellulose hydrolysis is 1% and 0.75%.

The activity of cellulose hydrolysis enzymes within the range of concentrations under study, was not inhibited due to their lack of saturation with cellulose concentrations. Hence, the activity of the enzymes depends on the concentration of the cellulose material and the special growth conditions. Plasma is a distinct state of matter due to its properties. It does not have a regular shape or size and can form strings and/or beams under magnetic fields. Depending on the generation method used, plasmas can display a wide range of systems, ranging from maximum equilibrium to near complete thermal equilibrium. Plasma can be found in the form of natural phenomena like light or man-made as in the production of fluorescent, neon lights, plasma TV etc. The areas of research in plasma technology are growing rapidly and have been specifically studied for use on biomedical materials [14].

The ability of a local bacterial isolate (*Bacillus subtilis*) into treating the agricultural materials and wastes, including the hydrolysis found in the cellulosic and semi-cellulosic materials. The possibility of using DBD plasma (cold plasma) technique in changing the metabolic balance of microorganisms, including *Bacillus subtilis*, has shown to increase its efficiency in the hydrolyzed cellulosic and semi-cellulosic materials. *Bacillus subtilis* bacteria shows a receptivity and ability to increase efficiency in the breakdown of cellulosic and semi-cellulosic materials. Increasing studies in the field of cold plasma techniques used for increasing micro-organisms efficiency in environmental pollutant treatments by manipulating and increasing gene expression to produce the enzymes and other products in microorganisms in the environmental pollutant's treatment, gives a beneficial production.

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