SUMMARY
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➢ Isolation and screening for bacterial xylanases conducted at three stages, including wheat bran agar plates, xylan agar plates and xylan liquid medium resulted in the selection of 10 strains from a collection of 200 isolates.

➢ SSP-34, the most potent one from 200 isolates was identified and named as Bacillus SSP-34, which produced 100 times more activity than the other isolates.

➢ Bacillus SSP-34 showed maximum xylanase activity at 102 hours and growth at 30 hours. The culture was having only trace levels of cellulase activity in the cell-free culture supernatant than the other nine isolates. It was proposed to be due to the action of xylanases on small chains of xylose residues present as contamination in the commercial celluloses. The bifunctional nature could also effect the cellulase/xylanase activity.

➢ Growth optimum of Bacillus SSP-34 was at pH 9.0 while maximum xylanase production occurred at pH 8.5.

➢ In studies with different initial pH values, all the media reached pH value 8±0.5 by 96th hour when the highest production occurred. When this alkaline condition was provided highest enzyme expression was the result. This kind of xylanase production has been seldom reported.

➢ Optimum xylanase production as well as highest growth occurred at a temperature of 35°C. The culture showed thermotolerant nature.

➢ Highest xylanase production occurred at 5% inoculum concentration. When the case was 1%, both biomass and xylanase production were low which could be due to the low cell density. Low xylanase production at higher concentrations was apparently due to the transfer of metabolically active compounds affecting the higher production of xylanases.

➢ Highest enzyme production occurred at the agitation of 300 rpm and at an aeration of 0.2 wv ratio, which was followed by 0.4 wv ratio and 300 rpm (76% xylanase production of that occurring at the highest case). Other
agitation and aeration conditions resulted lower xylanase production, all lesser than 49% possibly due to low efficiency in the transfer of dissolved oxygen to the microbial cell and transfer of oxygen from air into the medium.

- Of the 18 different carbon sources studied including monosaccharides, disaccharides, polysaccharides and lignonocellulosics, xylan was the optimum carbon source inducing highest levels of xylanase expression.
- Wheat bran and xylose induced xylanase production amounting 91% and 80% of that occurring at xylan. Lignocellulosics such as rice straw, bagasse, rice bran, coir and rice husk showed low levels of production (less than 23%).
- Growth in the CMC containing medium could be attributed to the constitutively expressed enzymes acting on the xylose residues present as contaminant in commercial celluloses, the utilisation of which maintained the growth rate of Bacillus SSP-34.
- Glucose resulted in the inhibition of xylanase production. The effect of glucose could be attributed to two mechanisms, first the catabolite repression occurring at the transcriptional level and second by inducer exclusion.
- Study with different concentrations of xylan showed maximum xylanase production was at 0.5% xylan while higher concentrations resulted in poor enzyme production. Maximum growth occurred at a level of 2.5%.
- Even though xylan and xylose resulted in xylanase expression, each when supplemented with glucose resulted in catabolite repression. But xylan along with xylose showed no inhibition of xylanase production confirming the inductive effect of xylose.
- The present study showed that the kind and level of nitrogen source used in the medium influenced xylanase production from microorganisms. The optimum nitrogen source was a combination of yeast extract and peptone,
each at a concentration of 0.25%. This medium resulted in low level of protease production i.e. 0.009 IU/ml.

- The reason for incorporating ammonium ion forming medium (with complex nitrogen sources) is to minimise the protease induction.

- When the level of nitrogen source increased (e.g. 2.0% YE and 2.0% Peptone) there occurred positive growth by the utilisation of minor carbohydrates found in the complex medium and xylanase production delayed up to 120 hours.

- From the present study it becomes evident that the nitrogen source in the medium should be optimum and promote growth as well as enzyme production.

- Inorganic nitrogen sources failed to effect good xylanase production.

- Different metal ions classified as Class A, Class B and borderline ions markedly differed in their influence on growth and enzyme production by *Bacillus* SSP-34. K$_2$HPO$_4$, MgSO$_4$, NaCl and ZnCl$_2$, Na$_2$HPO$_4$ and MnSO$_4$ did not inhibit both the biomass and xylanase production while CoCl$_2$ did. Co$^{2+}$ was inhibiting both growth and xylanase production. Class A ions (e.g. Mg, Ca, Na) are essential for the growth of microorganisms.

- Unique purification procedure was employed for purifying the xylanases from *Bacillus* SSP-34. After ultrafiltration and ammonium sulphate fractionation of the cell free culture supernatant, column chromatography was employed for further purification. The first stage in this was the gradient elution using DEAE Sepharode CL 6B column. In the second stage CM Sephadex column was used. Partially purified xylanase from the ion exchange column chromatographic analyses was further purified in size exclusion column chromatography with BioGel P100 (BioRad) column. Specific activity of the purified xylanase protein, i.e. 1723 U/mg was one among the highest values. The purification fold was 33.3 while the yield was 2.5%.
Electrophoretic analysis revealed the pattern of proteins present in crude and fractions from different stages of purification. The band pattern illustrates the justification of the incorporation of two ion exchanges (anion and cation) prior to GPC. The purification was almost 90% in the first two levels which minimises the task of GPC column considerably. The molecular weight of the single xylanase produced from Bacillus SSP-34 was 20-22 kDa and Zymogram analysis confirmed the xylanolytic nature of the purified low molecular weight protein.

The Bacillus SSP-34 xylanase was having optimum activity at a temperature of 50°C and pH of 6-8. The enzyme showed considerable thermostability (30 minutes at 50°C) and pH stability (4.5 to 9 for two hours).

The Bacillus SSP-34 xylanase was not a metalloenzyme and Hg²⁺ was a strong inhibitor.

The K_m value of the xylanase was 6.5 mg of oat spelts xylan per ml and V_max was 1233μmol/min/mg protein.

Hydrolytic pattern of the purified Bacillus SSP-34 showed the formation xylobiose, xylotriose and xylopentose. The hydrolysis pattern indicated that the enzyme was an endo-xylanase.

The prebleaching of pulp sample with Bacillus SSP-34 was highly effective with an ISO % brightness increase from 41.1% to 48.5%. This increase in brightness (7.4 %) was comparable to most of the reported cases.
Conclusive Remarks

Xylanases with hydrolytic activity on xylan, one of the hemicellulosic materials present in plant cell walls, have been identified long back and the applicability of this enzyme is constantly growing. All these applications especially the pulp and paper industries require novel enzymes. There has been lot of documentation on microbial xylanases, however, none meeting all the required characteristics. The characters being sought are: higher production, higher pH and temperature optima, good stabilities under these conditions and finally the low associated cellulase and protease production. The present study analyses various facets of xylanase biotechnology giving emphasis on bacterial xylanases. Fungal xylanases are having problems like low pH values for both enzyme activity and growth. Moreover, the associated production of cellulases at significant levels make fungal xylanases less suitable for application in paper and pulp industries.

Bacillus SSP-34 selected from 200 isolates was clearly having xylan catabolizing nature distinct from earlier reports. The stabilities at higher temperatures and pH values along with the optimum conditions for pH and temperature is rendering Bacillus SSP-34 xylanase more suitable than many of the previous reports for application in pulp and paper industries.

Bacillus SSP-34 is an alkalophilic thermostolerant bacteria which under optimal cultural conditions as mentioned earlier, can produce 2.5 times more xylanase than the basal medium.

The 0.5% xylan concentration in the medium was found to the best carbon source resulting in 366 IU/ml of xylanase activity. This induction was subjected to catabolite repression by glucose. Xylose was a good inducer for xylanase production. The combination of yeast extract and peptone selected from several nitrogen sources resulted in the highest enzyme production (379±0.2 IU/ml) at the optimum final concentration of 0.5%. All the cultural and nutritional parameters were compiled and comparative study showed that
the modified medium resulted in xylanase activity of 506 IU/ml, 5 folds higher than the basal medium.

The novel combination of purification techniques like ultrafiltration, ammonium sulphate fractionation, DEAE Sepharose anion exchange chromatography, CM Sephadex cation exchange chromatography and Gel permeation chromatography resulted in the purified xylanase having a specific activity of 1723 U/mg protein with 33.3% yield. The enzyme was having a molecular weight of 20-22 kDa. The $K_m$ of the purified xylanase was 6.5 mg of oat spelts xylan per ml and $V_{max}$ 1233 μmol/min/mg protein.

*Bacillus* SSP-34 xylanase resulted in the ISO brightness increase from 41.1% to 48.5%. The hydrolytic nature of the xylanase was in the endo-form.

Thus the organism *Bacillus* SSP-34 was having interesting biotechnological and physiological aspects. The SSP-34 xylanase having desired characters seems to be suited for application in paper and pulp industries.

Further work is necessary to enhance xylanase production and protein stabilities to still higher values. This requires genetic changes caused either by conventional mutation studies or gene manipulation.