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Enhanced Xylanase Production from *Bacillus safensis* MABS6 using Sorghum Straw Substrate: Optimization, Characterization, and Biotechnological Applications

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ABSTRACT

The objective of this study was to evaluate the synthesis of xylanase enzyme by *Bacillus safensis* MABS6 using sorghum straw as a substrate. A comprehensive investigation was conducted to optimize xylanase yield by examining various parameters such as sorghum concentration, inoculum size, culture age, pH, temperature, and agitation speed. Additionally, the impact of nutritional additives, metallic ions, organic solvents, and alkaline H_2O_2 treatment on xylanase production and activity was explored. Experimental trials were performed with specific parameters, including 4% w/v sorghum concentration, 2% inoculum size, 12 hours of culture age, pH 7, 35°C, and 250 rpm. Further improvements involved the addition of nutritional additives such as gelatin, xylose, and potassium nitrate. The effects of initial conditions, various metallic ions (Ca^{2+}, Mn^{2+}, and Fe^{3+}), and organic solvents (2-methyl propanol, methanol, and ethanol) on xylanase activity were evaluated. Additionally, saccharification investigations with a 4% alkaline H2O2 treatment assessed the enzymatic hydrolysis of sorghum straw. Optimized conditions resulted in a significant increase, with xylanase production reaching 3.49%. The partially purified xylanase exhibited over 60% relative activity within a pH range of 6 to 9 and demonstrated more than 55% activity between 45°C to 65°C after 1 hour. Certain metallic ions and organic solvents further enhanced xylanase activity. Notably, the xylanase derived from *Bacillus safensis* MABS6, utilizing sorghum straw, showcased desirable characteristics such as heat stability, alkali-solvent stability, and absence of cellulase activity. Its potential as a biocatalyst makes it valuable for various biotechnological applications, particularly in the efficient enzymatic hydrolysis of sorghum straw.
Keywords: *Bacillus safensis* MABS6; Biocatalyst; Enzyme optimization; Sorghum straw; Xylanase.

INTRODUCTION

Xylanase represents the prevailing non-cellulosic polysaccharide in terms of abundance (Simmons *et al.*, 2016). It is the large family of high molecular weight polysaccharides found in hemicelluloses that are insoluble in water but soluble in alkaline solutions (Xiao *et al.*, 2001). These polysaccharides aid in the development of plant cell walls by fusing with cellulose and lignin (Kang *et al.*, 2019). The main hemicellulose found in plant cell walls is xylan (Avci, 2022) and it is the second most common polysaccharide in nature (after cellulose) (Yousuf *et al.*, 2020; Šuchová *et al.*, 2022). Because of their wide range of structures, complexities, and polymerizations, endo-xylanases play a vital role in the industrial hydrolysis of xylan into short xylo-oligosaccharides and xylose (Kaushal *et al.*, 2021). Other auxiliary enzymes, such as β-xylosidase, α-L-arabinofuranosidase, and α-glucuronidase, also contribute to this process (Malgas *et al.*, 2019).

At the crux of a tropical plant biomass, a noteworthy revelation emerges-xylene, a polysaccharide, takes up a substantial 20 to 35% of the total dry weight (Oliveira *et al.*, 2020). Bacteria, fungi, actinomycetes, and yeast have all been found to produce xylanases (Bhardwaj *et al.*, 2019). Nonetheless, bacteria are often used for xylanase synthesis because of their enormous metabolic diversity. Many species of *Bacillus* are known to produce large amounts of extracellular xylanases that are either deficient in cellulase activity or completely cellulase-free (Baramee *et al.*, 2020). Biological fuels like ethanol and xylitol may be produced from lignocellulosic biomass using xylanases in combination with other enzymes (Patel and Shah, 2021).

Endoxylanase, xylosidase, glucuronidase, arabinofuranosidase, and acetylxylan esterase are all components of the multi-functional enzyme known as xylanase (Biely, 2002). Endoxylanases are enzymes that are responsible for catalyzing the random hydrolysis of xylan to produce xylooligosaccharides (Thirametoakkhara *et al.*, 2022). On the other hand, xylosidase is an enzyme that is responsible for releasing xylose residues from the non-reducing ends of xylooligosaccharides (Knob *et al.*, 2010). Because of its ability to convert xylan into useful products such as xylose, a sugar with fewer calories, and L-arabinose, a prebiotic, xylanase has garnered the interest of a significant number of researchers. Fungi, bacteria, and yeast are all capable of producing xylanases (Khan *et al.*, 1986). Bacterial genera include *Bacillus*, *Cellulomonas*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, *Arthrobacter*, *Microbacterium*, *Pseudoxanthomonas*, *and Rhodothermus* (Chakda *et al.*, 2016), as well as several species of fungi such as *Trichoderma*

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and *Aspergillus* (Polizeli *et al.*, 2005). It is still a tiresome effort to choose promising isolates, particularly when physiologically potential strains of *B. subtilis, B. stearothermophilus, B. amyloliquefaciens, B. circulans,* and *B. pumilus* need to be acquired in order to achieve maximal enzyme production (Logan *et al.*, 2011).

Xylanases, pivotal in food and feed industries, also contribute to global economy through single cell proteins (SCPs), syrups, and fuels. Beyond, they enhance dough, bleach pulp, boost detergent efficiency, and play roles in deinking, fuel alcohol, and flavor extraction (Tyagi and Sharma, 2021; Blasi *et al.*, 2023). Researchers study microbiological enzymes because they are important in biological and industrial processes. The hydrolytic enzyme market is dominated by xylanase. The greatest barrier to commercial application of xylanases is its high production cost. Industrial enzyme production is impossible due to the high cost of purified substrates (Immerzeel and Fiskari, 2023). Research on cost-effective substrates for efficient enzyme synthesis, such as agricultural waste, is imperative (Sadh *et al.*, 2023). Optimization of enzyme synthesis conditions, dependent on bacterial strains, is crucial for maximizing industrial production by adjusting physiological and nutritional factors (Su *et al.*, 2020). Additionally, addressing stability issues of known xylanases in extreme industrial conditions prompts efforts to discover more effective enzymes (Hauer, 2020).

The objective of this study was to assess the production capacity of xylanase in recently identified *Bacillus* species by utilizing cost-effective agricultural residues. Subsequently, a continous purification process was implemented to investigate the potential application of the enzyme in the saccharification of sorghum straw. Additionally, a comprehensive characterization analysis was conducted to gain insights into the enzymatic properties and performance of the xylanase.

MATERIALS AND METHODS

Screening of a xylanase-producing strain

Initial Screening

A diverse range of soil samples were collected, in mid-April 2022, from the operational compost heap at a depth of 10 cm. Each specimen was meticulously mixed, and 1 gram of the material was suspended in 50 ml of sterile distilled water. The suspension was allowed to settle, and subsequent serial dilutions were prepared. To determine xylanase activity, 0.1 ml of each dilution was incubated at 37℃ for 48 hours on 1% w/v oat spelt xylan agar plates (Chen *et al.*, 1997). Colonies exhibiting a distinct zone of xylan hydrolysis were selected for further screening.

Cultivation and assessment of xylanase activity of selected isolates

The distinct isolates obtained from the initial screening underwent further cultivation in specialized broth medium (Adhyaru *et al.*, 2014). The formulation of this medium, prepared in a 100 ml volume, consisted of oat spelt xylan (10 g), NaCl (5 g), beef extract (3 g), potassium nitrate (2 g), dipotassium hydrogen phosphate (1 g), and magnesium sulfate (0.5 g), dissolved in distilled water to achieve a total volume of 100 ml. The pH of the medium was adjusted to 7. The inoculated broth medium was incubated at 37°C in a shaking incubator at 150 rpm for 48 hrs. Following the incubation period, the fermented broth was subjected to centrifugation at 10,000 x g for 10 minutes, resulting in the separation of the supernatant. The obtained supernatant was utilized for the assessment of xylanase activity (Adhyaru *et al.*, 2014).

Identification the most potent isolate

Following a rigorous screening process, fifteen distinct bacterial cultures were selected for their ability to produce xylanase. Among these cultures, *Bacillus safensis* MABS6 demonstrated the most promising characteristics based on morphological and biochemical analysis, as well as 16S rDNA sequencing (Singh *et al.*, 2013). The identified *Bacillus safensis* MABS6 was deposited in the National Center for Biotechnology Information (NCBI) data bank under the accession number OQ788330 (https://ncbi.nlm.nih.gov/nuccore/OQ788330). In addition, to validate the identification and relationship of Bacillus safensis MABS6, the provided gene sequence was subjected to a thorough NCBI database search and dendrogram construction using Mega 5.2 software. This analysis ensured accurate classification and characterization of the selected strain.

Optimization of Xylanase Production from Various Agro-Residues

To examine and select the optimal agro-residues for xylanase production, selected residue were included sawdust, rice straw, barley straw, sorghum straw, wheat straw, and maize straw. To initiate xylanase synthesis, a 2% (w/v) of the chosen agro-residue was added to 100 ml of a specialized liquid medium, maintaining a pH of 7.0. The liquid medium contained the same components as the final screening, except for oat spelled xylan.

After sterilization at 121 °C for 15 minutes, the flasks were cooled and inoculated with a 1% (v/v) bacterial culture. The inoculated flask was then placed in a shaking incubation at speed of 150 x g and incubated for 48 hours at a temperature of 37 ℃. To assess xylanase activity, the fermented broth was centrifuged at 10,000 x g for 10 minutes at 4 ℃, the clear supernatant was taken and examined. The xylanase enzyme test was performed to evaluate the presence and effectiveness of xylanase production.

This methodology ensured the examination of different agro-residues and their impact on xylanase production, providing valuable insights into the optimization of the process. All studies were performed in triplicate. High-quality, locally purchased ingredients (Sigma- Aldrich and Merck) such as oat spelt xylan, carboxy methyl cellulose (CMC), bovine serum albumin (BSA), and dinitrosalicylic acid (DNSA) were used in this study. According to Bailey *et al.*, 1992 xylanase activity was determined. 450 milliliters of 1% oat spelled xylan in 50 mM of sodium phosphate buffer (pH 7.0) and 50 milliliters of the enzyme were mixeds and incubated for 10 minutes at 50 °C. The 3,5-dinitrosalicylic acid (DNS) technique, as proposed by Miller, 1959 was used for quantifying the reducing sugar released during the experiment. In accordance with the suggestion provided by IUPAC, the CMCase and FPase activities were analyzed (Ghose, 1987). Under the circumstances that were outlined, the quantity of enzyme that was necessary to free 1 mmol of xylose or glucose equivalent per minute was referred to as one unit of xylanase activity or cellulase activity, respectively. The protein quantity was ascertained utilizing the technique delineated by Lowry *et al.*, 1951, with bovine serum albumin (BSA) employed as the reference standard.

Characterization of cellulase-free xylanase production processes

Optimization of physiological parameters

Inoculum size

The production medium was inoculated with 12-hour-old bacterial culture broth at 0.5, 1, 2, 3, 4, and 5 % v/v to examine the influence of inoculum size. Moreover the inoculum size was compared with McFarland scale using as standard with same concentrations as the bacterial culture have 9.0 x 10^8 approximate bacterial count/ml. The bacterial inoculum preparation was done following the method of Hasegawa *et al.* (2000).

Age of inoculum

To investigate the impact of inoculum age on xylanase production, Bacillus safensis MABS6 was added to 50 ml of basal liquid medium. The inoculum was then incubated at 37 °C with shaking at 150 x g. At regular intervals of 6 hours, 1% v/v of the inoculum was transferred to 100 ml of fresh fermentation medium. The mixture was incubated at 37°C for 48 hours with shaking at 150 revolutions per minute. After centrifugation, the culture filtrate was collected for further enzyme assay.

Incubation period

To optimize xylanase synthesis, various incubation periods ranging from 12 to 60 hours were tested. Extraction and measurement of the crude enzyme were performed at regular intervals of six hours to monitor the enzyme activity and determine the optimal incubation period for maximum xylanase production.

pH

The fermentation medium initially exhibited a pH range of 3 to 10. To achieve the desired pH, the mixture was adjusted by adding either 1 M HCl or 1 M NaOH before undergoing autoclaving. This ensured the optimal pH conditions for the fermentation process.

Incubation Temperature

The influence of incubation temperature on xylanase production was examined across a temperature range of 25 to 50 °C. Various temperatures were analyzed to determine the optimal temperature for maximizing xylanase production.

Agitation Speed

The impact of agitation speed on xylanase production was studied by testing multiple speeds ranging from 0 to 300 rpm. Each speed was incremented by 50 rpm to evaluate the effect of agitation on maximizing xylanase synthesis.

Optimization of Nutritional Parameters

To optimize the nutritional parameters, the previously determined optimal conditions were applied.

Effect of Carbon Sources

The impact of various carbon sources on xylanase production was investigated using a concentration of 0.5% (w/v). This study included evaluation of different monosaccharides (glucose, fructose, xylose, galactose, mannitol), disaccharides (lactose, maltose, sucrose), and polysaccharides (cellulose, starch) to determine their influence on xylanase synthesis.

Effect of Nitrogen Sources from Organic and Inorganic Origins

To examine the impact of nitrogen sources on xylanase production, various organic sources (peptones, beef extract, yeast extract, malt extract, meat extract, casein, tryptone, gelatin, urea, and skimmed milk powder) and inorganic sources (diammonium hydrogen phosphate, diammonium dihydrogen phosphate, potassium nitrate, diammonium sulfate, ammonium nitrate, ammonium chloride, sodium nitrate, and ammonium sulfate) were investigated. The concentrations of these nitrogen sources were tested at 0.5% and 0.3% (w/v) to evaluate their potential in enhancing xylanase synthesis.

Characterization of partial-purified xylanase

A saturation solution of 70% was made by adding solid ammonium sulphate very gradually to the culture supernatant while it was being stirred constantly in an ice bath. After centrifugation at 10,000 rpm for ten minutes at 4 °C, the supernatant was discarded, and the precipitate was resuspended in 10 ml of a sodium phosphate buffer of pH 7.0 having a concentration of 50 mM. The resulting solution was dialyzed overnight against the same buffer at 4°C, with three changes of the buffer at sporadic intervals.

Temperature-Dependent Activity and Stability

To determine the optimal temperature for xylanase activity, the enzyme extract was incubated with a solution of 1% oat spelt xylan, varying the temperature between 35 and 75 °C. The thermostability experiment involved incubating the enzyme at different temperatures without a substrate for 120 minutes. Samples were collected at regular intervals to assess any remaining xylanase activity and evaluate the enzyme's stability at different temperatures.

pH-dependent activity and stability

The pH-dependent relative activity of xylanase was evaluated using a range of buffers. Buffers such as sodium citrate (pH 3-6), sodium phosphate (pH 6-8), and glycine-NaOH (pH 8-10) at a concentration of

50mM were employed to determine the optimal pH range for xylanase activity. Additionally, enzyme samples were incubated for 24 hours in various buffers to assess the stability of xylanase at different pH levels. The activity of the residual enzyme was measured by collecting samples at regular 3-hour intervals.

Effect of Metal Ions on Xylanase Activity

To investigate the influence of metal ions on xylanase activity, xylanase preparations were incubated for 30 minutes in 10 mM metal solutions. The enzyme assay test was employed to quantify the remaining enzymatic activity, providing insights into the effectiveness of different metal ions in either enhancing or inhibiting xylanase activity.

Effect of Solvents on Xylanase Activity

To evaluate the effectiveness of solvents on xylanase activity, various alcoholic and non-alcoholic solvents at a concentration of 10% (v/v) were employed. These solvents were chosen as they can serve as a medium for xylanase incubation. The incubation period lasted for 30 minutes. The residual enzymatic activity was measured using the standard test assay protocol (Bailey *et al.*, 1992) to assess the impact of different solvents on xylanase activity.

Chemical Pretreatment of Sorghum Straw

Prior to enzymatic hydrolysis, three different chemical pretreatments were performed on the sorghum straw. In each experiment, 10 g of dried and pulverized sorghum straw was used as the starting material for the pretreatment process.

Enzymatic Hydrolysis of Sorghum Straw

The enzymatic hydrolysis of sorghum straw was conducted in a 50 ml Erlenmeyer flask. Both untreated and pre-treated sorghum straw, at a concentration of 2.5% substrate, were combined with 20 ml of appropriately diluted crude enzyme. The hydrolysis process lasted for 48 hours at a temperature of 50 ℃ with an agitation speed of 100 revolutions per minute. To ensure reliable controls, enzymes that could not be activated by heat were used as substitutes for activated enzymes. Additionally, to prevent the growth of microorganisms, 0.005% sodium azide was added to the reaction system. Samples were collected at regular intervals over a period of 12 hours, and aliquots were obtained. After centrifugation, the supernatant was analyzed using the 3, 5-dinitrosalysilic acid technique to measure the concentration of total reducing sugar.

Statistical Analysis

The collected data was subjected to statistical analysis using GraphPad Prism 9 software. The results are presented as means ± standard error (S.E.).

RESULTS

Isolation and identification of Bacillus safensis MABS6

The newly isolated strain (Bacillus safensis MABS6) was found to be Gram +ve, spore-forming, rod-shaped lives in facultative aerobic environment. Tests for catalase and oxidase were positive. All above-mentioned results indicated the genus Bacillus of the current bacterium, furthermore, confirmation was carried out on 16S rDNA gene sequencing which was also submitted to the gene bank at NCBI, and the bacterium was assigned the name of B. safensis MABS6 (accession number OQ788330, https://ncbi.nlm.nih.gov/nuccore/OQ788330). The phylogenetic position within the genus and within the species has been shown in Figures (1) and (2) respectively.

Xylanase Production and the Impact of Agro-Residual Materials

The xylanase production of B. safensis MABS6 was evaluated using various agro-residual materials, including sawdust, rice, barley, sorghum, wheat, and maize. Among these materials, sorghum straw exhibited the highest xylanase production by B. safensis MABS6, with a value of 68.53±2.07 IU/ml. On the other hand, sawdust displayed the lowest xylanase production, with a value of 15.21±0.58 IU/ml. The xylanase activity against the remaining agro-residual materials is illustrated in Figure (3).

Additional experiments were carried out using submerged fermentation to examine the effects of varying amounts of sorghum straw, ranging from 2-8 % (w/v) under agitation condition. At 4% (w/v) of sorghum straw, xylanase synthesis reached its highest level (68.53±1.09 IU/ml). Figure (4) indicates the detailed effect of sorghum straw concentration on the xylanase activity. Beyond 4% sorghum straw concentration, xylanase production declines monotonically due to the formation of a thick suspension at higher substrate concentrations, was resulting in inadequate mixing under agitation conditions.

Optimizing Xylanase Production: Exploring factors and their Influence

Carbon and nitrogen source

The carbon sources were divided into three main classes: monosaccharides, disaccharides and polysaccharides. Among the other monosaccharides, xylose showed maximum xylanase production (159.01±3.98 IU/ml) followed by fructose, mannitol, galactose, and glucose (Table 1). Sucrose (118.98±3.56 IU/ml) among the disaccharides and starch (115.23±3.58 IU/ml) among the polysaccharides showed maximum xylanase activity. Minimum xylanase production was observed by Bacillus safensis MABS6 while glucose was used as a source of carbon in the current study.

The effects of different combinations of nitrogen sources on xylanase synthesis were investigated by adding them to the fermentation medium. Different organic and inorganic sources of nitrogen were used in the current study and found that maximum xylanase production (212.34±4.79 IU/ml) was observed by using gelatine followed by urea and others as organic nitrogenous source (Table 2).

Inoculum size, age, and incubation period

Maximum enzyme production requires a balance between the rapidly growing biomass and the limited supply of nutrients (Loeppmann *et al.*, 2020). 2% v/v inoculated media raised for 12 hrs. showed the

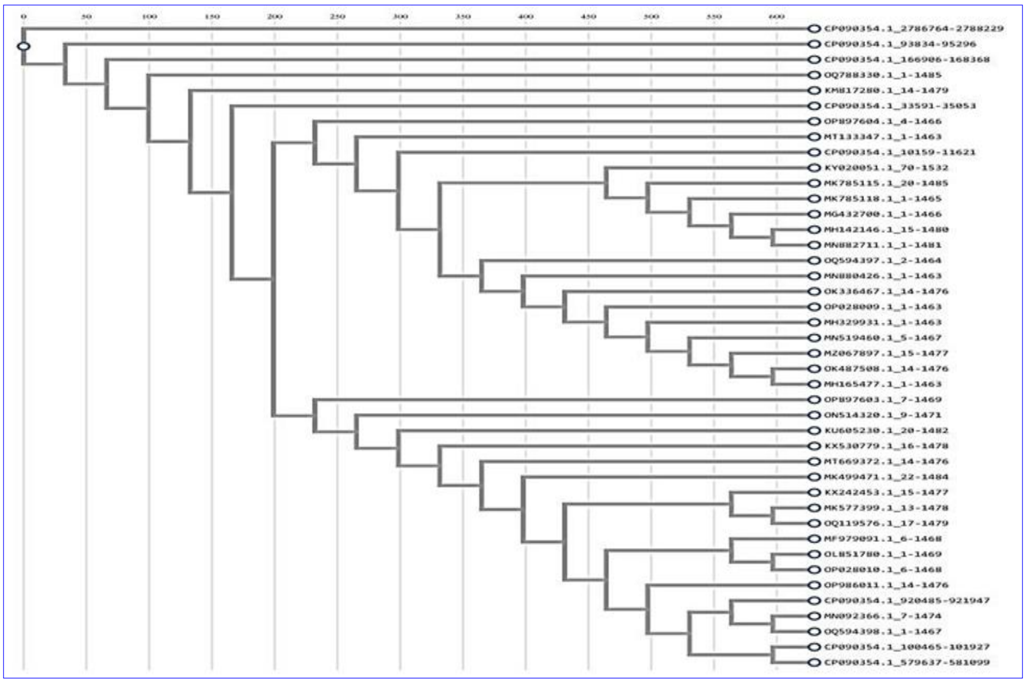

Figure (1): Phylogenetic Analysis of *B. safensis* MABS6 within the same genus.

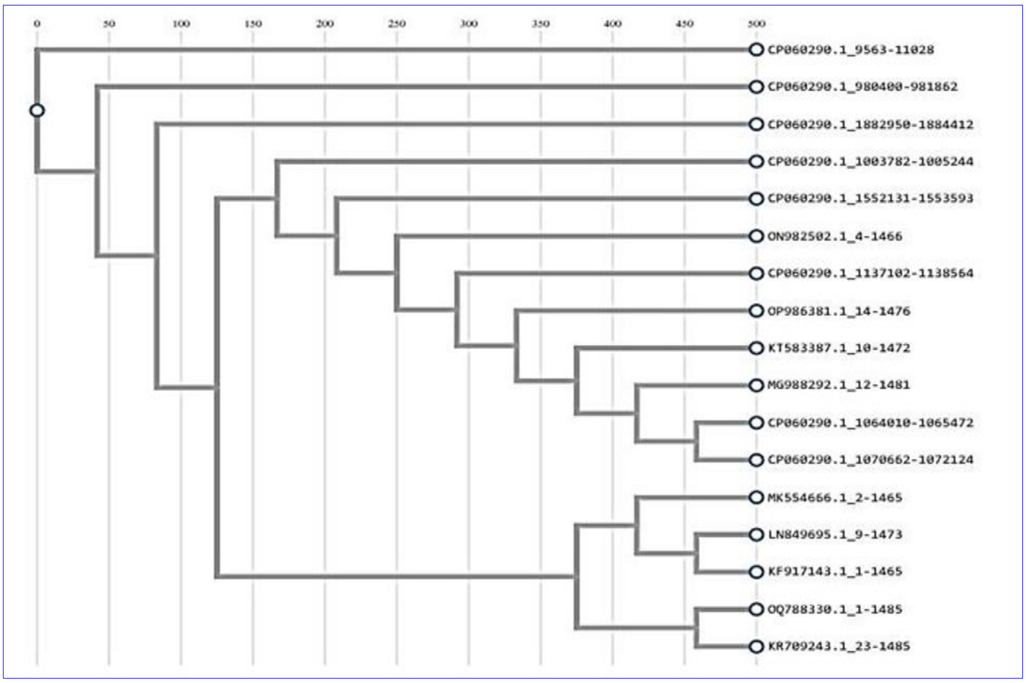

Figure (2): Phylogenetic Analysis of *B. safensis* MABS6 within the same specie.

maximum xylanase production (70.67±0.86 IU/ml) when 0.5 to 5% v/v inoculated media were subjected for determination of the highest yield of xylanase (Fig. 5) and this was comparable to 3% of McFarland scale with 9.0 x 10^8 approximate bacterial count/ml.

Investigating the influence of inoculum age on xylanase production, cultures of *Bacillus safensis* MABS6 were examined at various time points ranging from 6 to 48 hours. Experimental findings revealed that the maximum xylanase activity of 91.05±1.43 IU/ml was attained at the 12-hour inoculum age. Notably, the 18-hour-old inoculum displayed a comparable activity level of 89.95±2.08 IU/ml. Figure (6) provides a visual representation of the impact of different inoculum ages on xylanase production. Intriguingly, a decline in xylanase activity was observed beyond the 18-hour mark, suggesting a time-dependent effect on enzyme production. The highest xylanase activity (69.83±1.91 IU/ml) was observed after 36 hrs. of incubation time and decreased activity has been observed before and after that of this optimum incubation time (Fig.7).

Effect of different physiological parameters on the xylanase production

The impact of temperature, pH, and agitation speed on xylanase production revealed that xylanase production by *Bacillus safensis* MABS6activity found to be unsatisfactory below pH 5. However, at pH 6, a satisfactory level of xylanase production (62.03±1.56 IU/ml) was observed, which reached its maximum at pH 7 (88.17±2.03 IU/ml). Beyond pH 7, xylanase

Table (1): Effect of different carbon sources on xylanase production: enzyme activity, protein content, and specific activity. Data are presented as means ± standard error (SE).

Carbon source		Xylanase Activity (IU/ml)	Protein (mg/ml)	Specific Activity (IU/mg)
Control (No Carbon)		105 ±1.95	2.57±0.08	39.05±1.17
Monosaccharides				
	Glucose	59.08±3.24	2.07±0.06	27.16±2.39
	Fructose	92.92±2.56	2.16±0.12	36.14±1.55
	xylose	159.01±3.98	3.35±0.17	48.13±2.91
	Galactose	62.76±3.77	2.63±0.19	32.64±2.84
	Mannitol	84.13±3.96	2.89±0.09	31.11±1.37
Disaccharides				
	Lactose	59.11±1.87	2.39±0.19	28.89±2.96
	Maltose	79.39±2.15	2.18±0.06	32.17±0.92
	Sucrose	118.98±3.56	2.89±0.13	36.14±0.86
Polysaccharides				
	Cellulose	71.38±2.16	2.24±0.08	26.39±0.11
	Starch	115.23±3.58	2.18±0.06	32.17±0.92

Table (2): Effect of different organic and inorganic nitrogen sources on xylanase production: enzyme activity, protein content, and specific activity. Data are presented as means ± standard error (SE).

Nitrogen source		Xylanase Activity (IU/ml)	Protein (mg/ml)	Specific Activity (IU/mg)
Organic Source				
	Peptones	135.69±3.54	3.17±0.19	48.07±1.39
	Beef Extract	147.32±4.19	3.21±0.09	46.53±2.21
	Yeast Extract	184.44±5.51	3.48±0.07	49.96±0.86
	Malt Extract	105.05±2.46	2.4±0.16	31.19±0.41
	Meat Extract	91.27±3.11	2.17±0.16	38.89±1.11
	Casein	80.09±2.54	2.66±0.17	31.41±0.48
	Tryptone	157.61±2.69	3.79±0.11	47.13±1.51
	Gelatine	212.34±4.79	3.95±0.19	54.28±2.16
	Urea	196.54±6.13	3.27±0.1	59.89±1.67
	Skimmed milk powder	137.21±2.99	3.22±0.16	41.06±1.09
Inorganic Source				
	(NH4)2HPO4	194.56±7.19	3.44±0.12	49.76±0.87
	[NH4]H2PO4	102.97±4.33	3.18±0.15	32.48±0.96
	KNO3	254.41±3.26	3.96±0.18	62.23±1.98
	(NH4)2SO4	158.51±2.91	3.42±0.17	42.91±1.17
	NH4NO3	221.89±4.81	3.95±0.17	53.31±1.26
	NH4Cl	185.16±5.96	3.07±0.1	58.12±2.87
	NaNO3	186.31±2.08	3.18±0.15	55.17±1.78

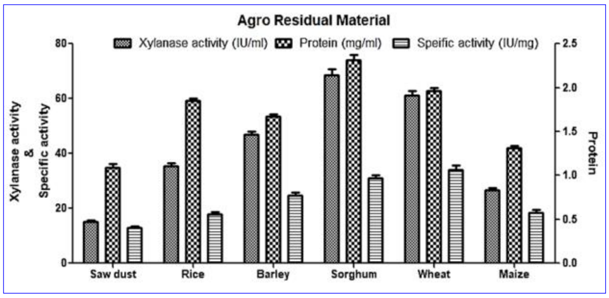

Figure (3): Influence of agro-residual materials on xylanase production: enzyme activity, protein content, and specific activity.

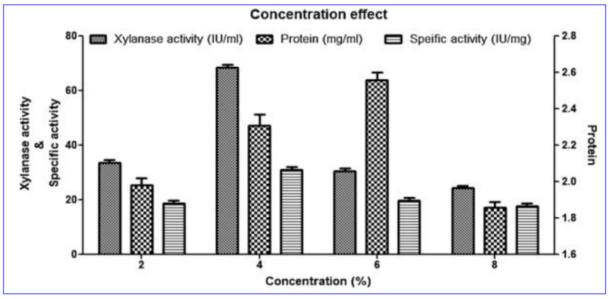

Figure (4): Influence of sorghum straw concentration on the xylanase production: enzyme activity, protein content, and specific activity.

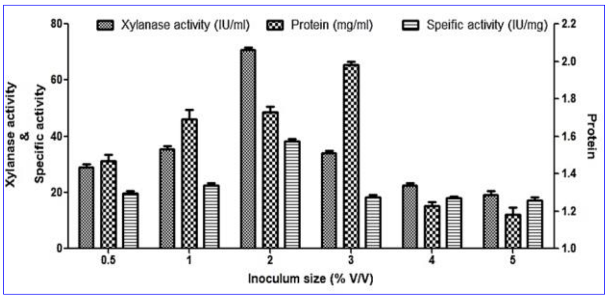

Figure (5): Influence of inoculum size on the xylanase production: enzyme activity, protein content, and specific activity.

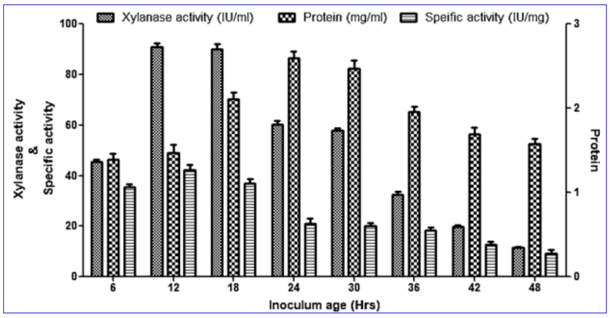

Figure (6): Influence of inoculum age on the xylanase production: enzyme activity, protein content, and specific activity.

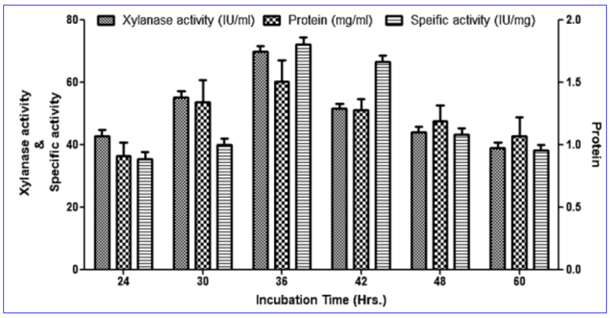

Figure (7): Influence of incubation period on the xylanase production: enzyme activity, protein content, and specific activity.

production gradually declined, with the lowest levels observed at pH 10 (Fig. 8). For the optimal temperature for enhancement xylanase production, the highest level of xylanase production (92.42±2.16 IU/ml) was observed at 35℃ (Fig. 9), and it significantly decreased beyond this temperature.

In submerged fermentation, effective mixing and maintenance of optimal oxygen levels are crucial for successful nutrient utilization. Agitation and aeration play pivotal roles in achieving these objectives. For *Bacillus safensis* MABS6, it was observed that agitation speeds ranging from 150 to 300 rpm resulted in substantial xylanase production.

For agitation speed, the optimal agitation speed for maximum xylanase production was determined to be 250 rpm, resulting in a peak activity of 105.68±2.57 IU/ml. This finding suggests that proper mixing and agitation are essential for optimal enzyme production. Conversely, lower agitation rates or static conditions led to minimal xylanase activity, as depicted in Figure (10).

Characterization of partially purified xylanase enzyme

Xylanase stability and activity along with temperature variation

The optimal temperature for the partly purified xylanase produced by *Bacillus safensis* MABS6 was found to be 50 ℃ where it showed maximum relative activity (99.66%), although it was active between 30℃ to 80 ℃ (Fig. 11).

Xylanase stability and activity along with pH variation

Enzyme activity is sensitive to changes in pH because of the role that charge distribution plays in substrate binding and catalysis (Pemberton *et al.*, 2020). The xylanase from *Bacillus safensis* MABS6 was most active at a pH of 7.0 and remained active with relative activity of more than 60% throughout a pH range of 6 to 9 (Fig.12). The findings showed that the alkali stability of the partly purified xylanase.

Influence of metallo-solvent additives on xylanase activity

The results of the xylanase activity test using various metallic solutions are presented in Table 3. Notably, the presence of Cu^{+2}, Mn^{+2}, and Fe^{+2} metallic ions led to a remarkable increase in xylanase activity, with values of 172.27±7.09%, 122.54±3.81%, and 103.96±3.44%, respectively. However, the addition of other metallic compounds, as shown in Table (3), resulted in a significant decrease in xylanase activity.

Effect of Solvent Additives on Xylanase Activity

The impact of various solvent additives, both alcoholic and non-alcoholic, on xylanase activity is illustrated in Table 4. Notably, the highest xylanase activity was observed with isopropanol as an additive, reaching 125.77±4.86%. This was followed by methanol with an activity of 115.18±4.93%, and ethanol with an activity of 107.96±3.65%. In contrast, toluene exhibited the lowest xylanase activity, measuring 66.12±3.17%.

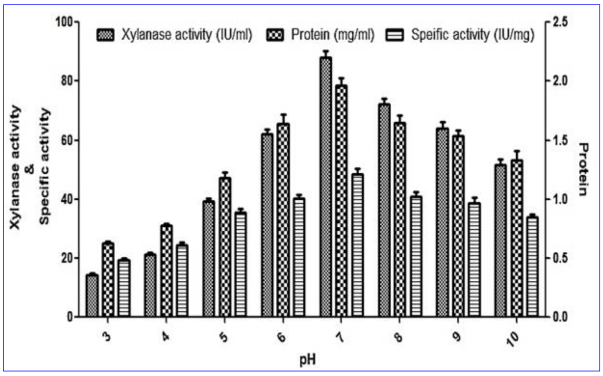

Figure (8): Influence of different pH on the xylanase production: enzyme activity, protein content, and specific activity.

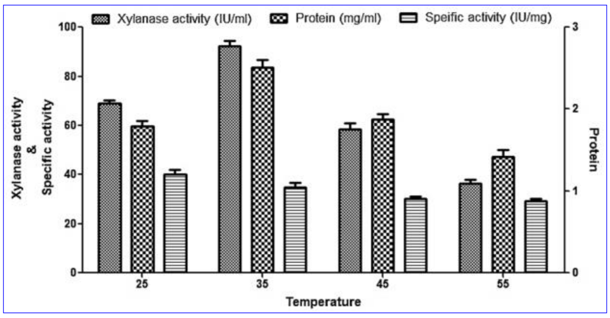

Figure (9): Influence of different incubation temperature on the xylanase production: enzyme activity, protein content, and specific activity.

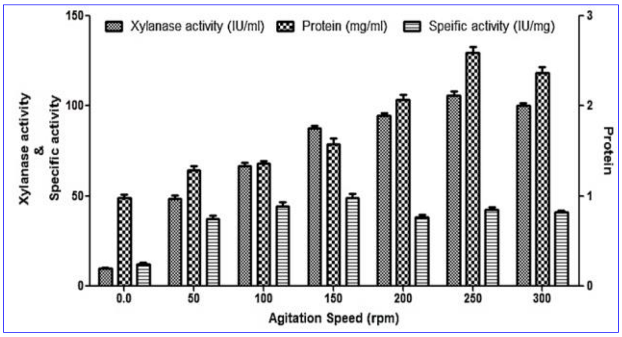

Figure (10): Influence of different agitation speed on the xylanase production: enzyme activity, protein content, and specific activity.

Impact of enzymatic hydrolysis on sorghum straw: a comparative study of alkali and acid pretreatment

Enzymatic hydrolysis has gained significant attention in modern biotechnology, particularly in the field of bio-solvent production, as a method for extracting sugars from agricultural residues. In this study, sorghum straw underwent a 4% alkaline H2O2 pretreatment, leading to the highest yield of reducing sugar (31.47 mg/g) after 36 hours of enzymatic hydrolysis. The results revealed that the biomass subjected to alkali pretreatment produced 27.84 mg/g of reducing sugar after 48 hours, while the biomass subjected to acid pretreatment produced 19.21 mg/g of reducing sugar. In contrast, the untreated biomass exhibited the lowest amount of reducing sugar (3.01 mg/g) within the same time period. After undergoing pretreatment with 4% alkaline H_2O_2, the sorghum straw showed an initial phase characterized by a rapid generation of sugars within 36 hours. However, the subsequent rates of hydrolysis displayed a decrease, which could be attributed to either the inactivation of enzymes or the depletion of a fraction of hemicellulose that is easily hydrolysable. This observation suggests the need for further investigation to determine the factors impacting the sustained efficiency of enzymatic hydrolysis and optimize the process for maximum sugar extraction from sorghum straw.

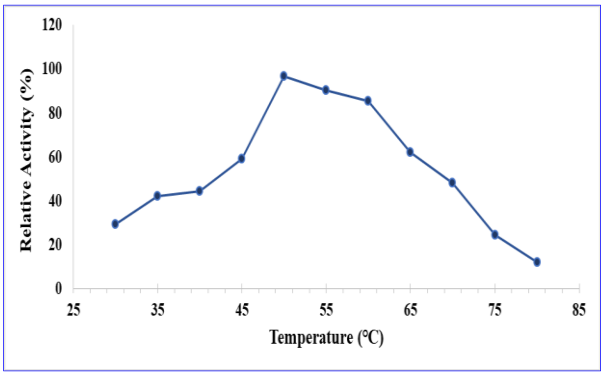

Figure (11): Thermal stability analysis of partially purified xylanase from *Bacillus safensis* MABS6

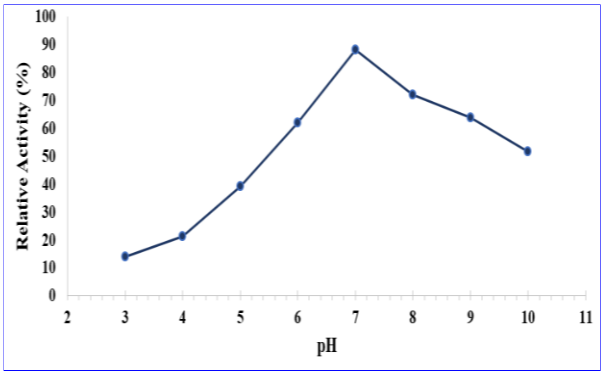

Figure (12): Acidic Stability Analysis of Partially Purified Xylanase from *Bacillus safensis* MABS6.

DISCUSSION

Xylan, a structurally intricate polysaccharide, serves as a carbon storage for microbes, namely bacteria. The enzymatic degradation of this substance results in the production of smaller molecular structures, which may be efficiently used as a source of energy. Carbon and nitrogen are essential elements for the growth of bacteria. They serve as building blocks for cellular structures and proteins. Xylan, with its combination of growth hormones, vitamins, and proteins, becomes a valuable resource for these bacteria. It implies that the bacteria are utilizing xylan as a source of nutrients to support their growth, development, and overall metabolic activities. The substantial presence of xylan in these vital nutritional constituents highlights its importance in facilitating the metabolic requirements of the bacterial species being studied (Revanker and Lele 2006; Sasmitaloka *et al.*, 2019).

Multiple bacterial species have been demonstrated to have pH-dependent xylanase synthesis (Li *et al.*, 2023).

Fungal xylanases thrive in acidic pH (4.0-6.0) ranges (Dhaver *et al.*, 2022) while bacterial xylanases prefer a more acidic (more than 6 pH) environment (Gupta *et al.*, 2022) and similar kind of results were observed during the current research. Elevated xylanase synthesis was also seen at pH 7.0 in *Bacillus pumilus* (Nagar and Gupta, 2021) *and Bacillus subtilis* (Torkashvand *et al.*, 2020), which is consistent with this observation. However maximum xylanase production was observed for *Bacillus mojavensis* AG137 (Akhavan *et al.*, 2011) and *Bacillus* NT (Han *et al.*, 2004) 9 at medium pH 8.0 and 10, respectively.

High temperatures generate physiological changes during enzyme synthesis; these changes are not well understood, but it has been hypothesized that at these temperatures microbes may manufacture less of the proteins necessary for growth and other physiological activities (Pold *et al.*, 2020). It's possible that the microbe's growth profile, in which no other temperature allowed for optimal growth and enzyme production, is consistent with our findings. Maximum xylanase synthesis from *Bacillus* was found by several studies to occur at a temperature of 37°C (Battan *et al.*, 2007; Sanghi *et al.*, 2008). The observed decrease in xylanase production under static to low agitation conditions may be attributed to various factors such as the limitation of dissolved oxygen (DO), inadequate mixing of medium components, and the formation of cell clumps. Researchers have shown that between 200 and 250 rpm is optimal for xylanase synthesis (Beg *et al.*, 2001; Taneja *et al.*, 2002; Kumar *et al.*, 2012; Kumar, 2020).

The enzyme was most active (above 55%) between 45 to 65℃. Least activity (less than 25%) was found at temperature 75 to 80℃. Enzyme thermal stability results from the favorable conformational structure promoted by hydrogen bonding (Sharma *et al.*, 2019), electrostatic and hydrophobic forces of attraction (Almeida *et al.*, 2022), disulfide linkage, and metallic binding (Gihaz *et al.*, 2020), which results in enhanced packing efficiency, decreased unfolding entropy, more easily released conformational strain, and increased α-helical stability (Rahban *et al.*, 2022). *Bacillus safensis* MABS6 xylanase has been shown to retain 85% to 97% of its original activity after 1 hour of incubation at temperatures between 50°C to 60°C. Even after being incubated at 70 °C for half an hour, 48% of the xylanase activity was observed showing that xylanase produced from *Bacillus safensis* MABS6 can withstand high temperatures. The majority of bacterial xylanases reach their peak levels of activity between the temperatures of 50 to 60℃ (Ketsakhon *et al.*, 2023). The optimum temperature for *Bacillus* xylanases was also found to be 50 °C (Saleem *et al.*, 2021). *B. halodurans* xylanase, on the other hand, showed activity from 30 to 100°C, with an optimal temperature of 80 °C (Glekas *et al.*, 2022), another strain PPKS-2 of the same bacteria showed maximum activity at a temperature of 70℃ (Prakash *et al.*, 2012).

Many studies have shown that the ideal incubation period for xylanase production is contingent upon the specific bacterial strain used and its corresponding growth duration. Gram-negative and Gram-positive bacteria, which fall under the category of non-actinomycetes, exhibit a comparatively shorter period of incubation. Many investigations have proven that *Bacillus subtilis* cho 40 only synthesizes xylanase during a four-day period of incubation (Khandeparker *et al.*, 2011). It is possible that a quicker food intake was to blame for the precipitous drop in enzyme titer that occurred when inoculum size was increased above the optimal levels. Nagar and Gupta (2021) also studied inoculum size ranges from 1 to 5% v/v for hyperproduction of xylanase. Considering industrial fermentations, a higher inoculum concentration is not a desirable trait (Vassileva *et al.*, 2021). The findings obtained may be attributed to the generation of maximal enzyme titer during the early to late exponential phase of the organism. Additionally, it hints at the existence of a partial relationship between the development of the organism and the pattern of enzyme synthesis it exhibited. Kumar *et al.*, 2012 researched the generation of xylanase utilizing an alkalophilic actinomycete isolate of *Streptomyces sp*. They discovered that inoculum 18 hours older demonstrated the highest level of xylanase activity. Similar kind of results were observed when Nagar *et al.*, 2010 conducted a similar kind of research for xylanase production using *Bacillus pumilus* SV-85S in submerged fermentation.

Comparable research demonstrated that *Bacillus* GRE7 xylanase was pH stable between 5 to 11 for 30 minutes (Kiddinamoorthy *et al.*, 2008). *Bacillus stearothermophilus* T-6 xylanase was stable at pH 6.5 to 10.0 (Huang *et al.*, 2014). Glycosylation and other post-transcriptional alterations in the xylanase excretion process may account for the discrepancies in pH and temperature stability for extracellular xylanases (De Carvalho *et al.*, 2019). Maximum enzyme synthesis during fermentation occurs at various times for different bacteria, depending on the kind of organism, its enzyme production pattern, culture circumstances, and genetic composition (Zikmanis *et al.*, 2020). Depletion of nutrients or proteolysis may be to blame for the drop in xylanase production (Ketsakhon *et al.*, 2023). In *Bacillus sp*. xylanase production was shown to be growth-associated, with a peak at 24 hours and a rather consistent rate of production up to 48 hours (Zambry *et al.*, 2021). *Bacillus halodurans* PPKS-2 (Prakash *et al.*, 2012) and *Bacillus* SSP-34 (Subramaniyan and Prema, 2000) reached peak xylanase production after 48 and 96 hrs., respectively.

Typically, bacterial cell counts exhibit a decline beginning on the eighth day of the exponential growth phase. This decline coincides with a time characterized by rapid bacterial proliferation, during which the cells demand a greater energy supply for their sustenance compared to earlier phases. Consequently, there was a substantial excretion of xylanase throughout the manufacturing process, aimed at maximizing energy acquisition via the degradation of xylan present in the medium. Furthermore, the concentration of xylan exhibited a significant correlation with enzyme synthesis. Consequently, this investigation also examined the impact of various xylan concentrations on xylanase activity. The findings of our study align with the research conducted by Guha *et al.*, 2013, which suggests that the optimal concentration range for xylanase production is between 0.25 and 1.0% (w/v) of the carbon source, namely xylan. According to the findings of Lawrence *et al.*, 2015, the addition of xylan at concentrations ranging from 0.5 to 1% (w/v) to the fermentation medium did not result in a substantial impact on the synthesis of xylanase, particularly when the concentration exceeded 1%. The supplementation of B. subtilis and B. megaterium with molasses at concentrations ranging from 0.5 to 3.0% has been seen to result in a decrease in xylanase production, which is proportional to the amount of molasses provided. The observed phenomenon might likely be attributed to the presence of nutrients in molasses, which are known to generate catabolite suppressants during the formation of xylanase (Irfan *et al.*, 2016).

The use of various supplementary carbon sources has been seen to have a significant impact on the activity of xylanase. The presence of sorghum straw may increase xylanase synthesis because of the carbon source's hemicellulose, activators, surface, pore size, and favorable degradability (Velvizhi *et al.*, 2022). Hemi cellulosic substrates from different natural sources like bran, wheat, rice, soyabean flakes, shells of groundnuts and bagasse from sugarcane were used from different research groups to determine the xylanase production (Battan *et al.*, 2007). The inclusion of fructose in the xylan production medium resulted in a notable reduction in xylanase output. This may be attributed to the inhibitory effect of fructose on xylanase synthesis. The reason for this inhibition is that fructose, being a simple sugar, is preferentially used as a carbon source by the bacterial cells, hence diverting resources away from xylanase production. According to Guan *et al.*, 2016, it is suggested that simple sugars, such as fructose, do not have a significant impact on xylanase production. This might be due to the potential inhibitory effect of these sugars on enzyme synthesis. This assertion is further substantiated by research done by Ajijolakewu *et al.*, 2016, which posited that simple carbohydrate molecules have repressive effects on the production of xylanase. Indeed, they were only used for the purpose of facilitating expansion. In contrast, a significant finding has emerged whereby *S. thermocoprophilus* TC12W shown the capability to generate 1204.8 U/g of xylanase via the use of alkaline pretreatment empty fruit bunch as a carbon source. This discovery, as reported by Sinjaroonsak *et al.*, 2020, is the first instance of such utilization.

One of the most crucial aspects of microbial development and metabolism is access to a carbon source (Liu *et al.*, 2020). The behavior of the bacteria in terms of enzyme synthesis was significantly influenced by the availability of carbon sources in the fermentation medium (Rabiya and Sen, 2022). Increased xylanase synthesis in microorganisms has been seen in the presence of xylose, sucrose, and starch (Javed *et al.*, 2019). However, the addition of other carbon sources might lead to a reduction in yield owing to catabolite suppression (Sun *et al.*, 2021).

It has also been observed that xylose as a carbon source showed the maximum activity among the various carbon sources and controls (Table 1). Catabolite inhibition by these other carbon sources is the most likely reason for the drop in xylanase production (de Assis *et al.*, 2020). Various microbial strains that produce xylanase have been seen to regulate enzyme synthesis in response to their carbon supply (Bhardwaj *et al.*, 2021).

The use of different nitrogen sources resulted in diverse impacts on xylanase activity, suggesting that yeast extract had the most favorable effect as a nitrogen source for enhancing xylanase activity. According to the review conducted by Bhardwaj *et al.*, 2019, nitrogen emerges as a pivotal ingredient in several metabolic processes, including enzymatic function. According to a research conducted by Zuhri *et al.*, 2013, it was shown that ammonium sulfate is the most effective nitrogen source for the growth of *Bacillus* sp. M123 in order to produce alkaline proteases. The inclusion of 1% casein was shown to have a significant impact on both the maximum xylanase activity and xylanase output, resulting in values of 1.78 U/mg and 3.69 U/mg, respectively (Tai *et al.*, 2019). According to a study conducted by Sudan and Bajaj, 2007, the inclusion of 0.3% ammonium sulfate in the production medium resulted in the synthesis of 15 U/mL of xylanase by *Aspergillus niveus* RS2 following a 5-day incubation period. This level of xylanase production was found to be the second highest, surpassed only by the use of yeast extract. Yadav *et al.*, 2018 discovered the significance of nitrogen, namely ammonium sulfate, derived by xylanase excretion in the context of *Anoxybacillus kamchatkensis* strain NASTPD13. In a study, Ravindran *et al.*, 2019 documented the production of the most significant amount of xylanase, reaching 6495.6 IU/g of dry SCW, with the effective utilization of a medium supplemented with a nitrogen source of 0.2g/g of yeast extract in *A. niger*. All nitrogen sources included into the production medium exhibited the ability to enhance cell biomass growth and xylanase production, with the exception of ammonium persulfate ($(NH_4)_2S_2O_8$), which hindered both cell biomass growth and xylanase production. The inhibitory effects of $(NH_4)_2S_2O_8$ on *S. costaricanus* 45I-3 cells and xylanase synthesis are likely attributed to its poisonous nature.

The impact of various inorganic phosphate supplies on the generation of xylanase has also been examined. The acquired results exhibited similarity to the findings published by Mandal, 2015, wherein it was asserted that NaH2PO4 serves as the most effective phosphate source for the manufacture of xylanase by *Bacillus cereus* BSA1. The xylanase production level was recorded at 5.53 U/mL. Phosphate salts of a certain concentration have been seen to promote the development of organisms and enhance the synthesis of extracellular enzymes in the production medium (Chellapandi and Jani, 2008). Based on the findings of this study, an optimally synthesized xylanase from Bacillus safensis MABS6 was successfully produced, demonstrating its cellulase-free, alkaliphilic, and thermostable characteristics. The utilization of sorghum straw as a substrate, which is known for its resilience in paper industry conditions, highlights the potential for sustainable xylanase production. One of the key innovations of this study is the efficient breakdown of sorghum straw through xylanase, resulting in the liberation of sugars for biofuel production. These findings suggest promising applications in the fields of biotechnology and sustainable biofuel industries.

Enzyme catalysis is facilitated by metal ions in a variety of ways (Dai and Zhang, 2021). They may be electron transporter (Schenk *et al.*, 2013), may exhibit electrophilic and nucleophilic nature (Wang *et al.*, 2020), may help enzyme substrate bond linkage (Sigel *et al.*, 2007) and may play a role in the stability of enzyme (Liu *et al.*, 2003). Mercuric chloride has been identified as a potent inhibitory reagent, exhibiting a significant inhibition rate of 64%. This inhibition suggests the presence of a crucial cysteine residue in the active region of the enzyme (Khandeparkar and Bhosle, 2006). Furthermore, xylanase activity in Bacillus halodurans was substantially reduced by the presence of stannous chloride, mercurous chloride, cupreous chloride, and cadmium chloride (Kumar and Satyanarayana, 2011). These findings highlight the importance of avoiding or considering the inclusion of specific ions in manufacturing procedures.

The impact of straight-chain alcohols on xylanase activity has been investigated by Li *et al*. (2010). Their study provides insights into the effects of these alcohols on the enzymatic activity, which can be valuable for optimizing the enzyme's performance in various applications. Performing biocatalysis in an organic medium offers several advantages, including reusability, reduced microbial contamination, and improved solubility of hydrophobic substrates (van Schie *et al*., 2021). These advantages make organic medium-based biocatalysis an attractive option for various biotechnological processes.

The development of solvent-stable xylanases has also been the focus of several published studies (Dahiya and Nigam, 2021). These studies shed light on the characteristics and potential applications of xylanases that exhibit stability in different solvent environments.

CONCLUSION

In conclusion, this study holds a great significance in the context of the increasing economic importance of xylanases. The study successfully optimized various parameters for xylanase synthesis from *Bacillus safensis* MABS6. The resulting xylanase enzyme exhibited favorable characteristics, such as being cellulase-free, demonstrating thermostability across a wide temperature range, and maintaining stability at high pH levels, indicating an alkaliphilic nature. The utilization of sorghum straw as a substrate for xylanase production was particularly noteworthy. Sorghum straw, a relatively lesser-known biomass material, was chosen due to its ability to withstand the harsh processing conditions commonly encountered in the paper industry. The findings of this study open avenues for the sustainable utilization of sorghum straw, which could otherwise be considered as agricultural waste. Furthermore, the application of an alkaline hydrogen peroxide treatment on sorghum straw biomass enabled the efficient breakdown of the material by the xylanase enzyme, resulting in the liberation of reducing sugars, primarily xylose. These liberated sugars hold significant potential for biofuel production through fermentation processes. Overall, the characterized xylanase enzyme from *Bacillus safensis* MABS6 possesses valuable properties and demonstrates its potential as a biocatalyst in various biotechnological industries.

The successful utilization of sorghum straw as a substrate, coupled with its ability to efficiently convert biomass into biofuels, further highlights the practical applicability and economic prospects of this research. Future studies can build upon these findings to explore the scalability and industrial applications of this xylanase enzyme, paving the way for sustainable and environmentally friendly solutions in biofuel and related industries.

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زيادة إنتاج إنزيم xylenase المحسّن باستخدام مخزون قش نبات الذرة Shorgum كمادة مغذية لبكتيريا *Bacillus safens*is MABS6. : تحسين، توصيف، وتطبيقات بيوتكنولوجية

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الملخص العربي

تهدف هذه الدراسة انتاج و تحسين إنزيم xylenase من عزلات مختلفة من البكتيريا لها القدره علي انتاج الانزيم واختيار احسنهم قي الانتاج وتعريفها بالطرق المتعارف عليها. واظهرت النتائج ان بكتريا *Bacillus safens*is MABS6. لها قدرة فائقة علي انتاج الانزيم موضوع الدراسة. يظهر الإنزيم الناتج صفات إيجابية، مثل خلوه من السليلوز، وقدرته على التحمل الحراري عبر نطاق واسع، والحفاظ على استقراره القلوي. يعتبر استخدام قش الذرة لنبات Shorgum كمصدرغذائي، الذي تم اختياره بسبب قوته في مواجهة ظروف صناعة الورق، ويعتبر مضدر مستدام لإنتاج xylenase من مادة حيوية نسبياً وصديقة للبيئة. واظهرت الدراسة ان تركيز النبات المستخدم من حيث الكمية يلعب دور كبير في كفائة الانزيم المنتج ببكتريا *Bacillus safens*is MABS6. ، كما اثبتت الدراسة حجمم وعمر حقنة البكتريا، وقيم الحموضة للوسط الغذائي، ودرجة الحرارة، وسرعة الهزات اثناء التحضين تؤدي الي تحسين ظروف الانتاج مع إضافة بعض المواد الغذائية وتقييم الأيونات المعدنية والمذيبات العضوية التي تؤدي الي زيادة نشاط الانزيم. كما اثبتت الدراسة ان معالجة قش الذرة يساعد بفعالية إلى تكسير المواد وتحويلها الي سكريات مختزلة، والتي بدورها تزيد من نشاط الانزيم. يتميز الإنزيم المنتج بواسطة *Bacillus safens*is MABS6MABS6 بخصائص قيمة، مما يجعله عاملاً حيويًا مشجعًا في مختلف الصناعات البيوتكنولوجية. ويمكن للدراسات المستقبلية البناء على هذه النتائج لاستكشاف قابلية التوسع والتطبيقات الصناعية، مما يساعد في التنمية المستدامة وصديقة للبيئة في مجال الوقود الحيوي والصناعات ذات الصلة.