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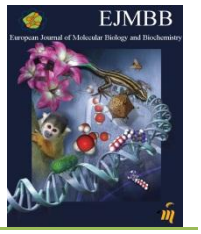

MICROBIAL PRODUCTION OF XYLANASE (EC 3.2.1.8) ENZYME USING BACILLUS SUBTILIS ISOLATED FROM GARDEN SOIL

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ABSTRACT
Xylans are polysaccharides almost as ubiquitous as cellulose in plant cell walls. In the present study xylanase producing *Bacillus subtilis* was isolated from garden soil using biochemical microbiological techniques; the isolates were subjected to xylan degradation. The influence of carbon, nitrogen sources, pH, temperature, NaCl, incubation and agitation in the enzymes production was characterized. Produced xylanase enzyme was partially purified by ammonium sulphate precipitation method. The optimum pH, temperature, additives, and carbon and nitrogen sources were as follows 8.0, 32°C, calcium chloride, xylan, and yeast extract respectively.

INTRODUCTION

Enzymes are biomolecules act as catalyst involved in the chemical reactions and they are specificity and selectivity. Xylanase (EC 3.2.1.8) is known as 1,4-β-D-xylan xylanohydrolase which cleaves the linear polysaccharide β-1,4-xylan into xylose [1]. Xylan, a highly complex polysaccharide and is a component of plant cell wall [2]. Xylanases are present in bacteria and fungi for the degradation of plant matter into usable nutrients [3-5]). Xylanse classified into endo-xylanase and ecto-xylanase [6].

Many species of microorganisms are known to have multiple xylanase production to utilize the xylan as a substrate. Each enzymes specialized function in the degradation of the complex polysaccharides. It is currently used in a broad array of industrial applications like food industry, animal feed, starch preparation, pulp and paper industry, biopulping, enzymatic bleaching, enhanced drainage, fiber modification, resin hydrolysis and pitch control [7-9]. Many fungi were used to large production of xylanase is often challenging because of slow growth profile, poor oxygen transfer and maintain proper levels of moisture.

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Bacteria were used more broadly than the fungi in industrial fermentations, since they excrete most of their enzymes. Bacterial xylanase have been found to be endo xylanase, producing xylobiase and xylotriose as the main end product. They are many xylanase producing bacteria are Bacillus stearothermophilus, Thermonospora fusca, Bacillus pumilus, Bacillus cereus, Bacillus circulans, Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis, Bacillus polymyxa, Bacillus sp, Flavobacterium sp, Dictyoglomus sp, Micrococcus sp, Pseudomonas sp etc.
In this study, xylanase producing bacteria was isolated and screened from garden soil by microbiological studies. The effect of physical and chemical conditions in the activity of xylanase enzyme like pH, temperature, incubation, agitation and substrate components were determined. The optimized parameters were used to maximum cultivation of xylanase with partial purification.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

Garden Soil Akilandeswari College, Wandiwash, South ndia was collected in a sterile container and it was brought to the laboratory for further processing. The collected sample was serially diluted up to 10^7 dilution using sterile saline as a blank and the diluted samples were plated into the sterile Nutrient agar using spread plate method. The NA plates were incubated at 37°C for 24

hours. The isolated colonies were further purified by streak plate method using sterile media plates. The pure cultures were inoculated into sterile nutrient agar slants and nutrient broth for further use.

Screening for xylanase producing organism

The isolated pure strains were screened for the production of extra cellular xylanase production using screening medium contains Beech wood xylan as a substrate [10]. The pure cultures were streaked at the center of the Sterile Xylan agar plates and the plates were incubated at 37^{O}C for 24 hours. The observation was made to see the Substrate utilized zone around the colony. Around 0.1% of Congo red solution was over layered on the medium and kept for 15 minutes. Destaining was made using 1M NaCl to make the zone visible and clear. Only positive and better zone formed strain was taken for further study.

Enzyme production

Preparation of Inoculum for bacteria:

The inoculum for further production of enzyme and other studies was prepared using Luria broth (LB). The pure culture was inoculated into sterile inoculum broth and was incubated at 37°C in a rotary shaker for overnight. The fresh over night culture was used as an inoculum for production of Enzyme.

Production

The enzyme production was carried out by shake flask fermentation using production medium which comprising of Glucose as a carbon source and amended with Peptone as a proteinacious substrate with pH 7.

500ml of sterile Production broth was prepared in one-liter conical flask and 5% inoculum was transferred aseptically in to the production medium. The inoculated medium was incubated at 37^{O}C for 48 hours. The medium was agitated at 200 rpm for better aeration and growth of the organism.

Xylanase Enzyme Assay

Bacterial crude culture preparation

After production of culture suspension was centrifuged at 5000rpm for 15 minutes and cell free extract was subjected to enzyme assay. This extract was stored at 4^{o}C for further analysis.

Plate assay

The plate assay was performed using Agar plates amended with Beech wood xylan. The Agar plates were prepared by mixing of 1% Beech wood xylan with 1.7% agar. After solidification of agar, Around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37^{O}C for overnight. The observation was made to see the hydrolytic zone around the well. For better appearance of the zone, around 0.1% of Congo red solution was over layered on the agar plates and kept for 15 minutes. Destaining was made using 1M NaCl to make the zone visible and clear [10].

Chemical Assay

Xylanase activity was determined by estimate the reducing sugar produced during enzymatic reaction by dinitro salicylic acid method [11]. Crude culture filtrate was used as enzyme sample; 1 ml of enzyme solution was mixed with 0.5% of xylan and incubated at 30^{O} C for 15 minutes to perform the enzyme substrate reaction. The contents were cooled and 1 ml of dinitro salicylic acid was added and heated at 90^{O}C for 5 – 15 minutes till slight reddish brown was developed. The contents were cooled and 1 ml of potassium sodium tartrate was added. The absorbency of the contents was measured at 540nm against reaction mixture prepared using distilled water as a blank.

Estimation of total protein

The chemical assay for the total protein content from the sample was determined using Bradford method. Brad ford reagent containing 100 mg of Coomassie brilliant blue G-250 dye in 50ml of 95% ethanol and 100 ml of 85% (w/v) phosphoric acid was added and the mixture was makeup to 1000ml with double distilled water. To estimation 1 ml of culture filtrate was taken and 5 ml of Bradford reagent was added. The tube was gently tilted once for mixing and the absorbency was taken at 595 nm in UV- vis spectrometer. The blank was prepared by mixing 1 ml of distilled water with 5 ml of reagent. The protein concentration was determined by comparing the value with standard graph prepared using Bovine serum albumin.

Parameter optimization studies

Effect of incubation time on xylanase production

Around 500ml of sterile production medium was prepared and 5% of bacterial inoculum was added aseptically. The inoculated medium was incubated at 37^{O}C temperature with shaking around 150 rpm. After incubation, around 20 ml of culture was aseptically with drowned periodically at 6 hours intervals up to 72 hours. The culture filtrate was examined for the total protein content and xylanase activity [10].

Temperature

To optimize the temperature, 100 ml of sterile production medium for bacteria was prepared in different conical flask with pH 7 and inoculated with 5% inoculum and incubated at different temperatures such as 28ºC, 32ºC, 37ºC, 42ºC, 47ºC, and 52ºC for 48 hours. The estimation of protein and enzyme activity was carried out [12].

pH

100 ml of sterile production medium was prepared for bacterial strain in different conical flasks and each flask was adjusted to different pH such as 5, 6, 7, 8, 9 using

0.1N NaOH and 0.1N HCl. After sterilization flasks were inoculated with 5% inoculum. The flasks were incubated at 32ºC for 48 hours. The estimation of protein and enzyme activity was carried out [10].

Carbon Sources

100 ml of sterile production medium with pH-8 for bacteria was prepared in different conical flasks. In each flasks were amended with different carbon sources such as Glucose, Xylose, xylan, sucrose and Maltose, The flasks were inoculated with 5% inoculum and incubated at 32ºC for 48 hours. The culture filtrates was collected and estimate the protein then the Enzyme activity was determined [10].

Nitrogen source

100 ml of sterile production media with pH.8 for bacteria was prepared in different conical flasks. In each flask were amended with different nitrogen sources such as Yeast extract, casein, peptone, potassium nitrate and ammonium nitrate. The flasks contain bacterial medium were inoculated with 5% inoculum and incubated at 32ºC for 48 hours. The culture filtrate was collected and Protein estimation, Enzyme activity was determined [13].

Additives

Sterile production media (pH.6) was prepared and 0.05% of different filter sterilized additives like SDS, Triton-X 100, EDTA, zinc sulphate and calcium chloride were added in separate flasks. Flasks appropriate additives were seeded with 5% bacterial inoculum. The bacterial culture was incubated at 32ºC for 48 hours. The total protein content and enzyme activity were estimated from the culture filtrate [10]**.**

Production of enzymes from crude substrates

Various natural products like Sugercane baggase, Filterpaper, Rice brawn, Wheat brawn, and Saw dusts were used as substrates for effective enzyme production. The production medium for both bacteria was prepared and the xylan was replaced by different crude substrates and pH was adjusted to 8. The appropriate media with different crude substrates were sterilized and seeded with 5% bacterial inoculum. They were incubated at 32ºC for 48 hours. The amount of total protein and Enzyme produced from different substrates were estimated by analyzing the culture filtrate [14].

Partial purification of enzyme

Separation of enzyme

The enzyme separation from the exhausted medium was done by Ammonium sulphate precipitation technique. The Bacterial and Fungal enzyme extraction was made from culture filtrate using 80% w/v of Ammonium sulphate saturation [15]. The mixture was then stored in cold room for 24 hours to precipitate all the proteins. Then the precipitation was separated by centrifugation around 10000 rpm for 10 minutes. Then carefully the supernatant was discarded and the remaining precipitation was dissolved with 2 ml of 0.5mM Tris-HCL buffer (pH-8). Then the mixture was subjected in dialysis.

The pretreatment of the dialysis membrane was done by immersing the membrane into the warm 0.5mM Tris-HCL buffer (pH-8) for 10 minutes (Sigma). One end of the membrane tube was closed tightly by fixing the clip. The precipitated bacterial and fungal protein was then transferred in the dialysis tube and other end was tied with a thread. The pack was suspended freely into a large beaker, which contains around 500 ml of 0.5mM Tris-HCL buffer (pH-8).

The buffer was stirred slowly using magnetic stirrer. The entire setup was placed in the cold room for 48 hours. Every 12 hours the buffer was changed periodically for better dialysis. After dialysis the clip from one end of the membrane was removed and the sample was transferred in to the clean plastic vials.

Table 1. Enzyme production and total protein content of bacterial isolate at different incubation time

S.No	Time of culture withdrawal (Hours)	Total protein content (µg/ml)	Enzyme activity (U/ml)
1	6	34	4
2	12	47	9
3	18	54	14
4	24	69	24
5	30	75	28
6	36	86	36
7	42	98	49
8	48	102	57
9	54	97	52
10	60	82	40
11	66	63	27
12	72	41	24

Table 2. Effect of Temperature on total protein and enzyme production by bacterial isolate

S.No	Temperature (^{O}C)	Total protein content (µg/ml)	Enzyme activity (U/ml)
1	28	101	56
2	32	107	61
3	37	109	59
4	42	76	42
5	47	61	37
6	52	47	21

Table 3. Effect of pH on total protein and enzyme production of bacterial isolate

S.No	pH	Total protein content (µg/ ml)	Enzyme activity (U/ml)
1	5	67	27
2	6	89	41
3	7	103	56
4	8	111	63
5	9	106	51

Table 4. Enzyme production and total protein production on different carbon sources

S.No	Name of Carbon sources	Bacteria	
		Total protein content (µg/ ml)	Enzyme activity (U/ml)
1	Glucose	48	15
2	Xylose	73	52
3	xylan	117	63
4	sucrose	86	57
5	Maltose	59	23

Table 5. Enzyme and total protein production on different nitrogen sources

S.No	Name of nitrogen sources	Bacteria	
		Total protein content (µg/ ml)	Enzyme activity (U/ ml)
1	Yeast extract	120	66
2	Casein	99	47
3	Peptone	116	61
4	Ammonium nitrate	46	17
5	Potassium nitrate	26	4

Table 6. Enzyme and total protein production on different additives

S.No	Name of the Additives	Bacteria	
		Total protein content (µg/ ml)	Enzyme activity (U/ ml)
1	SDS	45	19
2	Triton X- 100	39	9
3	EDTA	52	12
4	Zinc sulphate	96	54
5	Calcium chloride	118	63

Table 7. Enzyme and total protein production on different crude substrates

S.No	Name of substrates	Bacteria	
		Total protein content (µg / ml)	Enzyme activity (U/ ml)
1	Sugercane Bagasse	32	9
2	Rice bran	59	21
3	Wheat bran	69	45
4	Saw dusts	34	9
5	Filter paper	37	7

Figure 1. Enzyme production and total protein content of bacterial isolate at different incubation time

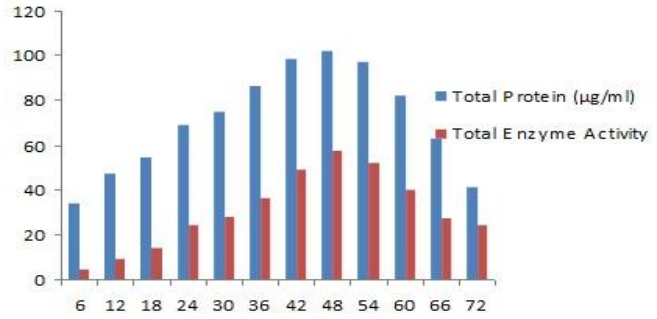


Figure 2. Effect of Temperature on total protein and enzyme production by bacterial isolate

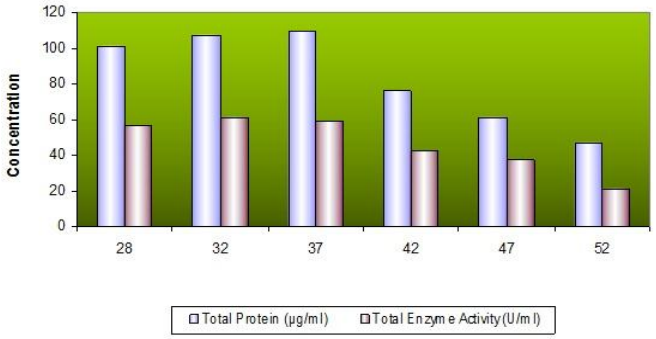


Figure 3. Effect of pH on total protein and enzyme production of bacterial isolate

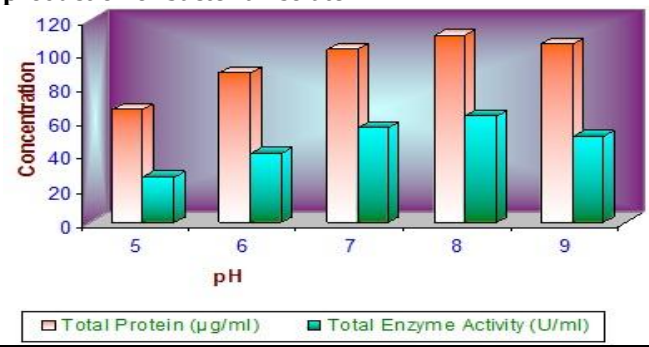


Figure 4. Enzyme production and total protein production on different carbon sources

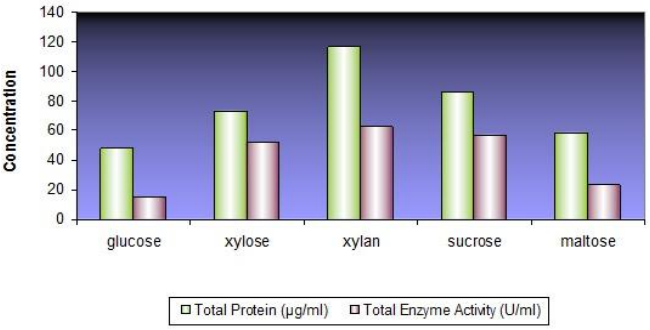


Figure 5. Enzyme and total protein production on different nitrogen sources

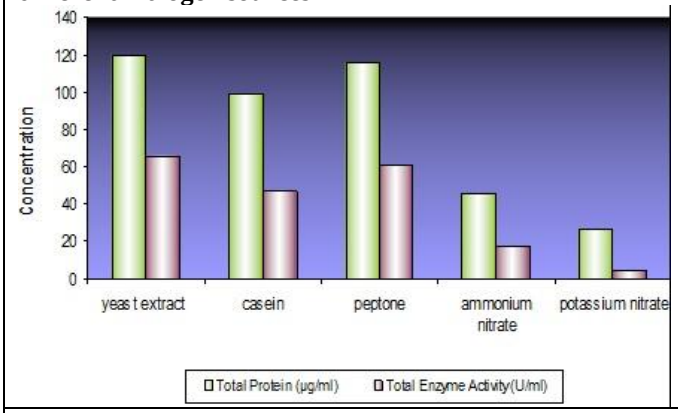


Figure 6. Enzyme and total protein production on different additive

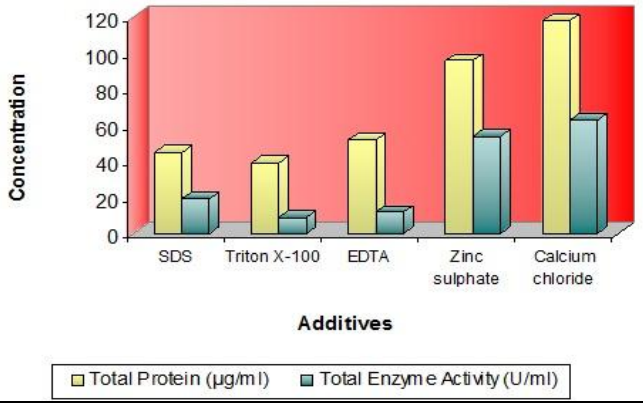
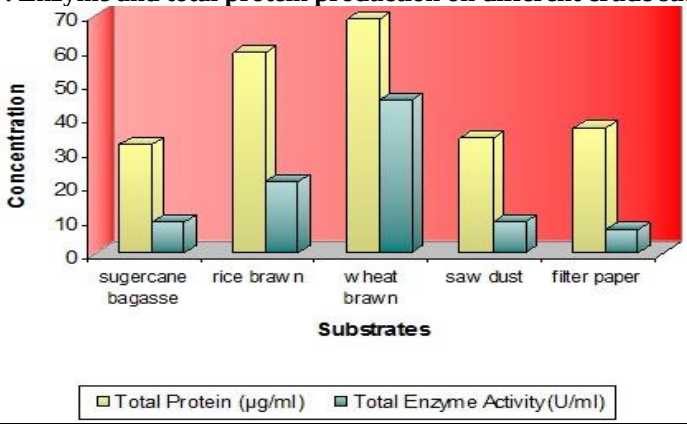


Figure 7. Enzyme and total protein production on different crude substrates



RESULTS AND DISCUSSION

Naturally occurring so many microorganisms are having ability to produce the various enzymes. Indeed, it is boon of god because now- a- days most of the enzymes are industrially important and human welfare. Xylanase is one of the important enzymes, which can be produced from microorganism.

In this study, the bacterial strain was isolated from garden soil from CAS in Botany, University of Madras, Chennai; because most of the plant wastes are degraded by the native microbes that are growing over that waste. In such a way, it is fact that the microbes which are isolated from the garden soil may have ability to produce xylanase. From the samples, around 13 bacterial strains were isolated. But later during screening it was found that 4 bacterial strains showed positive results on xylanase production. Between 4 bacterial strains the better zone formed bacterial strain was considered for further study

The growth study of the organism is essential for the production of enzyme because most of the extra cellular enzymes are produced during log phase of the organisms (Figure 1). Generally, during growth study, the bio moss of the cells will be estimated. Since it is an enzyme production, the modified growth study was carried out. The culture was withdrawn and checked up for enzyme activity. The bacterial cultures were with drawn every six hours once. The results revealed that there is gradual increasing of production has occurred from 24^{th} hrs to 97^{th} hrs and higher production has occurred at 48^{th} hours (Table.1). These shows that bacterial isolate should have maintained its log phase from around 24^{th} hours to 48hours. Besides, it is believed that the higher production of xylanase has occurred in extreme log phase because even though the log phase was maintained between around 24^{th} hrs to 48 hrs the followed drop of production has indicated that the organism should have entered in to stationary phase of growth. This variation of log phase timing is based on the nutrient present in the medium and the cultural condition of the organism. The environmental parameter also influencing the maintenance time of the Bacteria. Interestingly some Gram- positive bacteria also show the same kind of growth time like *Bacillus* sp [10].

The environmental parameters are showing great influence in the growth of the organisms and the production of enzymes. The main parameters like Temperature, pH are very essential parameters of the production. To optimize the optimum temperature for the better production, productions were made in various temperatures (Figure 2). It was found that like other mesophilic organisms, the higher xylanase activity was found (107U/ml) at 32^{O}C from bacteria (Table.2). These indicate that the optimum temperatures for better production of bacterial isolates are 32^{O}C. The temperature requirement of the organism is based on the nature of organisms. Many *Bacillus spp.* needs 32-37^{O}C for better production of xylanase [10]. Next to the temperature, the pH is the important parameter which determines the growth of the organism and xylanase production (Figure 3). Generally most of the bacteria require medium pH for its growth. The study results showed that the optimum pH around 8 is better for bacterial isolate (Table.3). Like temperature, Different microorganisms need different pH ranges for its xylanase production. Most of the *Bacillus spp.* has produced high amount of xylanase between pH 7-9 [10].

The carbohydrates are soul energy source for most of heterotopic organisms. These shows great influence on the production of many enzymes. In many of the other enzyme case, the production will be carried out by medium amended with xylan as a substrate and as carbon source for better growth and production. We can see the higher production of many other enzymes due to amending of glucose. But in this study, xylan was found to be a right carbon source for bacterial strain for higher production of xylanase (Table.4). Significant reduction in the production due to amending of glucose as a carbon source (Figure 4). The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we are using may stimulate or down modulate the production of enzymes. The yeast extract was found to be a better nitrogen source for bacterial isolate (Figure 5). In the case of inorganic nitrogen source, the ammonium nitrate was found to be more effective for bacterial strain (Table.5).

Generally, in the case of enzyme production, least quantity of additives like ionic detergents or minerals will be added to the medium to check the inhibition or enhancement of the production. In our study, we found that calcium chloride and zinc sulphate has enhanced the xylanase production by bacteria (Figure 6 and Table.6). This induction may be varying from organism to organism based on the mineral requirement and tolerance against the ionic detergents. The *Bacillus sp*. has produced considerable amount of xylanase from the medium amended with 0.8 ppm of Ca^{2+} [10]**.**

The real and beneficial production of enzyme is produced from the natural sources and industrial wastes. In this study, several natural and industrial wastes were used as substrates (Figure 7). The results revealed that the bacterial isolate has produced maximum quantity of xylanase from wheat bran (Table.7). This strain was isolated from bagasse and produced higher quantity from the same substrate [14].

CONCLUSION

The xylanase enzyme was produced by bacterial strain isolated from garden soil. The isolated bacterial stain was identified as *Bacillus subtilis*. Xylanase activity of *B. subtilis* was determined by plate method and chemical assay method. In the production optimization studies, the bacterial strain needs temperature around 32^{O}C, pH.8,

xylan as a carbon source and incubation time for 48 hours for its higher enzyme productivity.

Enhanced production of xylanase enzymes achieved using optimized conditions like yeast extract as nitrogen source and additives are calcium chloride and zinc sulphate The bacterial strain has produced higher enzyme production from wheat brawn as substrate.

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CONFLICT OF INTEREST: NIL

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