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Purification and Characterization of Xylanase Enzyme from Bacillus Substilis Isolated from Forest Soil

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KEYWORD:
Bacillus substilis, dialysis, ion-exchange chromatography, SDS PAGE, Zymogram

ABSTRACT:
The xylanase obtained from *Bacillus substilis* isolated from forest soil was purified and characterized to assess its future in industrial application. The purification process was by ammonium sulphate fractionation followed by dialysis, ion exchange chromatography and gel filtration chromatography. Solid ammonium sulphate salt was used in concentration of 80%.The precipitated protein was recovered by centrifugation and dissolving it in minimum quantity of buffer after which the protein were retrieved by dialysis. Xylanase further purified by DEAE cellulose ion exchange column. The yield and fold purification was 60.47% and 2.45% with specific activity of 7.495 U/mg protein. Column chromatography using Bio-Gel P-60 matrix indicate high purity yield of 53.46% and fold purification 6.25% with specific activity of 19.08 U/mg protein.The molecular weight was found to be 22Kd ,determined by SDSPAGE .Zymogram analysis of xylanase activity using purified protein resulted in a single band. There was a gradual increase in the enzyme activity from 2% to 3% of substrate concentration. The enzyme showed stability at alkaline pH7 and showed an increase in activity at 50°C.The xylanase activity was strongly inhibited by Hg2+ and Cu2+ and enhanced in presence of Mn2+.

INTRODUCTION

Xylanase are class of enzymes that degrade linear polysaccharide xylan in to xylose. Industrial interest in xylanases is for biofuel production, chemical and pharmaceutical industries, wood pulp bioleaching, paper making, the manufacture of food and beverages, and animal nutrition. The xylanase producing bacterial isolate was collected from soil of forest areas and dense plantation and screened on Birchwood xylan Agar media using Congo red assay If a new efficient xylanase-producing microorganism is isolated, it is essential to purify and characterize the enzymes to know the action towards substrates of each component of a xylanolytic complex, its regulation and biochemical properties in order to develop more competitive processes. The xylanase producing bacterial isolate *Bacillus substilis* was collected from soil of forest areas and dense plantation and screened on Birchwood xylan Agar media using Congo red assay

Enzyme purification is a complex and tedious process which involves a combination of methods but this step is far more important because enzymes should be available in their purified form if they are to be used for further studies or lest they should be employed for industrial applications. The choice of a suitable method ultimately depends on the nature of enzyme to be purified. In the present study, efforts were made to purify the xylanase enzyme produced from *Bacillus subtilis.* Crude enzyme extract (1000 ml) was prepared by growing the bacterial isolates independently under their respective optimized conditions. The purification process was initiated by ammonium sulphate fractionation followed by dialysis, ion exchange chromatography and gel filtration chromatography

MATERIAL AND METHODS

PURIFICATION AND CHARACTERIZATION OF XYLANASE

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(5), 739-746 | ISSN:2251-6727

Calculation of yield of protein, yield of enzyme activity, fold of purification

Yield of protein and enzyme activity of each fraction obtained during purification is the percentage activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be.

Yield of Protein= (Total protein content of the fraction)/(protein content of the crude extract) x100

Yield of activity= (Total activity of the fraction)/ (Total activity of the crude extract) x100

Specific activity= (Enzyme activity (U/mL))/ (mg of protein /mL) x100

Fold of Purification= (Specific activity of the fraction)/ (Specific activity of the crude extract) x100

PROTEIN ESTIMATION

Protein content was estimated according to the method of Lowry *et al*., 1951 using bovine serum albumin (BSA) as the standard.

ESTIMATION OF ENZYME ACTIVITY

Xylanase assay was done to determine the activity of the enzyme xylanase. The xylanase enzyme activity was done by measuring the reducing sugar released by the reaction on the birch wood xylan. Thus, the xylanase assay was done according to 3, 5 – dinitro salicylic acid (DNS) method.

The amount of enzyme produced by each isolate in liquid xylan medium was found. 1.0% of the birch wood xylan was dissolved in 50 mM Glycine- NaOH buffer (pH – 9.2) and this was used as substrate. 0.5 mL of the buffered substrate (1.0% birch wood xylan and 50mM Glycine NaOH buffer) was reacted with 0.5 mL of crude xylanase enzyme at a temperature of 55°C. The reaction was stopped after 10 minutes by adding 3 mLDNS reagent and then kept in a boiling water bath for 5 minutes. After cooling for a few minutes, the released xylose was quantified at 540 nm against a reagent blank. A reagent blank was made in the same manner except the crude enzyme was not added and 0.5 mL buffer was added. An enzyme blank was also made in which the reagent was added before the addition of enzyme so that only the reducing sugar is estimated. A standard of xylose (reducing sugar) was prepared using stock concentration 1 mg/mL. One unit of xylanase enzyme activity is defined as 1 µmole of xylose liberated per minute per mL of enzyme preparation under standard assayconditions.

Standard Curve:

ΔA540nm Std = A540nm Std - A540nm Std Blank

Prepare a standard curve by plotting the ΔA540nm Standard vs the µmoles of Xylose.

Sample Concentration Determination:

ΔA540nm Sample = A540nm Test - A540nm Blank

Determine the µmoles of xylose using the Standard Curve (µmoles of xylose liberated) x (df)

$$U/ml = \frac{(\mu moles\ of\ xylose\ liberated)(10)\ x(0.5)}{}$$

df = Dilution factor. 10 =Time of assay (in minutes) as per Unit Definition

0.5 = Volume (in millilitres) of enzyme used units

AMMONIUM SULPHATEPRECIPITATION

Enzyme production was carried out using submerged state fermentation under optimized conditions. Extraction of the enzyme was performed by ammonium sulphate precipitation method. Salting out by ammonium sulphate is the best-known method for concentration and purification of the enzyme. Ammonium sulphate precipitation was done according to Englard and Seifter, 1990. Crystal ammonium sulphate (Sigma Aldrich, Bengaluru) required to precipitate xylanase was optimized by its addition, at 80% saturation to the crude extracts.

The addition of ammonium sulphate was done under constant stirring at 4°C for 30 min and then stirring was continued overnight at 4°C. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4ºC. The precipitate was suspended in a 25 mM Na-phosphate buffer (pH-7.4).

DIALYSIS

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against (Sigma Aldrich, Bengaluru) 25 mM Na-phosphate buffer (pH-7.4). The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis tube (Sigma-Aldrich) was first treated to remove the humectants and protectants like glycerin and sulphur compounds present in it and to make the pores of the tube more clear. The pre-treatment involved washing of the tube in running water for 3-4 hours, dipping in 0.3 % (w/v) sodium sulphide at 80ºC for 1 min, further washing with hot water (60ºC) for 2 min followed by acid wash in 0.2 %

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(5), 739-746 | ISSN:2251-6727

(v/v) sulphuric acid. Finally the tube was rinsed with distilled water. Dialysis was conducted overnight and the buffer was changed several times to increase the efficiency of the dialysis. The precipitated protein was dialyzed in the pre-treated dialysis tube for 48 hours at 4ºC with several changes of buffer and assayed for xylanase activity, protein content, and specific activity. After dialysis, the sample was collected and stored under-20°C.

ION EXCHANGECHROMATOGRAPHY

Purification of xylanase from bacteria was carried out by the DEAE-cellulose (Sigma Aldrich, Bangalore) column chromatography. The column was packed with activated DEAE- cellulose equilibrated with 25 mM sodium phosphate buffer (pH 6.0). The height of the column was 20 cm with a 2.5cm diameter. The protein was eluted with the 0.1 to 0.5 M NaCl gradient. The 25 fractions were collected having 3 mL volume of each fraction with the flow rate of 1mL/min. All the steps were carried out at 4°C to prevent proteindenaturation.

GEL FILTRATION CHROMATOGRAPHY

Purification of xylanase was also carried out by Bio Gel P-100 (Bio Rad, India). The column was packed at the height of 60 cm having a 1 cm diameter and activated using 50mM TrisHCl buffer, pH 7.5. The void volume was calibrated by using blue dextran. The experiment was performed at the flow rate of 1mL/10 min. 25 fractions (1 mL each) were collected and analysed xylanase activity. All the procedures were carried out at4°C.

MOLECULAR WEIGHT DETERMINATION BY SDSPAGE

Purity of the fractions, showing xylanase activity, was checked by SDS- PAGE by the method of (Laemmli, 1970). The molecular weight of xylanase was determined by comparison with standard molecular marker proteins (Promega Corporation,USA).

ZYMOGRAM ANALYSIS

Zymography is a technique used to assess the enzymatic activity of proteins either in situ or by separating them with electrophoresis. The enzyme converts the substrate into a product which is detected by different staining methods. The zymogram analysis was performed by loading the concentrated partially purified xylanase fraction on a SDS- 12 % polyacrylamide gel containing 0.1 % xylan. After electrophoresis, the gel was incubated for 60 min at 80 C, soaked in 0.1 % Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed. On gel analysis, the activity band was seen as a clear colourless area of xylan hydrolysis

CHARACTERIZATION OF PURIFIED XYLANASEENZYME

Effect of substrate concentration on xylanaseactivity

The substrate specificity of the enzyme on xylanase activity was measured by considering different xylan substrate concentrations in Glycine-NaOH buffer (pH7.0) such as 0.5%- 4% at 37°C.

Effect of temperature on xylanaseactivity

The effect of temperature on xylanase activity was studied by incubating the enzyme at a range of 20°C to 60°C; using 3% (w/v) xylan as substrate in Glycine-NaOH buffer (pH7.0). Optimal temperature was defined as the temperature at which maximum hydrolysis occurred, detected by increase in activity.

Effect of pH on xylanase activity

The effects of pH on enzyme activity was determined over a pH range of 3.0 to 10.0; using 1 mL of 3% (w/v) xylan as the substrate prepared in citrate- phosphate buffer (pH 3.0-5.0), sodium phosphate buffer (pH6.0-8.0) and glycine- NaOH buffer (pH 9.0- 10.0). The enzyme suspension was incubated for 3 hours in various buffers having pH ranging from 3.0 to 10.0 at 50°C. Optimal pH was defined as the pH at which maximal enzyme activity was obtained in theassay.

Effect of metal salts on xylanaseactivity

Effect of various metal ions on enzyme activity was evaluated by incubating the enzyme along with different concentrations of 1mM of various metal salts in the enzyme reaction mixture for 1 hour. The metal salts studied included calcium chloride, mercuric chloride, ferric chloride, copper sulphate, manganese chloride, magnesium sulphate, zinc sulphate, which contributed the metal ions Ca^{2+} , Hg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+} , Mg^{2+} and Zn^{2+}respectively.

Analysis of Xylanase Activity

Xylanase assay was done to determine the activity of the enzyme xylanase. The xylanase enzyme activity was

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done by measuring the reducing sugar released by the reaction on the birchwoodxylan . Thus, the xylanase assay was done according to 3, 5 – dinitro salicylic acid (DNS) method. The amount of enzyme produced by each isolate in liquid xylan medium was found.

RESULTS AND DISCUSSION

Ammonium Sulphate Precipitation

Precipitation of the enzyme did occur when the crude extract obtained from *Bacillus subtilis* was treated with 80% ammonium sulphate salt concentration

Fig:1. Ammonium Sulphate Precipitation from crude extract

Dialysis

The crude enzyme obtained after ammonium sulphate precipitation was further purified by dialysis

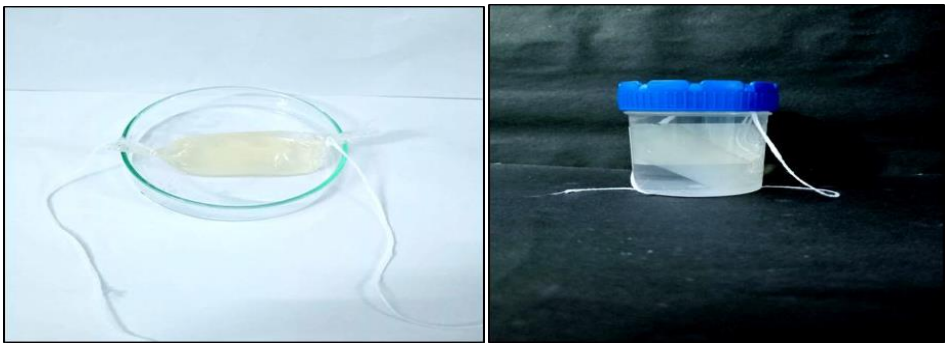


Fig:2. Dialysis of precipitated protein from ammonium sulphate precipitation

Ion exchange chromatography

Xylanase was further purified by the DEAE cellulose ion exchange column. The enzyme was eluted from DEAE cellulose column at a NaCl concentration of 0.1 to 0.5 M. The fractions having maximum specific activity were concentrated. The yield and fold purification of Xylanase enzyme was 60.47% and 2.45% respectively with specific activity of 7.495U/mg protein.

Gel Filtration

Dialysed xylanase enzyme obtained from dialysis with Sephadex G-100 matrix was purified by column chromatography using Bio-Gel P-60 matrix where enzyme was eluted out as a single sharp homogenous peak, as revealed from the chromatogram, indicating high purity. Xylanase activity was eluted at about 40-50 fractions using the sodium phosphate buffer (0.05 M, pH 7) as the eluent. The active fraction were pooled and concentrated by ammonium sulphate (80% precipitation) •and dialysed for 24 hrs and considered as pure xylanase for further studies. The yield and fold purification of Xylanase enzyme was 53.46% and 6.25 % respectively with specific activity of 19.08 U/mg protein. The purification reveals the presence of multiple isoforms ofxylanase

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(5), 739-746 | ISSN:2251-6727

Table:1. Showing Total protein, Total Enzyme activity, Specific activity, purification and yield of Xylanase by crude extract, Ammonium sulphate precipitation and dialysis, column chromatography and ion exchange chromatography

Xylanase enzyme	Total Protein (mg)	Total enzyme activity (U)	Specific activity(U/mg of protein)	Fold of purification	% yield of enzyme
Crude extract	1132.5	3452	3.049	1.00	100
Ammonium Sulphate Precipitation and dialysis	549	2746	5.001	1.63	79.54
Ion exchange chromatography	278.5	2087.5	7.495	2.45	60.47
Gel filtration	96.7	1845.5	19.08	6.25	53.46

Molecular Weight Determination by SDSPAGE

The SDS page was carried out to determine the molecular weight of the purified protein. A clear band was observed in the final purified product. The molecular weight was determined with the help of a protein marker and was found to be approximately 22kD

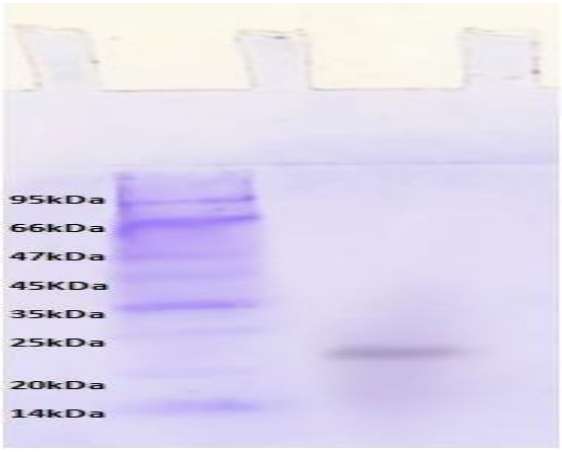


Fig:3. SDS-PAGE
Lane 1: Protein Marker Lane 2: 22kDa purified xylanase protein

ZymogramAnalysis

Zymogram analysis of xylanase activity using purified protein from *Bacillus subtilis*was carried out. Staining of the polyacrylamide gel with Congo red resulted in a single band.

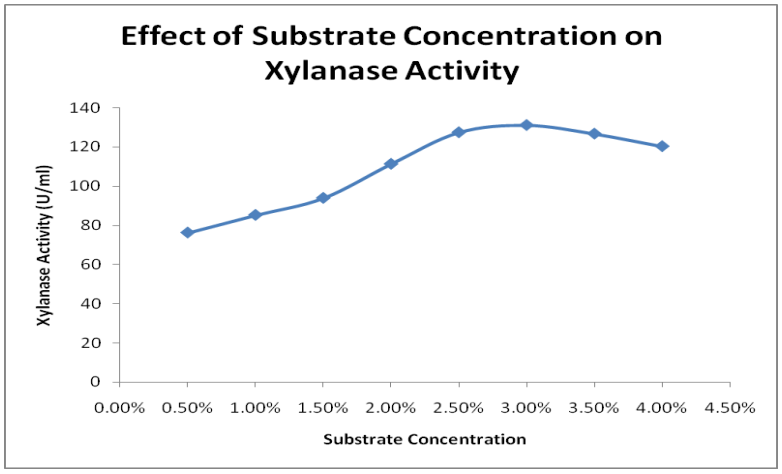
Fig :4. Zymography analysis of Xylanase showin clear zone

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(5), 739-746 | ISSN:2251-6727

Characterization ofenzyme

Effect of substrate concentration onactivity

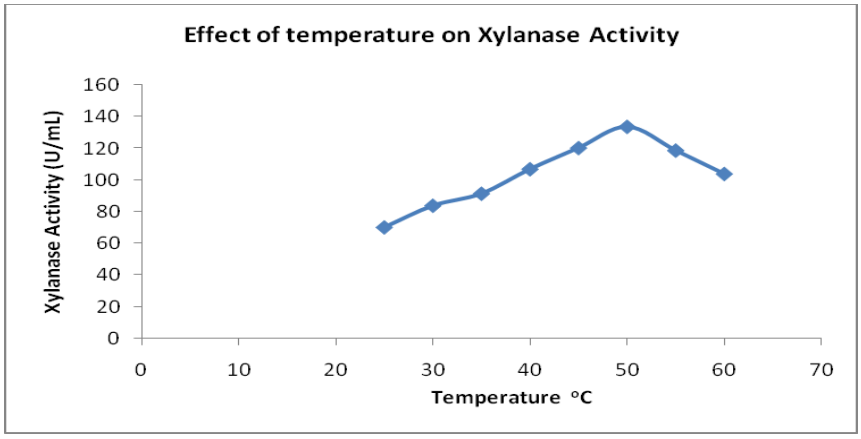
The effect of various substrate concentrations was determined in values ranging from 0.5% - 4% on the purified xylanase activity. Enzyme activity was found to be greatly affected by substrate concentration. The enzyme exhibited a gradual increase in enzyme activity from 2% to 3% (111.3 to 131.1U/ml) of substrate concentration, respectively and then decreased.



Graph: 5. Effect of Substrate Concentration on Xylanase Activity

Effect of temperature on activity

Measurement of the activity of purified extracellular xylanase from *B. subtilis*at different temperatures showed an increase in activity up to 50 ºC followed by a decline. Hence, the optimum temperature for purified *B. subtilis* extracellular xylanase was 50 ºC.

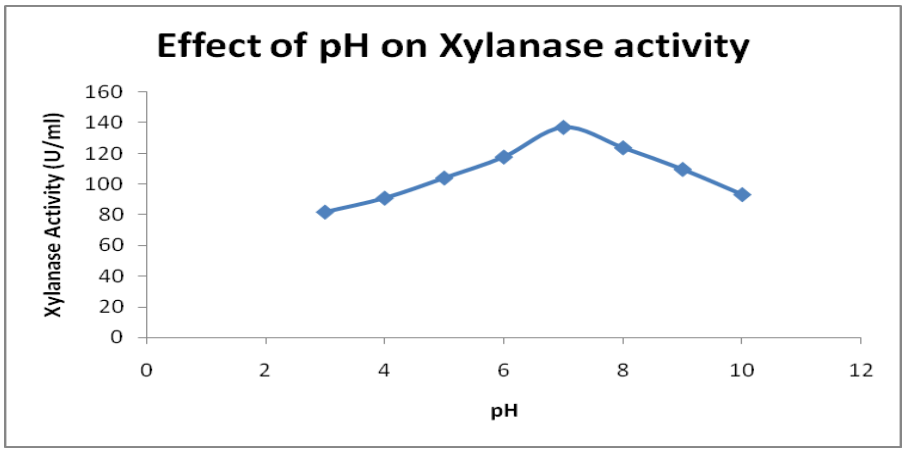


Graph: 6.Effect of Temperature on Xylanase Activity

Effect of pH on activity

The optimum pH of purified extracellular xylanase from *B. subtilis*was found to be 7.0 since maximum activity was observed at this pH.

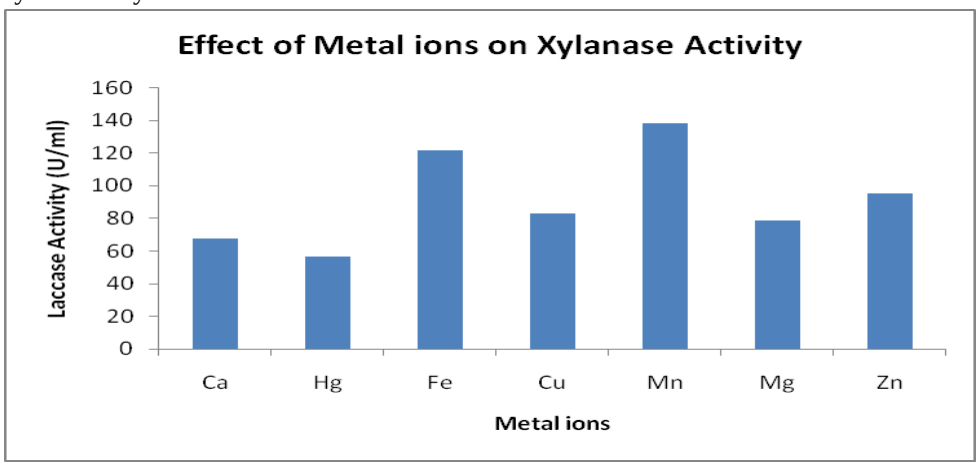
Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(5), 739-746 | ISSN:2251-6727



Graph:7. Effect of pH on Xylanase Activity

Effect of metal salts on activity

Xylanase activity was strongly inhibited by Hg2+ which might be due to its interaction with sulfhydryl groups present on the enzyme. Cu2+ ions were also found to be inhibitory in enzyme activity. Some other metal ions like Ca2+, Fe3+, Cu2+, Mg2+and Zn2+ decreased the enzyme activity but to a very less extent. In contrast, xylanase activity was enhanced in the presence of Mn2+ (138.3 U/ml).



Graph: 8. Effect of Metal ions on Xylanase Activity

CONCLUSION

The xylanase enzyme isolated can be purified by ammonium sulphate followed by dialysis, ion-exchange chromatography. The yield and fold purification of Xylanase enzyme was 60.47% and 2.45% respectively with specific activity of 7.495U/mg protein.Gel filtration chromatography reveals the presence of multiple isoforms of xylanase. The yield and fold purification of Xylanase enzyme was 53.46% and 6.25 % respectively with specific activity of 19.08 U/mg protein. molecular weight was found to be approximately 22kD by SDS PAGE.

Enzyme activity was found to be greatly affected by substrate concentration. The enzyme exhibited a gradual increase in enzyme activity from 2% to 3% (111.3 to 131.1U/ml) of substrate concentration, respectively and then decreased. Effect of temperature showed an increase in activity up to 50 ºC followed by a decline. The maximum activity was observed at Ph7.metal salt activity showed that Xylanase activity was strongly inhibited by Hg2+ and was enhanced in the presence of Mn2+ (138.3 U/ml). The optimized xylanase activity was 138.7U/ml.

Journal of Chemical Health Risks

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest

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