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Production, Optimization and Partial Purification of Xylanase from *Streptomyces Coelicolor* Using Agriculture Waste

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ABSTRACT

Xylan is widely distributed in plant cell walls and forms a main part of the hemicellulose fraction. In some higher plants and agricultural wastes, xylan constitutes 20-40% of the dry weight. In this study xylanase producing *Streptomyces coelicolor* colonies from soil sample was isolated, morphological and biochemical characteristics were examined. Various substrates were used for xylanase production. Comparative studies were evaluated with five different substrates such as sugarcane bagasse, pineapple, orange peels and pomegranate peels. Xylanase production was assayed by dinitro salicylic acid assay. Streptomyces sporulation medium containing sugarcane bagasse (substrate) was used for *Streptomyces coelicolor* growth and xylanase production as sugarcane bagasse gave satisfactory xylanase production. The medium contains various concentration of yeast extract (0.05, 0.10, 0.15gm) act as a carbon source, Tryptone (0.1, 0.2, 0.3gm) act as nitrogen source. The xylanase production and yield was performed by using yeast extract (0.10gm) and Tryptone (0.2gm). The optimization of pH and temperature was characterized by different assay conditions. Purification processes carried out by ammonium salt precipitation, dialysis and Ion Exchange Chromatography. The purified enzyme was immobilized in calcium alginate beads. The observed result shows that the optimum pH was found to be 8.0, the optimum temperature was 45^{o}C and purification fold of xylanase enzyme by ammonium sulphate precipitation was found to be 3.0, where as in dialysis it was 3.8 and in ion exchange chromatography it was 4.5. In conclusion the study proved that the agriculture waste sugarcane bagasse can be used as a substrate for the production of xylanase.

Key words: Xylanase, DNS assay, Ammonium Sulphate precipitation, dialysis, Ion exchange chromatography

1. INTRODUCTION

Xylanase (EC 3.2.1.8) an enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is a major component of the cell wall of plants[1]. Xylan consists of β-1, 4 – linked xylose with substituent's such as acetyl, arabinosyl and glucuronosyl residues[2]. Endo– 1, 4 – Xylanase is the main enzyme responsible for the cleavage of the linkages within the Xylan backbone[3]. The Xylan– degrading system includes endo-1, 4-xylanases, which release long and short xylo- oligosaccharides, or those that only attack longer chains and β-D-xylosidase, which remove D-xylose residues from short xylo-oligosaccharides[4]. Xylanases have been isolated from a wide range of microorganisms including fungi, actinomycetes and bacteria. Xylanases show great potential

for industrial applications mainly for the bioconversion of lignocellulose to sugar, ethanol and other useful substances, clarification of juices and wines, improving the nutritional quality of silage and green feed and de– inking processes of waste papers[5]. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites. Such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes like cellulase and xylanase used in waste treatment[6]. *Streptomyces coelicolor* is an actinomycetes, belonging to the family Streptomycetaceae. It is one of the best studied Streptomyces species at the genetical level with a well characterized chromosomal physical map, having more than 150 genes. It plays a major role in mineralization. In this study, xylanase was produced by *Streptomyces coelicolor* using agriculture waste. The Optimization of pH and temperature were characterized by different assay conditions. Purification of xylanase was carried out by using ammonium sulphate precipitation, dialysis and ion exchange chromatography. Finally the purified enzyme was immobilized in calcium alginate beads for future studies.

2. MATERIALS AND METHODS

2.1. Sample Collection and Treatment

Soil samples were collected from 5 different places from Namakkal district, Tamilnadu, India. The samples were collected aseptically and packed in sterile polythene bag. Approximately samples were taken from a depth of 10cm in and around field and carried to laboratory for further analysis. Collected soil samples were taken in a sterile beaker separately. Beaker containing l0g of soil samples were treated with 1.0gm of $CaCl_2$. This mixture was incubated at room temperature for 7 days. After incubation, it was used for the isolation.

2.2. Enrichment media Preparation and Isolation of micro organism

Actinomycetes broth was prepared under sterile aseptic condition. Followed by adding 1.0g of pre-treated soil sample into the actinomycetes broth and was incubated at 28ºC under shaking condition for 4 to 5 days. Streptomyces sporulation agar media were prepared for the isolation of xylanase producing micro organism. Media was prepared, poured on the Petri plates and labeled. Then take 0.1ml of culture from the enriched culture medium. Culture was spread on the sporulation medium plate by L-rod method. After spreading the plates were incubated at room temperature for 7 days.

2.3. Morphological Characteristic of *Streptomyces coelicolor*

The morphological characters like shape, size, elevation, margins, surface, edges, colour, Structure of *Streptomyces coelicolor* the strain were identified using standard methods.

2.4. Biochemical Characteristic of *Streptomyces coelicolor*

The *Streptomyces coelicolor* was tested for the following biochemical parameters such as Starch hydrolysis, Gelatin hydrolysis, Catalase test, Nitrate reduction test, Citrate utilization test.

2.4.1. Starch hydrolysis

Starch agar medium was prepared, poured on the Petri plates and labeled. A loop of single line streaking across the centre of the petriplate was transferred in to the starch agar medium. The plates were incubated at 37°C for 24 h. After that the plate was flooded with iodine solution.

2.4.2. Gelatin hydrolysis

Gelatin agar medium was prepared. Under aseptic conditions transferred a loop of culture to the gelatin containing medium. This was incubated at 37°C for 24 h and kept the tubes inside the refrigerator at 4°c for at least 4 hours[7].

2.4.3. Catalase test

In this test the plate were incubated at 37°C for 24 to 48 h. After the incubation period

hydrogen peroxide was poured on to the colonies[8].

2.4.4. Nitrate reduction test

Trypticase soy nitrate broth was Prepared, transferred a loopful of culture to the nitrate broth and incubated for 96 hours at 35°C. Following this minute amount of zinc was added and immediately observed for the sharp color change[9].

2.4.5. Citrate utilization test

Simmon citrate Agar medium was prepared. Culture was inoculated on to the simmon citrate agar medium, incubated at 37°Cfor 24 h. Color changes was observed.

2.5. Preparation of Vegetative inoculum

Streptomyces sporulation broth was prepared. Streptomyces organism was aseptically transferred to the broth from the master plates using loop inoculation. The broth was then incubated at room temperature for 72 h. 2.0gm of each powdered substrates were used for production of xylanase.

2.6. Processing and Effect of various Substrate on Xylanase Production

The sugarcane bagasse, pineapple, orange peels and pomegranate peels were obtained from a juice shop at Namakkal. It was washed several times in distilled water and then sliced. The sliced pieces were spread on the trays and shade dried. The dried slices were ground into fine powder and then sieved .The substrates were stored in the polyethylene bags at room temperature. 2g of each powdered substrates were used for production of xylanase. They were autoclaved at 15 lbs for 20 minutes before use.

The effect of different substrates (sugarcane bagasse, pineapple, orange peels, and pomegranate peels) on Xylanase production was studied by altering the different of substrates in the media.

2.7. Determination of xylanase activity

Xylanase activities were determined by DNS method. 0.5 % soluble oat spelt xylan in 50mM sodium phosphate buffer (substrate) was prepared. Pipette out 0.5ml - 3ml of substrate in different test tubes and equalize the volume to 3mL with dist water in all the tubes. Then add 3.0mL of DNS reagent. The mixture was incubated in a water bath at 60^0C for 5 minutes. When the contents of the tubes are still warm, add 1mL of 40% potassium sodium tartarate. A reddish brown colour developed at room temperature. The absorbance was measured at 575 nm using distilled water as blank[10].

Production of xylanase using sugarcane bagasse was high compared to other substrates (pineapple peels, orange peels and pomegranate peels). So the optimization was carried out in sugarcane bagasse containing streptomyces sporulation medium.

2.8. Optimization of culture conditions for enzyme production

Xylanase production was studied at different pH (6.0, 7.0, 8.0), temperature (25^o C, 37^o C, 45^o C), yeast extract as carbon source (0.05gm, 0.10gm, 0.15gm) and Tryptone (0.1gm, 0.2gm, 0.3gm) as a nitrogen source was assayed[11].

2.8.1. Determination of pH optima

The optimum pH for Xylanase production was determined by altering the pH of media. The pH 6.0 pH 7.0 and pH 8.0 were chosen for the experiment. The 100ml of the different pH adjusted media was distributed in Erlenmeyer flask under sterile condition and then 1.0ml of 72h old *Streptomyces coelicolor* culture was inoculated separately. It was then subjected to incubation at 30^0C. Xylanase production was assayed .The effective pH for the maximal Xylanase production was determined.

2.8.2. Determination of Temperature optima

The effect of Temperature on Xylanase production was studied by altering the incubation temperature. The temperatures chosen for this experiment were 25^0C, 37^0C and 45^0C respectively. The 100 ml of the different temperature adjusted media was distributed in Erlenmeyer flask under sterile conditions and then 1.0ml of 72hours old

Streptomyces coelicolor culture was inoculated separately, incubated at the above temperatures. Xylanase yield in the medium was assayed. The effective temperature for Xylanase production was determined.

2.8.3. Determination of Carbon source (Yeast extract) optima

The effect of different concentration of yeast extract on Xylanase production was studied by altering the concentration of carbon of the media. Three set of concentrations of the order of 0.5gm, 1.0g and 1.5g were chosen for the experiment. The 100 ml of the different yeast extract concentration containing media with standard was distributed in Erlenmeyer flask under sterile condition and then 1.0ml of 72hs old *Streptomyces coelicolor* culture was inoculated separately, incubated at 30^0C. Xylanase yield in the medium was assayed. The effective carbon source concentration for the Xylanase production was determined.

2.8.4. Determination of Nitrogen source (Tryptone) optima

The effect of different concentration of Tryptone on Xylanase production was studied by altering the concentration of tryptone in media. The chosen concentrations were 0.1gm, 0.2gm and 0.3gm respectively. The 100 ml of the different tryptone concentration containing media was distributed in Erlenmeyer flask under sterile condition and then 1.0ml of 72h old *Streptomyces coelicolor* culture was inoculated separately, incubated at 30^0C. Xylanase yield in the media was assayed. The effective yeast extract concentration for the Xylanase production was determined.

2.8.5. Determination of protein concentration by lowry method

Theprotein concentration was determined using folin phenol reagent by lowry method[12].

2.9. Enzyme Extraction and Purification

After optimization period the mixture was grounded and filtered using Whattman No: 1 filter paper. The filtrate was subjected to centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected and used for purification.

The partial purification of xylanase was carried out by ammonium sulphate precipitation, dialysis and ion exchange chromatography. The purification fold was calculated by using the following formula

Fold of purification = specific activity recovered/starting specific activity

2.9.1. Ammonium persulphate precipitation

The Solid ammonium persulphate was slowly added to the supernatant of crude enzyme preparation so as to reach 20% saturation. Addition of ammonium sulphate was carried out with continuous stirring in an ice bath, and then it was kept at 4^oC for overnight. The precipitated protein was removed by centrifugation at 10,000 rpm for 30 minutes at 4^oC. Ammonium sulphate was added to the supernatant to 80% saturation. The precipitated protein was again separated by centrifugation at 10,000 rpm for 30 minutes at 4^oC. The precipitated protein was dissolved in 15ml sodium acetate buffer. The purification fold of protein was calculated.

Enzymes can be concentrated by precipitation, and this is generally used as the initial step of purification. Salting out by ammonium sulphate is the best-known method for concentration and purification of the enzyme.

2.9.2. Dialysis

4.0gm of sodium carbonate was dissolved in 200ml of distilled water. The sodium carbonate solution was heated to boil. Five dialysis membranes each of 5cm length were kept in the boiling solution for 5-10 minutes. This will activate the dialysis membrane. Then the bags were removed with forceps and washed with distilled water. One end of the bag was tied with a twine. Then the bags were loaded with the sample through the open end. After loading, the open end was also tied with a twine. The twine in a glass rod was carefully tied at equally distances. Kept the glass rod over a beaker containing water. The rod was adjusted in such a way that the bag is

suspended enough to be completely immersed in water. Likewise five dialysis bags were set up for 5 samples.

2.9.3. Ion Exchange Chromatography

1.0gm of DEAE cellulose was mixed with 6ml of Phosphate buffer using magnetic stirrer. It was poured into the clean column, allowed to settle for 20 mins and phosphate buffer was eluted. 5ml of Activated buffer was added slowly without disturbing the settled cellulose. It was allowed for activation for 20mins and eluted the activated buffer. 2.0 ml of partially purified sample was added and eluted the impurities. 5ml of Elution buffer was added and the elution is collected and stored.

2.9.4. Immobilization of Xylanase by Entrapment Method

2% calcium chloride in 100ml water was prepared and transferred to three-fourth volume of a neat clean Petri plate. 2% sodium alginate in 10ml water was prepared and 0.5ml of Xylanase sample was added into it. Stirred the mixture continuously in a magnetic stirrer for 5 min. Drawn this mixture into a syringe and added drop wise slowly into the above Petri plate to form the enzyme beads. Leave the immobilised Xylanase beads to harden in the calcium chloride solution for 5–10 minutes. The alginate will be ionically cross-linked by the calcium ions.

3. RESULTS AND DISCUSSION

3.1. Identification of *Streptomyces coelicolor*

Soil samples were treated with Calcium Chloride. The treated soil sample was used for isolation of *Streptomyces sp*. by spread plate method using *Streptomyces* sporulation agar medium. The isolated organism was identified based on morphology (Table-1) and biochemical characterization (Table-2). Finally the morphological and biochemical test indicated that the suspected organism was *Streptomyces coelicolor*.

3.2. Processing and Effect of various Substrate on Xylanase Production

Different concentrations (2%) of substrates (sugarcane bagasse, pineapple, orange and pomegranate peels) were optimized for the enhancement of xylanase production by *Streptomyces coelicolor*, Xylanase activity was analyzed (Table-3). Sugarcane bagasse as a substrate was found to be the best one at 2 % for xylanase production.

Table.1. Morphological characteristics of *Streptomyces coelicolor*

Shape	Irregular
Size	Millimetres
Elevation	Elevated
Margin	levelled
Surface	Rough
Edge	Undulate
Colour	Dull white
Structure	Opaque

Table.2. Biochemical characteristics of *Streptomyces coelicolor*

Starch Hydrolysis	Negative
Gelatin Hydrolysis	Positive
Catalase test	Negative
Nitrate reduction test	Negative
Citrate utilization test	Positive

Table.3. Effect of various substrate concentrations

Name of the Substrates	Amount of the Substrates (gm / 100ml)	Amount of the Substrates (gm / 100ml)
Sugarcane bagasse	2.0	188
Pineapple peels	2.0	098
Orange peels	2.0	071
Pomegranate peels	2.0	112

3.3. Determination of pH optima

The optimum pH for Xylanase production was determined by altering the pH

of media. The pH 6.0 pH 7.0 and pH 8.0 were chosen for the experiment, Xylanase activity was analyzed (Table-4). The results revealed that optimum of p^H 8.0 to be the best for Xylanase production.

Table.4. Xylanase activity in different pH

Different PH of medium	Xylanase Concentration mg /ml
pH 6.0	142
pH 7.0	156
pH 8.0	198

3.4. Determination of Temperature optima

The optimum temperature for Xylanase production was studied by altering the incubation temperature .The temperatures chosen for this experiment were 25^0C, 37^0C and 45^0C respectively, Xylanase activity was analyzed (Table-5). The results revealed that the optimum temperature of xylanase was found to be 37^0C.

Table.5. Xylanase activity in different Temperature

Different temperature of medium	Xylanase Concentration mg /ml
25^0 C	171
37^0 C	185
45^0 C	200

3.5. Determination of Carbon source (Yeast extract) optima

The effect of different concentration of yeast extract on Xylanase production was studied by altering the concentration of carbon of the media. Three set of concentrations of the order of 0.5g o.10g and 0.15g were chosen for the experiment, Xylanase activity was analyzed (Table-6). The results revealed that the optimum Yeast extract concentration for Xylanase production was found to be 0.10g.

3.6. Determination of Nitrogen source (Tryptone) optima

The effect of different concentration of Tryptone for Xylanase production was studied by altering the concentration of tryptone in media. The chosen concentrations were 0.1g, 0.2g and 0.3g respectively, Xylanase activity was analyzed (Table-7). The results revealed that the optimum Tryptone concentration for Xylanase production was found to be 0.2g.

Table.6. Xylanase activity in different concentration of Yeast extract

Concentrations of Yeast Extract	Xylanase Concentration mg /ml
0.05 gm	173
0.10 g	296
0.15 g	242

Table.7. Xylanase activity in different concentration of Tryptone

Concentrations of Tryptone	Xylanase Concentration mg /ml
0.1 g	144
0.2 g	196
0.3 g	126

3.7. Purification of xylanse

The partial purification of xylanase was carried out by ammonium sulphate precipitation, dialysis and ion exchange chromatography (Table-8). The purification fold was calculated by using the following formula Fold of purification = specific activity recovered/starting specific activity

Table -8

Purification of Xylanase by various methodsifferent Purification process	Fold of purification
Ammonium persulphate precipitation	3
Dialysis	3.8
Ion Exchange Chromatography	4.5

3.8. Immobilization of Xylanase by Entrapment Method

The purified enzyme was immobilized by entrapping calcium alginate beads.

Immobilization is one of the method to store the enzyme without losing enzyme activity.

The present study contributed towards the screening, optimization of the nutritional parameters and culture conditions. Important findings included optimization of substrates (Sugarcane bagasse, Pineapple, Orange peels and Pomegranate peels), pH, Temperature, Carbon and Nitrogen source. However, not only the production level of xylanase, but also the ability to produce enzyme on low cost using agricultural residues such as Sugarcane bagasse. Xylanase produced by *Streptomyces coelicolor* seems to be more efficient for xylanase production. So, the use of xylanase enzyme as an effective bio-reagent to achieve bio-bleaching place of toxic chlorine compounds conventually used today, is should be encouraged.

4. CONCLUSION

The present study proved that soil taken from Namakkal district is abundant in xylanase producing actinomycetes and on strain improvement. The *Streptomyces coelicolor* micro organism may be used for high production of xylanase in industries.

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