Production and Optimization of Xylanase Enzyme from Bacillus cereus by Submerged Fermentation Method

Ashraf Abd Alhussien[1], Rahman Laibi Chelab[2]

[12]*Biology Department, College of Education for pure science, Thi -Qar University, Thi-Qar, Iraq*

KEYWORDS

Bacillus Cereus, Xylanase, Submerged Fermentation, Lugol's solution.

ABSTRACT

Three strains of Bacillus cereus were isolated from soil samples in this study in an effort to enhance the circumstances for the production of xylanase by the bacteria. The bacteria released extracellular xylanase throughout the submerged fermentation process. In a 250 ml conical flask, the whole fermentation process was conducted at 150 rpm stirring speed. The highest amount of xylanase synthesis was demonstrated by the isolated strains at 45°C, 48 hours of incubation, pH 5, 1.5% oat splet xylan base concentration, lactose carbon source, and beef extract source.

1. Introduction

Enzymes are biological materials or biological macromolecules that can be created by living things. They are biological substances that catalyze particular biochemical reactions (Mohamed et al.,2023). The preservation of enzyme activity in settings with temperature fluctuations (55 °C to 121 °C and -2 °C to 20 °C), pressure (>500 atm), pH (pH > 8, pH < 4), and salinity (1-5 M NaCl or NaCl) are requirements for industrial activities. One common use of industrial biotechnology is the synthesis of bacterially derived enzymes, such as cellulase, protease, xylanase, and amylase. Since the US Food and Drug Administration (FDA) has classified the Bacillus genus as generally recognized as safe and it has been demonstrated via study, using it to produce biomolecules is a promising approach. It has been discovered that this genus is capable of producing and secreting enzymes with a wide range of uses(Bruno et al.,2020). three organic substances are found in plant cell walls: cellulose, hemicellulose, and lignin. The primary component of hemicellulose, a complex polysaccharide made up of a chain, is xylan. Because of its intricate structure, xylan necessitates the coordinated action of multiple enzymes to completely break down. the most significant of which is xylanase, which contributes to the degradation of xylan (Mahesh et al.,2021). The most effective method for generating microbially derived commercial xylanase is fermentation (Veerakumar et al.,2022) . It is well known that bacteria produce xylanase, which is suited for industrial needs since it develops more quickly than fungus and is stable at high pH and temperatures (Sampaio et al.,2018). In biotechnology, xylanase is used in a variety of processes, including feed digestion, food preparation, and wastewater treatment. The pulp and paper sector, textiles, biofuels, animals, and fermentation technologies (Muhammad et al.,2022).this research study evaluates the ability of microorganisms present in soil collected from the Al-Chibaish marshes in Thi-Qar Governorate, Iraq, to produce xylanase from B.cereus isolates and characterize the enzyme. It also studied the ideal conditions: pH, temperature, incubation period, carbon source, and nitrogen source.

2. Methodology

Soil samples were collected from the Al-Chibaish marshes in Thi-Qar Governorate. the samples were kept in sterile Polythene bags and stored at 4℃ in laboratory for further experimental analysis(Ameer *et al.*, 2016).

Microbial Cultures

Serial dilutions (10^{-1} – 10^{-6}) were performed on all soil samples, where 1 gram of soil was taken and placed in test tubes containing 9 ml of distilled water, and the tubes were placed in a water bath device at a temperature of 80 ℃ for 10 min. 0.5 ml of each dilution was taken, then spread on a plate of

nutrient agar, then incubated for 48 h, at a temperature of 37 ℃(Fakhraddin *et al*., 2014).

Identification of Isolated Microorganisms

The isolated microorganisms were identified by morphological examination, biochemical characteristics, morphological test, gram staining and spore staining Biochemical tests Indole test Methyl red test, Voges proskar test, Citrate consumption test, Catalase test, Oxidase test, Motility test, Gelatin hydrolysis test, Hemolysis test, Starch hydrolysis test(Mohamed *et al*., 2023).

Produce the Enzyme Xylanase

The bacterial isolates were grown on oat spelt xylan agar medium, which consists of (oat spelt xylan 0.5% , Yeast extract 0.5% , Nacl 1%, Agar-agar 2%) the dishes were placed in the incubator at 37℃ for 48h, after which the dishes were immersed in a Lugol's solution. It was noted that there was a clear transparent area around the bacterial colony producing the enzyme xylanase (Bruno *et al*., 2020).

Production Medium

The medium for xylanase production consists of (oat splet xylan 0.5%, yeast extract 0.5%, Nacl 1%). the broth was prepared in 100 ml in a conical flask. The broth was sterilized and left to cool. the medium was then inoculated with 100 μl of bacterial inoculum. the medium was then incubated 37℃ for 48 h on a rotary shaker at 150 rpm. after incubation, the enzyme was separated by centrifugation at 10,000 rpm for 10 minutes. the supernatant fraction representing the source enzyme is taken (Moorthy *et al*., 2019).

Enzyme Assay

Xylanase enzyme was assayed by adding 1 ml of enzyme to 1 ml of 1% soluble oat solution and 1 ml of 0.1 M phosphate buffer solution and incubated at 50 °C for 30 min. the reaction was stopped by adding 2 ml of 3,5-dinitrosalicylic acid reagent, then the mixture was placed in a water bath at boiling temperature for 10 min, then left to cool, after which the absorbance was measured by a spectrophotometer at a wavelength of 540 nm.

Optimal Conditions of Xylanase Production

Effect of incubation period on xylanase activity: In order to determine the effect of incubation period on extracellular enzyme production, the selected bacterial isolate was grown in oat splet xylan broth and incubated at 37℃ for 24 , 48 , 72 , 96 , 120h . The culture broth was then centrifuged at 10,000 rpm for 10 min to obtain the supernatants in which extracellular xylanase activity would be measured.

Effect of temperature on xylanase activity: In order to determine the effect of temperature on extracellular enzyme production, the selected bacterial isolate was grown in broth and incubated at 32, 37, 45, 50 and 55°C for 48h. The culture broth was then centrifuged at 10,000 rpm for 10 min to obtain the supernatants in which extracellular xylanase activity would be measured.

Effect of oat splet xylan concentration on xylanase activity: Under optimal temperature, pH, and incubation period. Five concentrations of the main substrate represented by oat splet xylan (0.5%, 1%, 1.5%, 2%, 2,5%) were tested. The production of the enzyme xylanase was measured.

Effect of pH value on xylanase activity: Effect of initial media pH on xylanase production was performed by adjusting oat splet xylan broth to pH 5.0, 6,7, 8, 9,10 and 11 before bacterial inoculation. after 48 h of incubation at 37°C, the culture broth was then centrifuged at 10,000 rpm for 10 min to obtain the supernatants from which extracellular xylanase activity was measured.

Effect of carbon sources on xylanase activity: under optimum temperature, pH, and incubation period. Five different carbon sources (Lactose, Maltose, Dextrose, Fructose, Glucose) with the same concentration of 0.5% were tested for xylanase production.

Effect of nitrogen sources on xylanase activity: The effect of different nitrogen sources was selected by adding 0.5% of the nitrogen sources (peptone, Beef extract, Meat extract, Urea, KNO3). all of these media were sterilized separately at 121℃ for 1h. The flasks were incubated at 37℃ for 48h. The amount of enzyme produced was estimated.

3. Result and Discussion

Samples Collection and Identification of Bacteria

Soil samples were collected from the Al-Chibaish marshes in Thi-Qar Governorate, and the samples were diagnosed using morphological tests based on the shape, color, and size of the colonies, and biochemical tests. Most of the results indicated that the isolates belong to *B. cereus*, and they were studied as shown in Table 1.

Table 1 : morphological and biochemical characteristics for the identification of isolates

morphological and biochemical	***Bacillus cereus* RAT5**	***Bacillus cereus* RAT3**	***Bacillus cereus* RAT1**
Gram stain	+	+	+
Endospore Stain	+	+	+
Shape	Rod	Rod	Rod
Starch test	+	+	+
Blood hemolysis	+	+	+
Catalase test	+	+	+
Oxidase	-	-	-
Gelatin	-	-	-
Mannitole	-	-	-
Klikler Iron test	K/A	K/A	K/A
Indole test	-	-	-
Methyl Red test	+	+	+
Voges proskar test	+	+	+
Motility	+	+	+

Screening of Xylanase Activity.

Xylanase activity was used to examine the rate of enzymatic activity using Lugol's solution , as in Figure1.

Figure 1: Cellular activity of *B. cereus RTA5* Isolation of xylanase-producing bacteria using agar medium containing 0.5% (wt/vol) oat splet xylan. Samples were incubated at 37℃ for 48h. Plates were stained with Lugol's solution. the clear area indicated hydrolysis of oat splet xylan to produce xylanase.

Effect of Incubation period on Xylanase activity

As shown in Figure 2, the isolates *B. cereus* RAT5, *B. cereus* RAT3, and *B. cereus* RAT1 were incubated for varying lengths of time (24 h–120 h). The maximum enzyme activity was displayed (0.645 OD, 0.456 OD, and 0.513 OD, respectively). The best time for enzyme production was 48 hours, and xylanase production fell at 120 hours.

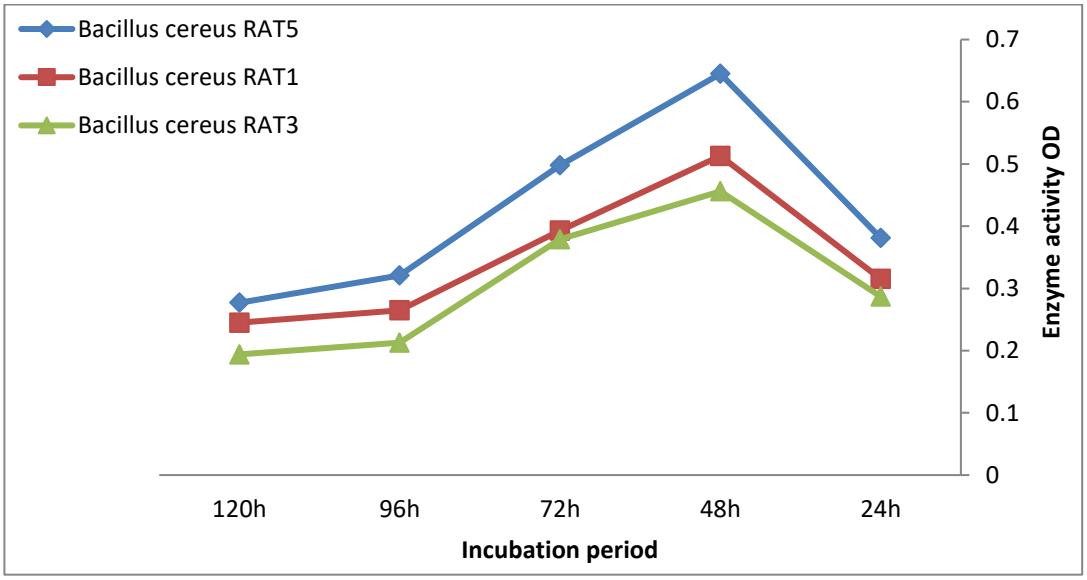

Figure 2: Incubation period study of xylanase production by *B.cereus* RAT5,*B.cereus* RAT3,*B.cereus* RAT1 in submerged fermentation at 37℃

Effect of Temperature on Xylanase activity

According to Figure 3, the greatest activity of the xylanase enzyme was observed at 0.851 OD, 0.685 OD, and 0.994 OD, respectively, at 45°C, which was shown to be the optimal temperature for enzyme synthesis.

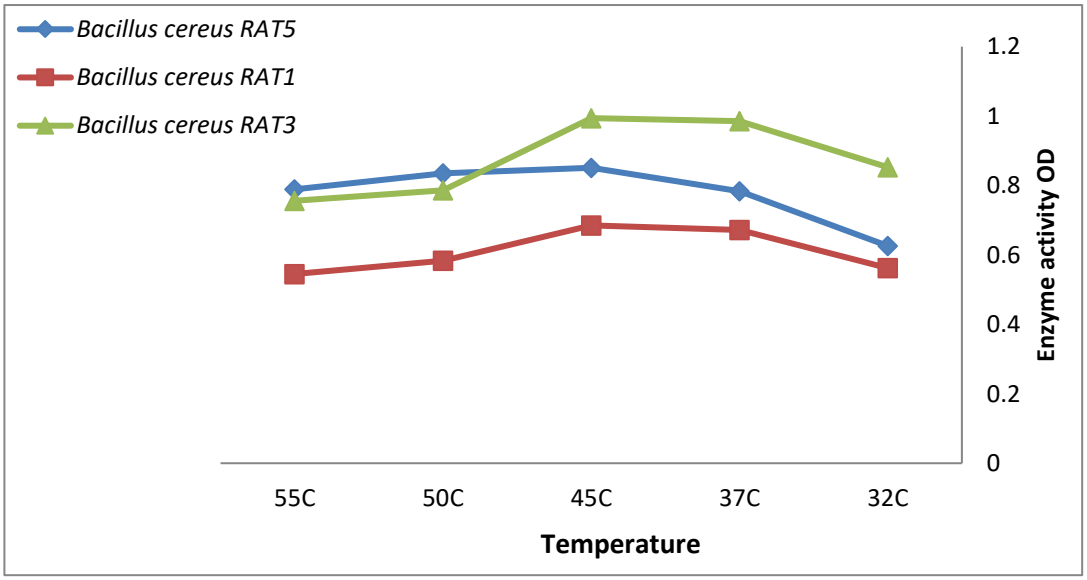

Figure 3: Effect of temperature on the activity of xylanase. Enzyme activity was assayed in phosphate buffer pH 6 at different temperature.

Effect of PH on Xylanase activity

The isolates, were incubated for 48 hours at varying pH levels of 5 to 11. The results indicated that pH

5 was the optimal pH for xylanase enzyme production, with maximum limits of enzyme activity (0.601 OD, 0.627 OD, and 0.768 OD, respectively). This is illustrated in Figure 4.

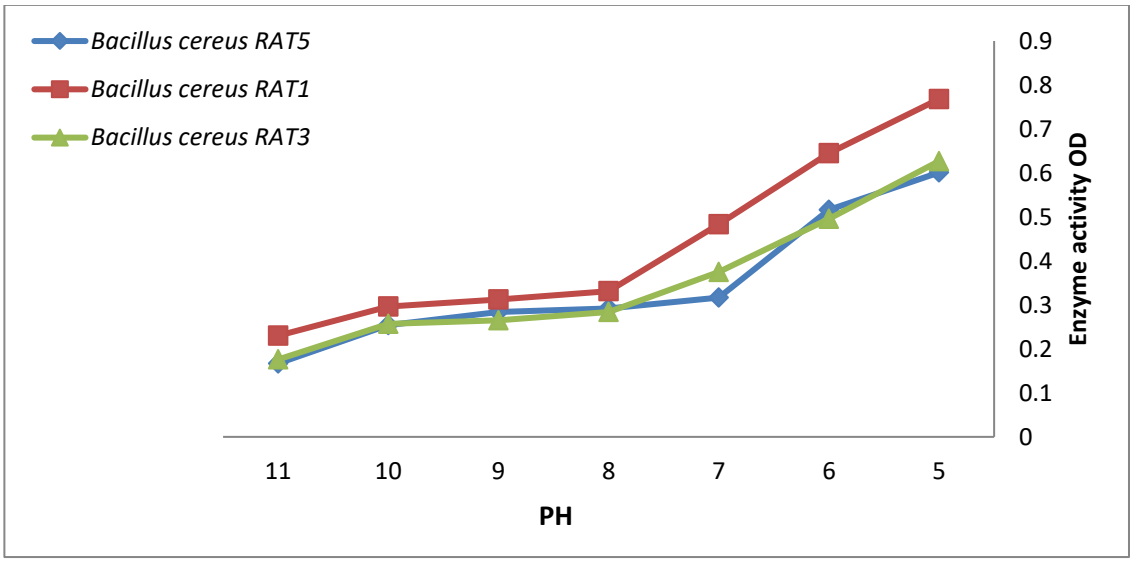

Figure 4: Effect of pH on activity of xylanase produced by *B. cereus RAT5*,*B. cereus RAT3*,*B.cereus RAT1*. in submerged fermentation**.**

Effect of Concentration of Substrate Oat Splet Xylan on Xylanase activity

The impact of varying substrate (oat splet xylan) concentrations, ranging from 0.5% to 2.5%, on the xylanase production by *B. cereus* RAT5, *B. cereus* RAT3, and *B. cereus* RAT1 is depicted in Figure (5). during submerged fermentation. The findings showed that 1.5% substrate concentration was the greatest xylanase production (0.922 OD, 0.980 OD, 0.907 OD), and that increasing substrate concentration further did not significantly impact xylanase production.

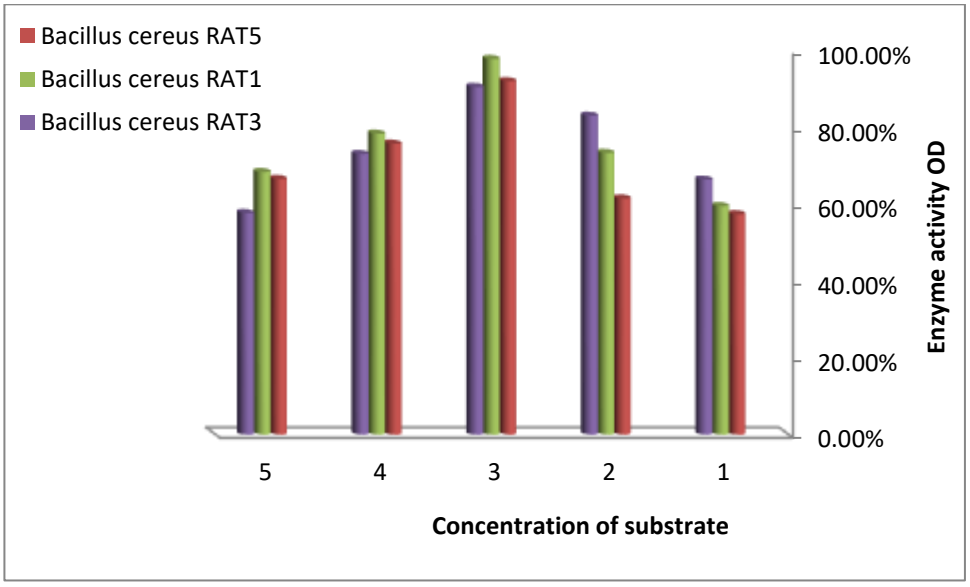

Figure 5: Effect of substrate concentration on xylanase production by *B. cereus* RAT5,*B. cereus* RAT3,*B. cereus* RAT1. in submerged fermentation (initial pH 6, 48 h period).

Effect of Carbon Source on Xylanase activity

By adding an appropriate carbon source to the fermentation medium, xylanase output was significantly

increased. The maximum reported enzyme activity was (0.734OD, 0.863OD, 0.995OD) for *B. cereus* RAT5, *B. cereus* RAT3, and *B. cereus* RAT1 in submerged fermentation, indicating that lactose at a concentration of 0.5% was the optimum catalyst for xylanase. Regarding other carbon sources, xylanase production was reduced by dextrose, fructose, glucose, and maltose as shown in the Figure (6).

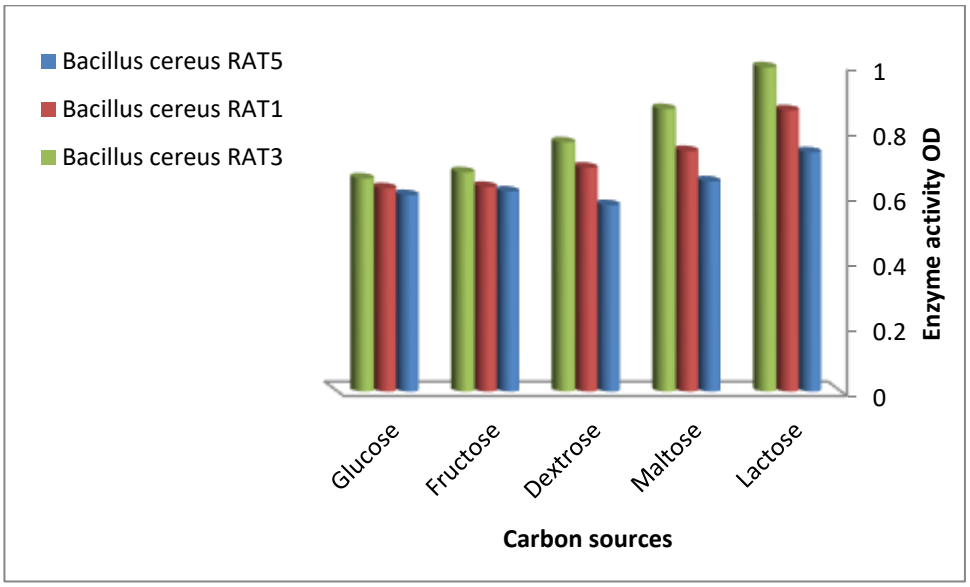

Figure 6: Effect of supplementation of additional carbon sources on xylanase production by *B. cereus RAT5,B. cereus RAT3,B. cereus RAT1* . in submerged fermentation (initial pH 6, 48 h period).

Effect of Nitrogen Source on Xylanase activity

The best organic and inorganic nitrogen source for producing the xylanase enzyme by B.cereus RAT5, B.cereus RAT3, and B.cereus RAT1 bacteria was found to be beef extract. The maximum activity of the enzyme was (0.851 OD, 0.706 OD, and 0.545 OD), respectively, and Figure 7 illustrates how enzyme production decreases at KNO3, as shown in the Figure (7).

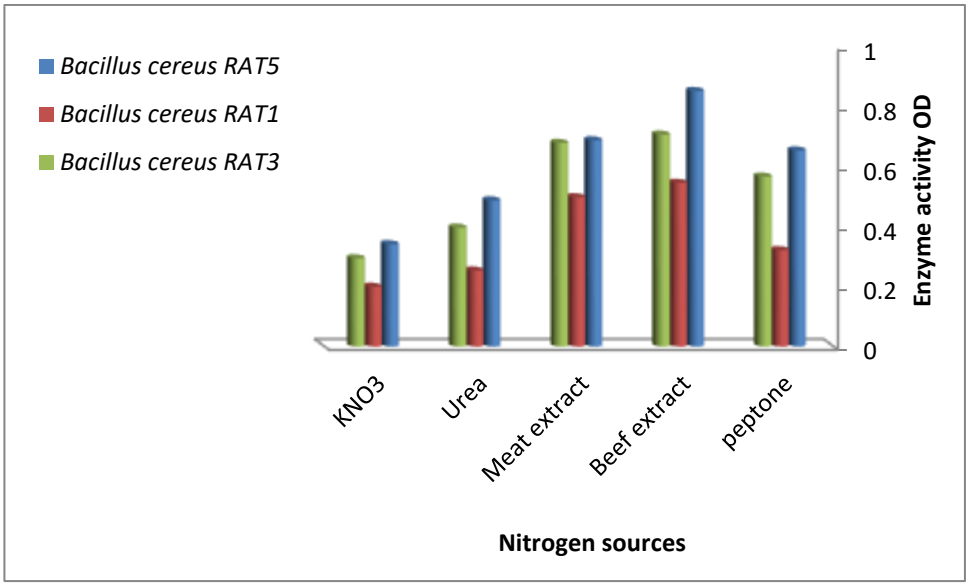

Figure 7: Effect of different nitrogen sources on xylanase production by *B. cereus RAT5, B. cereus RAT3, B. cereus RAT1*. in submerged fermentation (initial pH 6, 48h period).

DISCUSSION

The study was carried out in the Microbiology Laboratory at the College of Education for Pure Sciences/Department of Biology, Thi-Qar University, Iraq, using soil samples that were taken from the Al-Chibaish Marsh in the Thi-Qar Governorate. The study's findings demonstrated that, when the isolates of Bacillus cereus were grown on oat xylan agar medium under optimal conditions—37°C for 48 hours and a pH of 6—the soil was rich in these isolates. After applying Lugol's solution to the bacterial colonies, a translucent halo measuring 2.3 cm in diameter developed around them, and the isolates also demonstrated their vitality. on the medium's breakdown by the xylanase enzyme's synthesis, and these outcomes agreed with the findings published by (Bruno *et al*., 2020). After growing on nutritional agar medium for 24 hours at 37°C and stained with Gram stain, the bacterial isolates appeared violet in the shape of groups, according to the results of light microscopy examination. Gram positive status is indicated by its rod-shaped or single form. The biochemical test results were in line with the findings reported by (Srikant *et al.*, 2020). and indicated that the bacterial isolates were *B. cereus*. The 48-hour fermentation duration proved to be optimal for yeast, according to the results of the xylanase production time period. After that, a further extension of the fermentation period resulted in a decrease in the production of enzymes because microbial growth produces toxic compounds that prevent the synthesis of enzymes. These outcomes concur with those of (Muhammad *et al*., 2016). concerning the pH effect, which demonstrated that all *Bacillus cereus* isolates exhibited a peak in enzyme activity at pH 5 and a decline in enzyme output at pH 10,11. The bacteria could withstand the medium's acidic conditions, according to these data. The results of all the isolates also demonstrated that the temperature at which enzyme production was highest, 37°C, was significantly lower at 50°C and 55°C. Concerning the impact of substrate concentration on xylanase synthesis, the findings demonstrated that enzyme production peaked for all *B.cereus* isolates at 1.5% concentration, and that further concentration increases had no discernible influence on enzyme production. by adding an appropriate extra carbon source to the fermentation medium, xylanase output was significantly increased. In submerged fermentation, lactose at a concentration of 0.5% was the most effective inducer of xylanase by *B. cereus*. There was less enzyme production from other carbon sources, such as fructose and glucose. Regarding the nitrogen source's influence, xylanase production by *B.cereus* isolates is best catalyzed by beef extract among all the organic and inorganic nitrogen sources that were chosen; nevertheless, production was reduced when nitrogen sources such as urea and kNO3 were used.

4. Conclusion and future scope

In conclusion, the results obtained from the present work indicate a significant amount of xylanase production from a new isolate B. cereus using selective growth and nutritional conditions. The xylanase produced by the test organism due to its wide activity, temperature range and cellulose-free nature appears to have great use in the paper and pulp industry.

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