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Partial purification and optimization of xylanase from *Bacillus circulans*

Dhruti Pithadiya[1]*, Deep Nandha[2] and Aarti Thakkar[3]

[1]*School of Science, Gujarat University, Ahmedabad, Gujarat, India*
[2] *Disha Life sciences Pvt. Ltd., Ahmedabad, Gujarat, India*
[3]*President Science College, Gujarat University, Ahmedabad, Gujarat, India*

ABSTRACT

Bacillus circulans was isolated for xylanase production from paper effluent collected from paper mill. Extracellular Xylanase production was observed in the culture medium with pH 7 at 30˚C. Culture medium amended with 1% birch wood xylanwas found to be suitable source to enhance Xylanase yield in shaking condition at 7 pH at room temperature in submerged fermentation. Partial purification of Xylanase was done from crude enzyme extract by three steps (ammonium sulphate precipitation, TCA-Acetone precipitation and Ion exchange chromatography).The molecular mass of Xylanase was ~ 20 KDa and was observed by SDS-PAGE Zymography technique. Optimum Enzyme activity was recorded at 30˚C in standard conditions.

Key words: Xylanase, SDS – PAGE, Zymography

INTRODUCTION

Xylanase [E.C.3.2.1.8] is the name given to a class of enzymes which degrade the linear polysaccharide β-1,4xylan to Xylose, thus breaking down hemicelluloses which is a major component of the cell wall of plants [1].The substrate of Xylanase, xylan, is the second most-abundant polysaccharide in nature, accounting for approximately one-third of the renewable organic carbon on Earth [2] and It constitutes the major component of hemicellulose, a complex of polymeric carbohydrates, including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-Galactose, D-glucose and D-mannose) and arabino galactan (hetero-polymer of D-Galactose and arabinose)[3]. Xylan is distributed in several types of tissues and cells and is present in a variety of plant species[4], being found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants(<30%) [5]. Biodegradation of xylan requires action of several enzymes, among which xylanase play a key role [6]. In 1985 microbial Xylanase was isolated by Biely [7]. Xylanase is produced by many bacteria, actinomycetes [8] and fungi and some of the most important xylanolytic enzyme producers include *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia and Bacillus* [9,10,11]

Xylanase can be classified as endo- and exo-xylanases [12]. Xylanase have been classified in at least three ways: based on the molecular weight and isoelectric point (pI) [13], the crystal structure [14] and kinetic properties, or the substrate specificity and product profile. As the first classification is not sufficient to describe all xylanases, several exceptions have been identified [15] because not all xylanase have a high molecular mass (above 30 kDa) and low pI or a low molecular mass (less than 30kDa) and high pI [16] Therefore, a more complete system, based on the primary structure and comparison of the catalytic domains, was introduced analyzing both the structural and mechanistic features [17,18]. The majority of the studied xylanases have been classified into the GH10 or GH11 families, whereas studies of the xylanases in families 5, 7, 8and 43 are still emerging[19]. The xylanolytic enzyme system that carries out the xylan hydrolysis is normally composed of a repertoire of hydrolytic enzymes, including

endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72) [20]. All of these enzymes act cooperatively to convert xylan into its constituent sugars [21]. Among all xylanases, endoxylanases are the most important due to their direct involvement in cleaving the glycosidic bonds and in liberating short xylooligosaccharides [22]. *Bacillus* species secrete appreciable levels of extracellular Xylanase [23]. Many of the xylanase producing microorganisms express multiple iso-forms that have been ascribed to a variety of reasons that is, heterogeneity and complexity of xylan structure [24]. Filamentous fungi are industrially important producers of xylanase due to higher yield compared to yeast and bacteria [25]. Xylanase are produced by either solid state or submerged fermentation [26].

Column chromatographic techniques, mainly ion exchange and size exclusion are the generally utilised schemes for xylanase purification, but there are also reports of purification with hydrophobic interaction column chromatography [27]. There are several reports regarding the purification of xylanase to electrophoretic homogeneity, however, the yield and purification fold varies in different cases. In all the cases the culture supernatants are initially concentrated using precipitation or ultrafiltration techniques. A moderately thermostable xylanase was purified from *Bacillus* sp. Strain SPS-0 using ion exchange, gel and affinity chromatographies [28].

Xylanase are of industrial importance, which can be used in paper manufacturing to bleach paper pulp, increasing the brightness of pulp and improving the digestibility of animal feed and for clarification of fruit juices. Applications of xylanase avoid the use of chemicals that are expensive and cause pollution [29]. In Food Industry, Xylanase improves maceration and juice clarification, reduces viscosity and improves extraction yield and filtration, process performance and product quality. It improves elasticity and strength of the dough, thereby allowing easier handling in baking product. In Feed industry, it decreases the content of non-starch polysaccharides, thereby reducing the intestinal viscosity and improving the utilization of proteins and starch. It improves animal performance, increases digestibility and nutritive value of poorly degradable. A large quantity of raw materials is processed in pulp and paper, textile, and in feed industries, and thus the volume of effluents released is very high. In order to deal with industrial waste and waste water, research and development departments have worked toward the establishment of strategies that are totally free from the use of hazardous chemicals and provide the same results as are achieved through conventional methods. Treatment with xylanase does not pose any environmental threat and therefore provides a glimmer of hope to environmentalists[30].

This study primarily aims at the isolation of native xylanase producing bacterial strains. Selection and identification of those able to produce maximum levels of Xylanase, along with the optimization of the process parameters and partial purification of it.

MATERIALS AND METHODS

2.1 Collection of Sample:-
Paper effluent sample was collected from Dhanlakshmi paper mill located in Bapunagar, Ahmedabad (Gujarat). Sample was collected in sterile plastic bottle.

2.2Isolation of Organisms on Medium-I [31]: -
Isolation was carried out in Medium I (Birch wood xylan, 0.5gm; Yeast extract, 0.2 gm; NaCl, 0.25gm; KH_2PO_4, 1.5 gm; NaH_2PO_4, 3.0 gm; NH_4Cl, 0.5 gm; $MgSO_4.7H_2O$, 0.025gm; Distilled water, 100 ml and pH was adjusted at 7.0). Xylanase producing bacteria were isolated from paper effluent in the Medium I by using Birch wood xylan as the sole carbon source.

2.3 Identification of Microorganisms:-
(i) Microbial Identification by Gas Chromatographic Analysis of Fatty Acid Methyl Ester (GC-FAME)
Selected isolates were identified on the bases of various biochemical tests and further identification was done by GC-FAME analysis by the method described in MIDI Sherlock® Microbial Identification System catalogue. The method consists of saponification in dilute Sodium hydroxide/Methanol solution followed by derivatization with dilute Hydrochloric acid/Methanol solution to give respective Fatty Acid Methyl Esters (FAMEs). The FAMEs were then extracted from the aqueous phase by the use of an organic solvent and the resulting extract was analyzed by gas chromatography.

2.4 Enzyme production from submerged fermentation [32]
Selected bacterial strain was inoculated in culture media and incubated in shaking condition at 150 rpm for 24-48 hrs at ambient temperature. The cell free supernatant was recovered by centrifuging samples at 15,000 rpm at 4 ˚C for 10 min. Supernatant was filtered using whattman filter no.1 and few drops of 0.1 % Formalin were added.

Supernatant was used as a crude enzyme for measuring the total soluble protein and reducing sugar. For Estimation of Enzyme activity, 1.0 ml of extracellular crude enzyme was taken and mixed with 0.5 ml substrate (0.6% xylan in 50mM Phosphate buffer) along with 0.5 ml 50mM Phosphate buffer. The residual enzyme activity was carried out according to DNS method.

Estimation of total soluble protein, 0.5ml of crude enzyme was taken and mixed with 0.5 ml substrate solution (0.6% xylan in distilled water). The residual protein content was estimated by using Lowry method (Lowry et al. 1951).

2.5 Optimization of Culture condition for Xylanase production [33]
2.5.1 Effect of pH on Xylanase production
Isolates were grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) in shaking conditions (150 rpm) having various pH like 4.0, 6.0, 8.0, 10.0. The enzyme assay was carried out after 48 hours of incubation.

2.5.2 Effect of temperature on xylanase production
Effect of temperature on enzyme production by bacteria was analyzed by varying the incubation temperature at 30˚C, 37˚C, 60˚C, 80˚C. After incubation, absorbance was measured at 600 nm. Enzyme was extracted and estimated reducing sugar and total soluble protein by DNS method and Folin Lowry method respectively.

2.5.3 Effect of Incubation time on Xylanase production
Isolates were grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) in shaking condition (150 rpm). Few ml of culture was removed from it at different intervals like 24, 48, 72 and 96 h and enzyme activity was checked.

2.6 Effect of various parameters on enzyme activity [34]
2.6.1 Effect of temperature & pH on enzyme activity
Effect of temperature on enzyme activity was measured by incubating the enzyme and substrate mixture at different temperature (37˚C, 60˚C, 80˚C). Effect of pH on enzyme activity was determined at different pH such as 4, 5, 6, 7, 8 and 10 and enzyme activity was carried out by DNS method.

2.7 PARTIAL PURIFICATION OF ENZYME:-
For the partial purification of enzyme was done by two methods 1.Fractional ammonium sulphate precipitation, 2. TCA (Trichloroacetic acid): Acetone precipitation.

2.7.1 Fractional ammonium sulphate precipitation [35]
Maximum production of Xylanase was optimized and ammonium sulphate precipitation was carried out for it. The amount of ammonium sulphate was required calculated for precipitation. The precipitation was carried out at 4°C in stirring condition by using various percentage of ammonium sulfate such as 45%, 70%, 75%, 80% and 85% respectively. Precipitates were collected by centrifugation at 8000 rpm for 20 min at 4°C and dissolved in 4 ml of sodium phosphate buffer pH 7. Total protein content of each fraction was carried out by Folin Lowry method. Enzyme activity of each fraction was carried out by using 1% xylan as a substrate.

2.7.2 TCA (Trichloroacetic acid): ACETONE PRECIPITATION [35]
The 10.0 ml protein solution was taken in centrifuge tube. 100 ml 10% TCA: Acetone solution was added in the beaker and mixed by vortex. The mixture was allowed to stand in cold at 4˚C overnight. The pellets were collected by centrifugation at 10,000 rpm for 10mins at 4˚C. The pellets were dissolved in 50 mM phosphate buffer. Enzyme activity was checked by DNS method and total soluble protein was analyzed by Lowry method.

2.7.3 Ion Exchange Chromatography (GeNeiTM) [34]
The crude enzyme was loaded on to equilibrated CM-cellulose column. The top and bottom caps of column were replaced and incubated for 1 hr at R.T. with intermittent mixing. After an hour, the column was allowed to settle. Then, slowly the supernatant was pipetted out or decant without disturbing the column. The column was washed with approximately 30 to 40 ml of 1X Wash buffer. Enzyme was eluted from the column using 15 ml of 1X elution buffer. The elute was collected and enzyme activity total soluble protein was determined by DNS Folin-Lowry method respectively.

2.8 SDS-PAGE and Zymography [34]
Protein sample and 2X Non reducing SDS-gel loading buffer was taken in the ratio 1:1 and mixed well. This mixture was heated at 100˚C for 10 min and centrifuged at 1000 rpm for 10 min at 4˚C. 50 µl samples were loaded with the help of micro pipette and allowed for electrophoresis at 50V. The gel washed with distilled water and

placed in petriplate containing silver nitrate stain and allowed to stand for 1 hr. The gel was agitated slowly on shaker. The staining solution was decanted and washed with distilled water. The gel was kept in destaining solution for 2 to 3 hr. The molecular weight of visualized protein bands was compared with molecular weight markers. Gel from cassette was removed and placed in plastic tray containing 250 ml of renaturing solution (Triton X-100).The gel was incubated for 30 min at 4˚C and gentle agitation was given. After washing the gel, the gel was incubated in activation buffer at 4˚C for overnight. The activation buffer was decanted and gel was stained in staining solution for at least 2 hr. Decanted the staining solution and kept the gel in destaining solution for 1 hr. After the destaining, the Xylanase activity was detected as unstained white bands on a blue stained background of the gel, indicating the proteolysis of the xylan substrate. These zymograms were quantitated using Bio-Rad gel documentation system (Bio-Rad G-800). The relative proteinase activity was determined for each proteinase by multiplying the area of each lysed band by its optical density (O.D.*mm^2).

RESULTS

3.1 Isolation of Microorganism:

For the preliminary experiment of this study, Thirty five xylanase producing bacteria were isolated from paper effluent in the Medium I by using Birch wood xylan as the sole carbon source. The plates were incubated for 24 hrs at 37˚C, Clear zone was observed around the cremish white colonies (Figure 1). One isolate was selected on the bases of the zone size of xylan degradation and was further identified as *Bacillus* spp. from various biochemical analyses by the method of Bergay's Manual of systematic bacteriology.

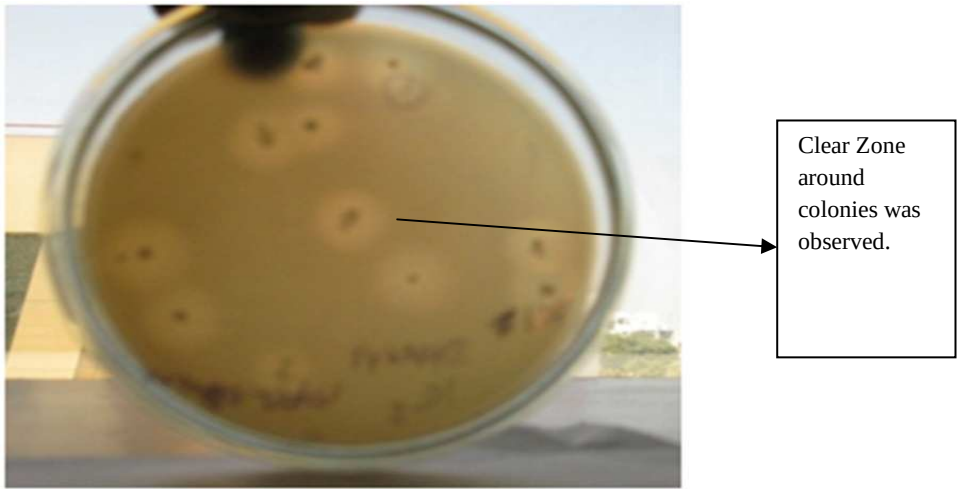

Figure 1. The plates showing colonies of Xylanase producing bacteria isolated from paper effluent

3.2 FAME analysis by Gas chromatography:-

For further identification up to species level FAME analysis was done and two isolates were identified as *Bacillus ciruculans* GC subgroup B and *Marinibacillus marinus*. Out of the two, *Bacillus circulans* showed higher resemblance with the isolated organism. The isolate was identified as *Bacillus ciruculans.*

Matches:

Library	Sim Index	Entry Name
RTSBA6 6.00	0.262	Bacillus-circulans-GC subgroup B
	0.179	Marinibacillus-marinus (Bacillus)

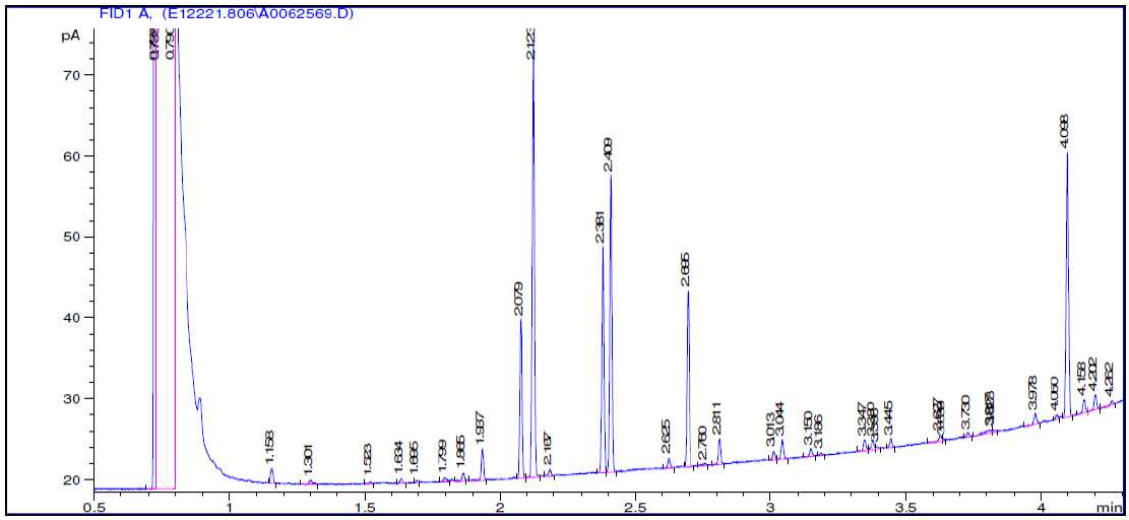

Figure 2. Chromatograph showing the Fatty Acid Methyl Ester profile on Gas Chromatography

3.3 Crude Enzyme Preparation:

Extracellular xylanase was produced from *Bacillus circulans* in submerged fermentation. Crude enzyme was prepared from the submerged fermentation broth by centrifuged it and filtered it through Whattman filter paper no.1.The enzyme activity of Xylanase was done by DNS method.

3.4 Optimization of Culture condition for xylanase production:-

Isolate was grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) in shaking condition (150 rpm). Enzyme activity was checked at different intervals like 24, 48, 72 and 96 h. Highest amount of xylanase production was found after 48 hours of incubation. Isolates was grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) shaking conditions (250 rpm) having various pH like 5.5, 7.0 and 8.5. The enzyme assay was carried out after 48 hours of incubation. The isolate give higher enzyme activity at pH 8which is shown in Figure 4.Effect of temperature was checked on xylanase production. Growth was carried out in 100ml of medium incubated in shaking condition. Highest amount of enzyme was produced at 30˚C and at pH 6.0 (Figure 3& Figure 5).

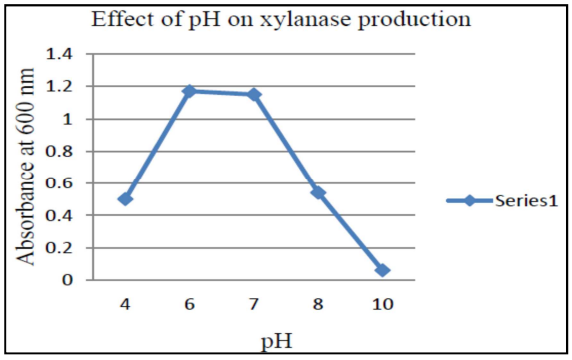

Figure 3. Influence of culture pH on Xylanase production by *Bacillus circulans* under SmF

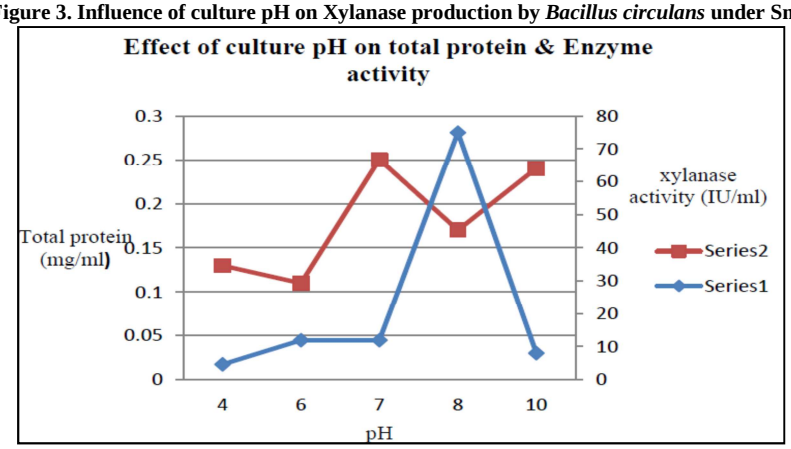

Figure 4. Influence of culture pH on total protein and Enzyme activity

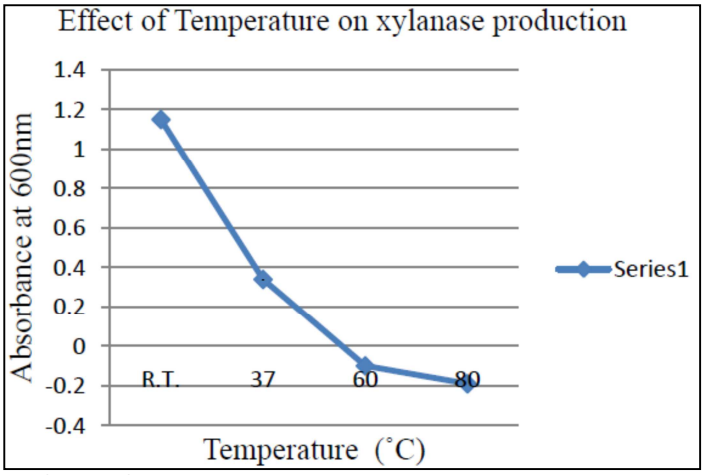

Figure 5. Influence of temperature on Xylanase production by *Bacillus circulans* under SmF

3.5Effect of various Parameters on Enzyme activity:-

Enzymes are sensitive to changes in the environment in which they work and temperature can profoundly affect the activity of enzyme. Optimum enzyme activity was recorded at 30˚C (Figure 6) and enzyme activity was reduced in the same way as the temperature was increased. The changes in the hydrogen ion concentration (pH) can profoundly affect the enzyme activity. At pH 8.0, Maximum Enzyme activity was obtained (Figure 7).

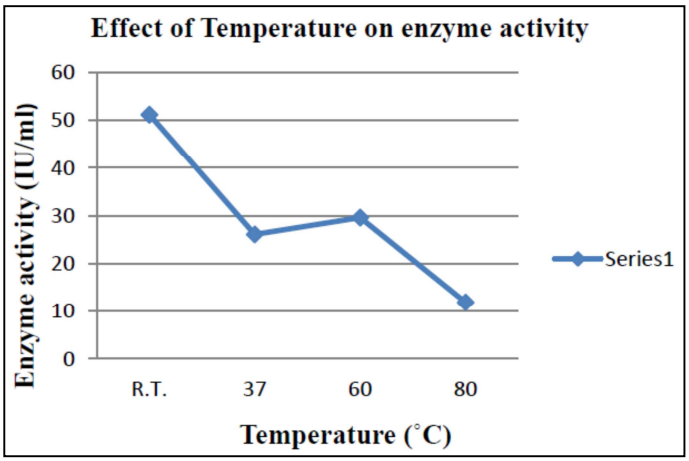

Figure 6. Influence of temperature on Enzyme activity

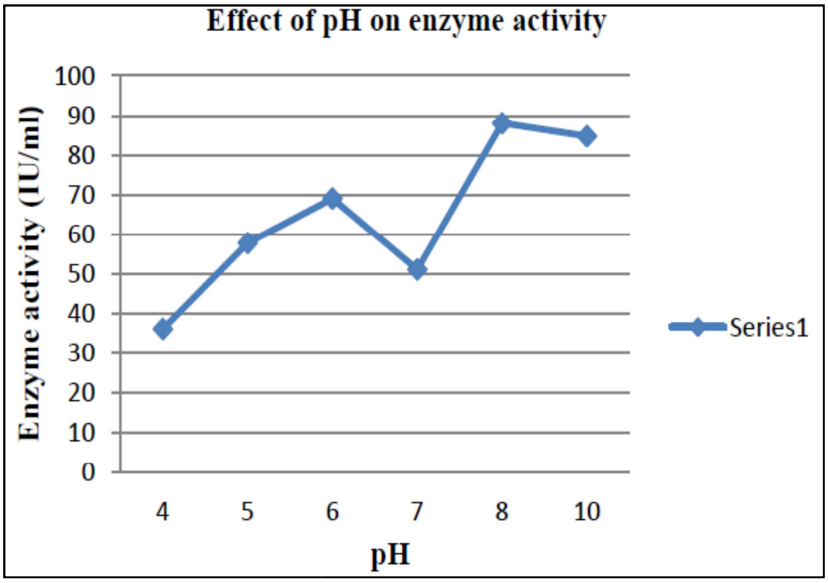

Figure 7. Influence of pH on Enzyme activity

3.6 Partial Purification of Enzyme:-
After Ammonium sulphate precipitation, Xylanase activity in fraction sample (10 ml) was 204 IU with 32.1% recovery and 5.42 fold purification with specific activity of Xylanase was 127.5IU/mg protein.

TCA: Acetone precipitation test was also used to further partial purification of enzyme. The Xylanase activity in fraction sample (12 ml) was 288.0 IU/ml with 45.3 % recovery and 5.10 fold purification with specific activity of xylanase was 120 IU/mg protein. Higher enzyme activity was observed from TCA: acetone precipitation than ammonium sulphate precipitation Table I.

After the ion exchange chromatography step, the Xylanase activity in the elution buffer (6.0 ml) was 306 IU with 1.5 % recovery and had 7.48 fold purification and specific activity of Xylanase was 175.8 IU/mg protein.

Table 1. Data analysis of Purification of Xylanase from Bacillus circulans produced by SmF

No.	Sample	Protein content (mg/ml) (P)	Total volume (ml) (V)	Total protein (mg) (TP)	Enzyme activity (IU/ml) (A)	Total Enzyme activity (IU) (TA = A × V)	Specific enzyme activity (IU/mg protein) (SA = TA step / TP step)	Fold purification (FP = SAstep / SA crude)	Yield or recovery (%) (Y or R = TAstep / TA crude ×100
1.	Crude Filterate	0.18	150	27.0	4.23	634.5	23.5	1.0	100
2.	Precipitation Test of crude enzyme								
	(a) Ammonium sulphate precipitation	0.16	10.0	1.6	20.40	204.0	127.5	5.42	32.1
	(b) TCA:Acetone precipitation	0.20	12.0	2.4	24.0	288.0	120.0	5.10	45.3
3.	Ion exchange chromatography (CM-cellulose)	0.29	6.0	1.74	51.0	306.0	175.8	7.48	1.5

3.7SDS PAGE and Zymography:-
Nature of enzyme was studied after partial purification by SDS-PAGE. The band appearing in the crude sample (lane 5) was not clear and also single band was not observed, where as single band was obtained from purified fraction after ion exchange chromatography (lane 3, 4)and was compared with protein marker (Bangalore GeNei). The SDS-PAGE study showed the presence of xylanase protein ~ 20 kDa in both samples (Figure 8).

Zymography helped for detection of enzyme activity on SDS PAGE incorporated with 1% xylan solution. Partial purified enzyme obtained from ion exchange chromatography was loaded on SDS-PAGE incorporated with xylan solution (lane 1 and 2) (Figure 9).

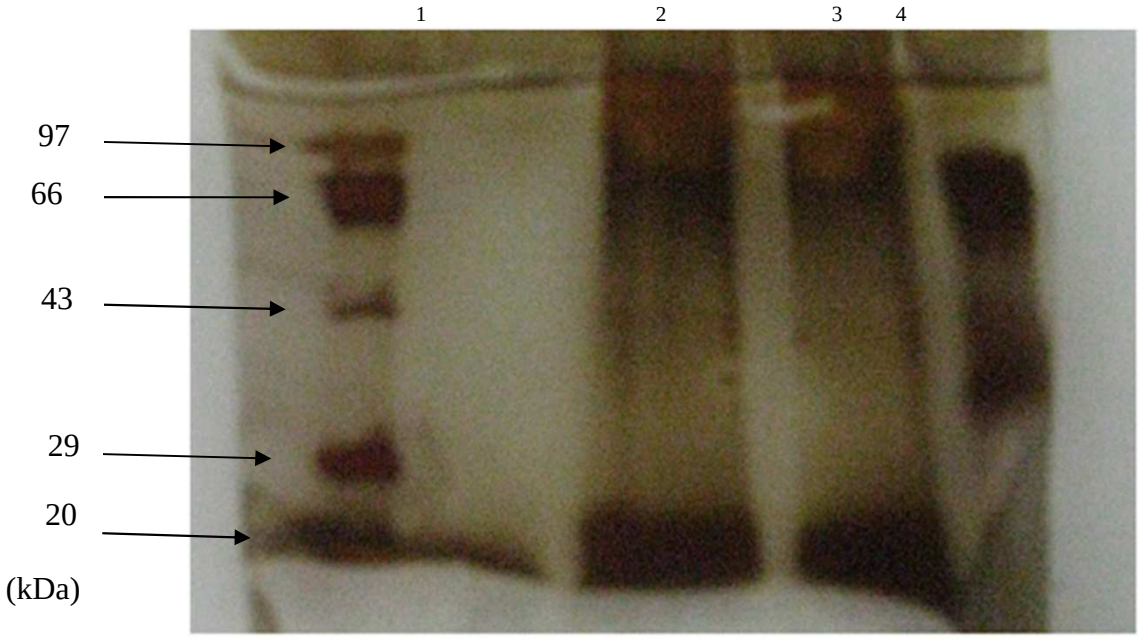

Figure 8. SDS-PAGE

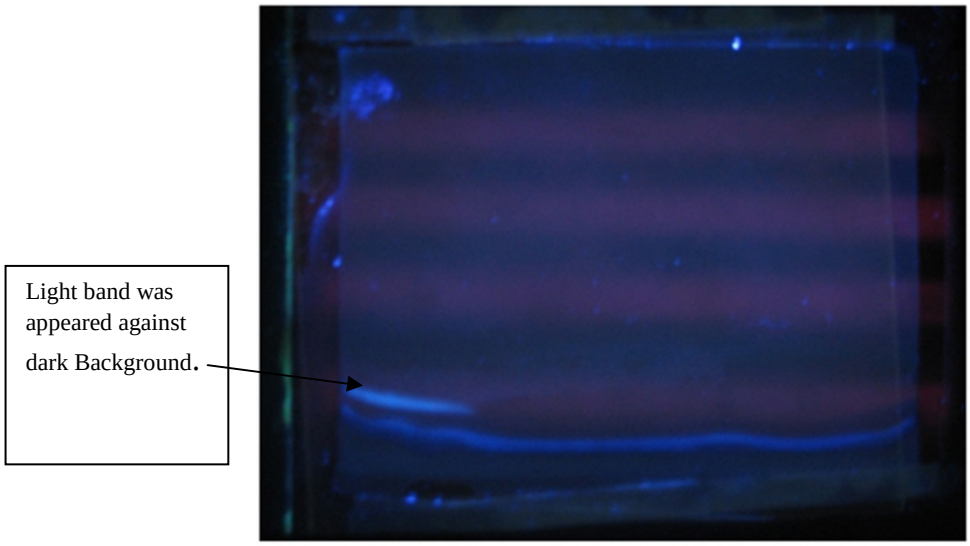

Figure 9. Zymogram

DISCUSSION

The Characteristics of Microorganisms are similar to those described for *Bacillus* sp in the Bergey's Manual of Systematic Bacteriology. Rajashri Kamble and Anandrao Jadhav (2011) found 25 bacterial strains forming clear halos around their colonies on xylan agar plates. *Bacillus Circulans* was used for extracellular enzyme production. Crude Enzyme preparation was done by using Submerged Fermentation. Enzyme activity by DNS method was done and 51 IU/ml required releasing 1 micromoles of xylose in 1 min. Total soluble protein was 294.5µg/ml in a 1ml crude extract.51 IU/ml of enzyme activity was observed by Arunachalam et al. (2001) isolated from *A. flavus* isolates, while Bailey & Viikari (1993) isolated cellulase-free xylanase from *A. fumigatus.* Most researchers have used submerged cultures for xylanase production, which allows control over the degree of aeration, pH and temperature of the medium as well as control over other environmental factors required for optimum growth of organisms. Haltrich et al. (1996) reported that purified xylans can be excellent substrates for xylanase production and are frequently used for small-scale experiments. In a number of organisms, this pure and defined substrate increased the yield of xylanase, caused a selective induction of xylanase, either with complete absence or with low cellulase activities (Biswas et al. 1990; Gilbert et al. 1992).*Bacillus circulans* was given maximum xylanase production at 30˚C and at pH 6.0. Similar findings were also reported by Sepahy et al. (2011) showing optimum pH of 8.0 for Xylanase production by *B. mojavensis* AG137 in submerge fermentation. Simphiwe et al. (2011) reported

that eight different strains of *Bacillus* sp. showed maximum xylanase production at pH 8.0. *Bacillus pumilus* showed maximum xylanase production at pH 7.0 (Monisha et al. 2009). Sepahy et al. (2011) reported optimum temperature of 37 °C for xylanase production by *B. mojavensis* AG137 in submerged fermentation. Different strains of *Bacillus* sp. gave maximum yield of xylanase production at incubation temperature of 45 °C and 55 °C (Simphiwe et al., 2011). Monisha et al. (2009) reported that maximum xylanase production by *B. pumilus* at 37 °C. Maximum enzyme activity from *Bacillus circulans* was found at 30˚C temp and 8.0pH. The optimum pH of xylanase enzyme from different Bacillus sp. was reported at pH 5 (Pratumteep et al. 2010) pH 7 (Sanghi et al. 2010), pH 8 (Kamble&Jadhav2012) and pH 9 (Annamalai et al. 2009). Xylanase enzyme from various *Bacillus* species has optimum temperature of 40–60 °C (Khandeparker et al. 2011; Kumar et al. 2011; Pratumteep et al. 2010; Roy & Rowshanul 2009; Sanghi et al. 2010; Sa'-Pereira et al. 2002). Xylanase from *Bacillus* sp precipitated with 90% ammonium sulphate, showed 1.25Umg-1specific activity, with 2.01 fold purification and 80.0% yield (Breccia et al. 1997). Recently, in *Bacillus pumilus* sp. where 21-fold purity was observed with 2% yield by using TCA:Acetone precipitation method. The specific activity of xylanase produced by *Bacillus pumilus* was previously reported as 298 U/mg by Panbangred et al. (2010). Sanghi et al. (2010) reported one step purification and characterization of an extracellular cellulase free xylanase from a newly isolated alkalophilic *Bacillus subtilis* ASH. Xylanase was purified to homogeneity by 10.5-fold with ~43% recovery using ion-exchange chromatography through CM-Sephadex C-50. The Molecular weight from *Bacillus Circulans* was ~ 20KDa. Xylanase obtained from *Bacillus* sp strain K-1 also has a molecular weight of 23 kDa. (Ratanakhanokchai et al. 1999). The molecular weight of the purified xylanase was found to be 20 kDa by SDS-PAGE and Zymogram analysis (Shrinivaset al, 2010).

CONCLUSION

This study proved that the paper effluent is a rich source of Xylanase producing bacteria. Medium I proved to be optimum media for growing xylanase producing bacteria. Xylanase production under culture condition was optimized by studying effect of various parameters like pH and Temperature. Xylanse production was maximum at pH 8.0 at 30˚C.Maximum xylanase activity was obtained after Ion Exchange chromatography was 306 IU with 1.5 % recovery and had 7.48 fold purification having specific activity of 175.8 IU/mg protein which was higher than crude enzyme. Specific activity of Crude Enzyme was 23.5IU/mg and total Enzyme activity was 634.5 IU. The molecular weight of Xylanase was found to be 20 kDa using SDS-PAGE. Low molecular weight is the important property of industrial Xylanase. Enzyme activity with molecular weight was obtained on the single gel through Zymography.

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