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STUDY OF THE IMPACT OF MINERAL SALTS, INCUBATION TIME AND AERATION ON BY BACILLUS SP. (STRAIN RL1) ON XYLANASE PRODUCTION

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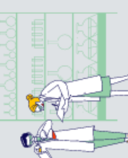
Abstract
This study used Bacillus sp. (Strain RL1), six distinct mineral salts (LiCl, K2HPO4, KCl, CaCl2, ZnSO4, and CuSO4), and five incubation times (24, 48, 72, 96, and 120 hours) to investigate the xylanase enzyme synthesis. The method of measuring xylanase activity involved assaying dinitrosalicylic acid. the highest enzymatic activity was obtained from the use of K2HPO4, which reached, 0.5722 U/ml, followed by CaCl2, as the enzymatic activity reached 0.5682 U/ml, and CuSO4 gave the lowest, Enzymatic effectiveness reached 0.2816 U/ml, while the enzymatic effectiveness of using LiCl, KCl and ZnSO4 was (0.2644, 0.3588 and 0.362 U/ml), respectively. the enzymatic activity of the xylanase produced increased with increasing incubation period, reaching 0.486 U/ml after 24 hours, reaching its maximum level of 0.681 U/ml after 96 hours of incubation. It returns and decreases as the incubation period increases, reaching 0.376 after 120 hours of incubation. While aeration, the highest enzymatic effectiveness of the xylanase enzyme was 0.681 U/ml when the shaking incubator was used at 200 rpm after 96 hours of incubation time.

Keywords: xylanase enzyme, Bacillus, mineral salts, incubation time, Aeration.

Introduction

A class of hemicelluloses known as xylanase enzyme (E.C.2.8.1.8) is required for the hydrolysis of 1,4-xylans found in lignocellulose materials (Jae *et al*., 2009). Because of its alternate technique of chemical hydrolysis, it plays a significant function in nature in preventing contamination of the environment (El Shamy *et al*., 2016). The capacity of xylanase to hydrolyze xylan, a plentiful natural polysaccharide, is essential to its industrial uses (Polizeli *et al*., 2005). A growing number of industrial processes, including hydrolytic production, food additives, poultry, vegetable oil extraction, biomanufacturing, bleaching of soft tissues in the paper industry, and better handling of dough used in coffee, vegetable oil, and starch extraction, have shown interest in the enzyme xylanase, which is produced by microorganisms like fungi, bacteria, and a few kinds of yeast (Shabeena *et al*., 2017 ; Nitin *et al*., 2017). The most notable microbes that generate the enzyme xylanase include *Aspergillus*, *Trichoderma*, and bacterial strains like *Bacillus* spp (Sapaq *et al*., 2002).



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Materials and Methods

Chemicals and cultures media were obtained from Sigma Company, while all analytical chemicals were obtained from the USA and the remaining chemicals were obtained from the Chinese Shanghai Company.

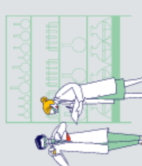
Microorganism and culture : Employing pour plate and repeated dilution procedures up to 10–9 techniques were used to isolate the xylanase-producing bacterial strain (*Bacillus* sp. RL1) from soil (Jin Yun Mountain, 1800 m). Five transfers of the isolated single colonies onto CMC agar plates were made in order to purify and isolate them. The isolate underwent a 24-hour growth period at 30°C for primary screening. The medium used for the growth consisted of KH_2PO_4, 2, $(NH_4)2SO_4$, 4, $MgSO_4$, 0.5, Peptone, 10, agar agar, 20, and D.W. Additionally, 1% carboxymethyl cellulose (CMC) was added to the media.

Identification of the isolates: Pure cultures of the bacterial isolates found during the first screening were maintained on CMC agar slants. All agar slants were stored at 4 °C until they were used. After examining the colony shape of the isolated culture, Gram's staining and endospore staining were carried out. The separated colonies underwent physical and biochemical assessment using conventional methods (Kannan, 2002). For identification, the seventh edition of Burges' Manual was utilized.

Secondary screening: Using the Congo red test once more, cellulolytic activity was examined. On successive CMC agar plates containing (g/L), KH_2PO_4, 2, $(NH_4)2SO_4$, 4, $MgSO_4$, 0.5, Peptone, 10, agar agar, 20, and distilled water, supplied with 1% CMC, at 30°C for 24 hours, the bacteria were cultivated in order to boost the secretion of xylanase. Once the agar substrate had been incubated, it was covered with an aqueous Congo red solution (1% w/v) and allowed to settle for fifteen minutes. The stain was then scraped from the plates, and the solution was drained off before being cleaned for 20 minutes with 1M NaCl. (Teather and Wood, 1982) observed the appearance of a distinct hydrolysis zone, which indicated the breakdown of cellulose.

Confirmation of xylanase activity: We created this method in order to assess the xylanase production that takes place in our lab. 0.5 ml of 1% substrate (CMC) in 0.05 M citrate buffer (pH 4.8) was added to the supernatant-free cell and left for 30 minutes at 50°C. 0.1 cc of 1% naphthol was added, and the mixture was gently mixed to terminate the reaction after 15 minutes at 100 °C. The solution should turn brown upon adding 0.5 ml of concentrated H_2SO_4, indicating that the test was successful.

Fermentation experiment: For seventy-two hours, the culture was cultivated aerobically at 35 °C and pH 7.0 in a 50 ml Erlenmeyer flask that held 30 ml of CMC medium (g/L) KH_2PO_4, 2, $(NH_4)_2SO_4$, 4, $MgSO_4$, 0.5, Peptone, 10, D.W, and 1% carboxymethyl cellulose (CMC) without water. After the period of incubation, the culture was centrifuged for 10 minutes at 10,000 rpm. The culture supernatant was then used to measure the activities of xylanase, FPaes, and extracellular proteins. This information is the standard error plus the average of three replicates.



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Xylanase assay: Bailey *et al.* (1992) reported that the amount of reducing sugars released was assessed using the dinitrosalicylic acid technique (Miller, 1959) and that the xylanase activity was tested using a 1% solution of oat spelt xylan as the substrate. One mole of xylose equivalent generated per minute was considered to be one unit of enzyme activity under the given circumstances.

Effect of mineral salts : mineral salts were used in the production of the xylanase enzyme : LiCl, K_2HPO_4, KCl, $CaCl_2$, ZnSO4 and $CuSO_3$.

Effect of incubation of time : the optimal incubation time for the production of the xylanase enzyme was determined by monitoring its production over different periods of time, which were (24, 48, 72, 96 and 120 hour), respectively.

Effect of aeration : a shaking incubator was used to produce the xylanase enzyme at a speed of 200 rpm.

Results and Discussion

The enzyme xylanase is produced by microorganisms, which are fungi, bacteria, and yeasts, such as *Bacillus*, *Aspergillus, Penicillium, Trichoderma spp, Aureobasidium, Thermomyces* and *Schizophyllum* (Wong *et al*.,1988 ; Gomes *et al*.,1992 ; Li *et al*., 2000 ; Beg *et al*.,2001).

Effect of the mineral salts: the production of the xylanase enzyme from the *Bacillus* sp. (Strain RL1) isolate was monitored through the use of different mineral salts. It is clear from observing Figure. 1 that the highest enzymatic activity was obtained from the use of K_2HPO_4, which reached, 0.5722 U/ml, followed by $CaCl_2$, as the enzymatic activity reached 0.5682 U/ml, and $CuSO_4$ gave the lowest, Enzymatic effectiveness reached 0.2816 U/ml, while the enzymatic effectiveness of using LiCl, KCl and $ZnSO_4$ was (0.2644, 0.3588 and 0.362 U/ml), respectively.

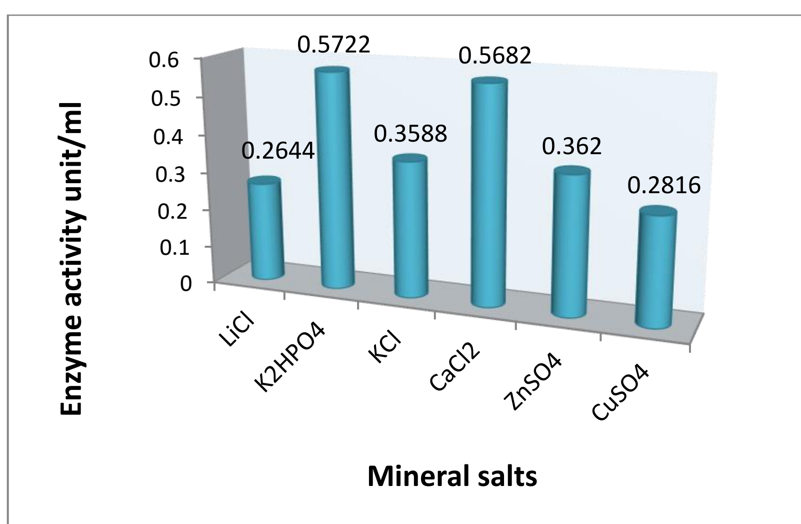

Figure 1 : The effect of the mineral salts on the production of xylanase enzyme.

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Cells need salts and some mineral elements for multiple purposes. They either enter the cellular structures, and this is usually what they need in large quantities, or they enter into the synthesis of some enzymes, while they need a portion of them to obtain a state of ionic balance that is important in the biochemical reactions of the cell. Ellaiah *et al*., (2002), was used NaCl, K_2HPO_4, $MgSO_{4}.7H_2O$ and $FeSO_{4,7}H_2O$ while Anto *et al*., (2006) were used K_2HPO_4, KH_2PO_4, $FeSO_{4,7}H_2O$ and $MgSO_{4}.7H_2O$.

Some mineral salts are added to the production medium to maintain the stability of the enzyme, such as $CaCl_2$, or to maintain the pH, such as K_2HPO_4 and KH_2PO_4 (Adimarayana *et al*., 2003). The production of the microbial xylanase enzyme is affected by the presence of metal ions, as it has been found that some positive ions have a supportive effect on the effectiveness of the enzyme's stability (Vijay *et al*., 2010).

The production of bacterial xylanase depends on the presence of calcium, as $CaCl_2$ stimulates the production of the enzyme Haddar *et al*., (2010), Karan *et al*., (2011), also found that the presence of $MgCl_2$, $MgSO_4$, K2HPO4 and KH_2PO_4 supports the production of extracellular xylanase in some types of microorganisms, while other metal ions such as $MgCl_2$, $BaCl_2$, MnSO4, $ZnSO_4$ and $CuSO_4$, have been proven to have an inhibitory effect on xylanase production Ramesh *et al*., 2009 ; Pillai *et al*., 2011).

Effect of incubation time : the production of the xylanase enzyme from the *Bacillus* sp. (Strain RL1) was monitored over different incubation time. It is clear from observing Figure. 2 that the enzymatic activity of the xylanase produced increased with increasing incubation period, reaching 0.486 U/ml after 24 hours, reaching its maximum level of 0.681 U/ml after 96 hours of incubation. It returns and decreases as the incubation period increases, reaching 0.376 after 120 hours of incubation.

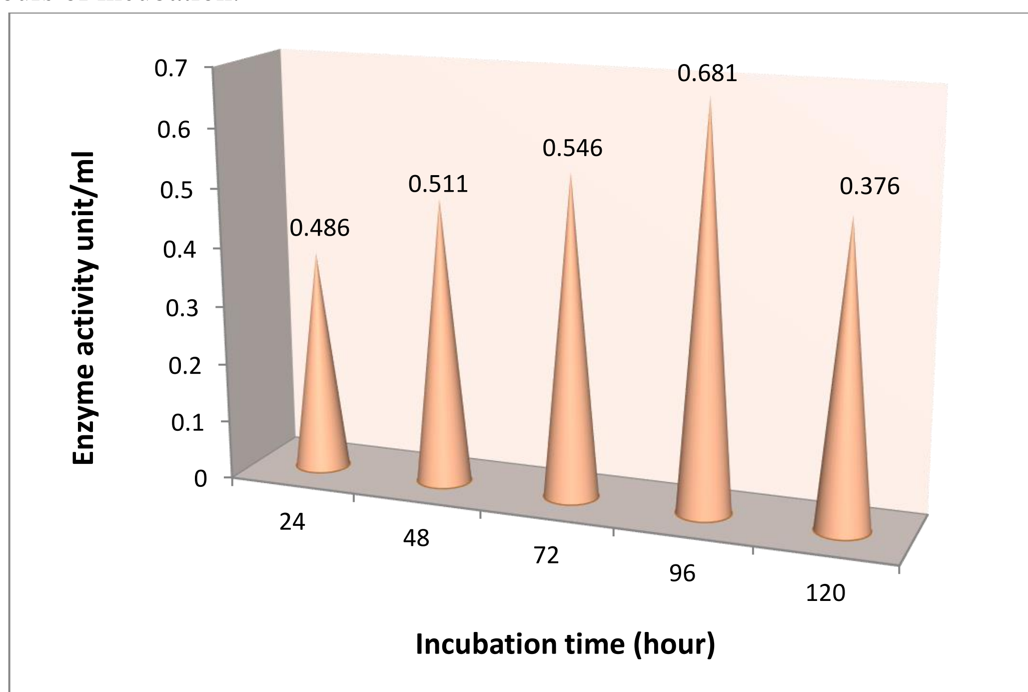

Figure 2 : The effect of the incubation time on the production of xylanase enzyme.

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The decrease in enzymatic activity with increasing incubation time can be attributed to the self-decomposition of the cells and the release of their contents into the medium and the occurrence of self-digestion of the enzyme, or due to a change in culture conditions due to the products of metabolic processes formed with the continued growth of bacteria, which negatively affects production (Lazazzera, 2000).

The deterioration of the productivity of enzymes from microorganisms after the ideal incubation period is attributed to the bacteria entering the stage of numerical stability or the stage of destruction, especially in batch cultures due to a decrease or depletion of the components of the medium. A set of changes were identified in them that negatively affect the biomass and cause cell decomposition and the release of enzymes. A protein degrader that may affect the enzyme required to be produced and thus decrease its effectiveness. Therefore, it is recommended to stop fermentation processes before reaching the stage of cell lysis. Studies vary in determining the incubation period necessary to produce enzymes depending on the type of microorganism and production conditions, especially the temperature and pH of the medium (Kastner *et al*., 2006).

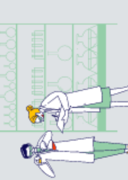
These results are consistent with what Gugi et al., (1991), stated, that the incubation period of 96 hours was the best for enzyme production from *P. fluorescens*, which is what Mu et al., (2009), confirmed, stating that the best enzyme production from *P. fluorescens* Rm12 was after 96 hours.

Effect of aeration and agitation : the highest productivity of the xylanase enzyme was 0.681 U/ml when the shaking incubator was used at 200 rpm after 96 hours of incubation time. The process of stirring is an important factor in the production of microbial enzymes by constantly providing nutrients and making them ready for the microorganism (Saran *et al*., 2007).

Moving the bacteria with the subject material not only leads to the spread of bacteria in the medium, but also leads to the breakdown of solid food materials. Also, the high speed affects the microscopic organism and then causes morphological changes and thus a difference in growth and metabolic products of the microscopic organism. It may also cause damage to the cell structure (Mantzouridou *et al*., 2002).

Aerobic fermentations lead to providing the microorganism with oxygen, and when the speed of aeration increases, oxygen becomes a limiting factor, as the consumption of carbohydrates increases through metabolic pathways that lead to the formation of biomass at the expense of other metabolic products, including enzymes (Oncu *et al*., 2007 ; Gupta *et al*., 2008), However, the increase in the speed of ventilation leads to the agglomeration of the microorganisms into separate masses, which limits the rate of access of nutrients (Palaniyappan *et al*., 2009).

The importance of aeration and agitation is due to the microorganisms' need for dissolved oxygen, the homogeneity of the components of the medium, and the distribution of the substrate in the development medium. In general, the xylenase enzyme is produced under aerobic conditions, and there is a linear relationship between the enzyme and aeration, as the production of the extracellular xylanase enzyme in aerobic microorganisms is affected by the availability of oxygen. Solute in the medium (Nadeem *et al*., 2009).



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The process of aeration and stirring enables the transfer of oxygen into the growth medium, which is the basis for the production of xylanase, as oxygen has different effects on the formation of the enzyme during aerobic fermentations due to its effect on the metabolic pathways of the microorganisms that produce it (Potumarthi *et al*., 2007).
Naidu and Devi, (2005), confirmed the existence of a relationship between enzyme synthesis and energy metabolism, which is controlled by heat and the untake of oxygen.

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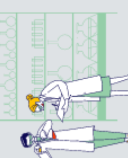

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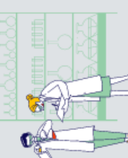

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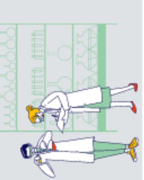

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