SUMMARY
Summary

The major findings reported in the present thesis are summarised below:

1. **Isolation of microorganisms:** Fifteen microbial strains (including 10 bacterial and 5 fungal isolates) were isolated from forest soil around Shimla. The fungal strains were identified at "The Division of Mycology and Plant Pathology", Indian Agricultural Research Institute New Delhi-110 012 as Aspergillus niger, Penicillium sp., P. aurantiogriseum, P. lanoso-coeruleum and Trichoderma viride. Out of ten bacterial isolates, (XBM-1 to XBM-10) the hyper xylanase producing XBM-2 strain was identified as Flavobacterium sp. at "The Institute of Microbial Technology (IMTECH)", CSIR complex, Chandigarh-39.

2. **Screening:** All fifteen microbial isolates were screened for their potential to release extracellular xylanase. Among the bacterial strains, Flavobacterium sp. exhibited maximum release of xylanase (3.87 IU/ml) while the strain XBM-9 could produce only 1.7 units of xylanase. Aspergillus niger exhibited highest extracellular production of xylanase (37.62 IU/ml) among the fungal strains whereas only 1.65 units of xylanase were released by P. aurantiogriseum. Since Flavobacterium sp. and A. niger emerged as hyper xylanase producing strains, therefore these two organisms were selected for subsequent studies.
3. **Factors affecting xylanase production:** The production of extracellular xylanase by *Flavobacterium* sp. and *A. niger* was studied in different media and under varied conditions of pH, temperature, size of inoculum and concentrations of xylan. The *Flavobacterium* sp. produced maximum enzyme (3.87 IU/ml) in Riviere's medium. The optimum pH and temperature for enzyme production were recorded as 6.8 and 28°C respectively. A continuous increase in the production of xylanase was observed with increase in the concentration of xylan in the medium with maximum yield (6.77 IU/ml) of enzyme at 2 per cent xylan in the growth medium. Release of extracellular enzyme by the bacterium was significantly affected by the size of inoculum and the highest xylanase production (7.23 IU/ml) was recorded by addition of $5 \times 10^6$ cfu/ml in the medium.

Maximum production of extracellular xylanase by *A. niger* was observed in John and Schmidt's (1988) medium (37.62 IU/ml). A wider range of pH (4.0-5.0) and temperature (25-35°C) was recorded for higher production of xylanase by this mold. Maximum xylanase production was at pH 5.3 (37.62 IU/ml) and temperature 30°C (42.26 IU/ml). A gradual increase in the production of xylanase with increase in the concentration of xylan from 0.25 per cent to 2.5 per cent (9.40 IU to 42.50 IU/ml) was also recorded. Size of inoculum also influenced the xylanase production by *A. niger* and it was maximum (65.60 IU/ml) with addition of $1 \times 10^7$ spores/ml in the medium.

4. **Biodegradation studies:**

a. **Untreated biomass materials:** The degradation of forest biomass by hyper xylanase producing organisms was studied by growing these organisms in the medium containing lignocellulosic forest biomass as sole source of carbon. The biodegradation index (total sugar released + proteins formed/2) was taken as a criterion for assessing degradation of materials by these microbial isolates. The forest lignocellulososes used
in the present study were wood and barks of *Populus ciliata*, *Quercus incana*, *Robinia pseudoacacia* and wood, bark and needles of *Cedrus deodara*, *Pinus roxburghii* and *P. wallichiana*. Based on the values of BI it was observed that needles and barks were more prone to the degradative action of both *Flavobacterium* sp. and *A. niger* in comparison to woods. Poor degradation of woody materials by both bacterium and mold may be due to intimate association of hemicelluloses with other components.

b. **Physico-chemically pretreated materials:** The forest biomass materials were subjected to various physical (gamma-irradiations and grinding) and chemical pretreatments (*H*$_2$O, l per cent as such; *H*$_2$O$_2$: MnSO$_4$; *H*$_2$O$_2$: MnCl$_2$ and *H*$_2$O$_2$: CoCl$_2$ (all in 100:1 M ratio). The degradation of physicochemically treated lignocelluloses by *Flavobacterium* sp. and *A. niger* showed that, all treatments enhanced the degradability of the materials.

The per cent increase in the BI of *H*$_2$O pretreated materials was maximum (181.12) in *P. wallichiana* wood while it was only 26.55 per cent in *C. deodara* woods when *Flavobacterium* sp. was used as degradative agent. The degradation of *H*$_2$O$_2$: MnSO$_4$ pretreated materials by *Flavobacterium* sp. resulted in 230.07 per cent increase in the BI of *P. wallichiana* wood followed by 184.81 in *C. deodara* needles and 158.67 per cent increase in *C. deodara* bark. The per cent increase in the BI of *H*$_2$O$_2$–MnCl$_2$ pretreated samples was highest (298.60) in *P. wallichiana* wood followed by 201.84 in *P. roxburghii* needles. The increase was 178.32 and 159.91 per cent in *H*$_2$O$_2$–CoCl$_2$ pretreated materials of *P. wallichiana* wood and *P. roxburghii* needles respectively. The gamma irradiation pretreatment drastically enhanced the degradation of all the materials by *Flavobacterium* sp. and the percent increase in the BI was maximum (456.64) for *P. wallichiana* wood.

Various physico-chemical pretreatments of the forest biomass materials greatly enhanced the susceptibility of the
materials to the degradative action of *A. niger*. The per cent increase in the BI of \( \text{H}_2\text{O}_2 \) pretreated lignocelluloses was recorded highest (104.27) in *P. wallichiana* bark however it was 213.28 per cent in \( \text{H}_2\text{O}_2\text{-MnSO}_4 \) pretreated needles of *C. deodara*. The \( \text{H}_2\text{O}_2\text{-MnCl}_2 \) and \( \text{H}_2\text{O}_2\text{-CoCl}_2 \) pretreatments also influenced the susceptibility of different materials and the increase in the BI was maximum in *C. deodara* needles (199.59 per cent) and *Robinia pseudoacacia* wood (199.17 per cent) respectively for these treatments. As in case of *Flavobacterium* sp. the degradation by *A. niger* was also highest in gamma irradiated materials with 390.77 per cent increase in BI for *P. ciliata* wood.

5. **Bioconversion of pretreated lignocelluloses into proteins:**

The conversion of pretreated lignocellulosic materials into protein (SCP) was studied by growing several yeast strains in co-culture with xylanolytic organisms (*Flavobacterium* sp. and *A. niger*) and also by growing these food yeasts on culture supernatants of these xylanolytic microorganisms grown on pretreated lignocelluloses.

a. **SCP production by co-culture system:** The conversion of lignocelluloses into protein was highest (12.44 per cent) when *Flavobacterium* sp. was grown in co-culture with *Candida utilis* (NCIM-3055) on gamma irradiated *P. ciliata* wood. However, the *A. niger, C. utilis* co-culture resulted in 16.12 per cent protein production from \( \gamma \)-irradiated *Q. incana* wood. Maximum conversion of pretreated materials into proteins by *Saccharomyces cerevisiae* var. *ellipsoideus* (NCIM-3281) was recorded in gamma irradiated woods of *P. ciliata* (9.80 per cent) and *Q. incana* (13.16 per cent) when grown in co-culture with *Flavobacterium* sp. and *A. niger* respectively.

b. **SCP production by step-wise fermentation:** The SCP production was also studied by growing different yeast strains
on the culture supernatants of Flavobacterium sp. and A. niger grown on various pretreated wood samples. The culture supernatants of the bacterium and the mold grown on γ-irradiated woods of P. ciliata and Q. incana supported maximum growth of C. utilis (NCIM-3055) and produced 2.56 and 2.07 per cent proteins respectively. However, the protein production by growing C. tropicalis (NCIM-3319) on culture filtrates of these xylanolytic organisms was 3.23 per cent and 2.68 per cent respectively from gamma irradiated woods of P. ciliata and P. wallichiana.

The SCP production in culture supernatants of the xylanolytic organisms (Flavobacterium sp. and A. niger) grown on γ-irradiated woods of P. wallichiana and Q. incana was maximum (3.35 and 2.02 per cent) with S. cerevisiae (NCIM-3116) whereas growth of S. cerevisiae var. ellipsoideus on the culture supernatants of the bacterium and mold grown on irradiated wood samples of Q. incana yielded 3.35 and 2.45 per cent proteins respectively.

6. Production of ethanol: Seven yeast strains namely C. utilis, C. tropicalis, S. cerevisiae, S. cerevisiae var. ellipsoideus, Candida shehatae (ATCC-22984), Pachysolen tannophilus, (ATCC-32961) and Pichia stipitis (NCIM-3497) were screened for their ability to produce ethanol from the culture supernatants of the xylanolytic organisms (Flavobacterium sp. and A. niger) grown on various pretreated woods and needles. C. shehatae produced maximum ethanol from culture supernatant of the bacterium grown on gamma irradiated C. deodara needles (10.2 per cent). The hydrolysates of γ-irradiated Q. incana wood and needles of C. deodara and P. roxburghii were effectively fermented by all yeast strains to yield higher levels of ethanol.

Fermentation of the biomass hydrolysates resulting from the growth of A. niger on these materials, by several ethanogenic yeasts revealed higher ethanol production from
P. wallichiana needles (8.7 per cent by P. stipitis) and C. deodara needles (7.2 per cent by C. shehatae), while the wood hydrolysate of P. ciliata supported maximum growth of all yeast and yielded maximum ethanol among the woody materials.

7. Partial purification of xylanases:

a. Purification: The extracellularly released xylanases from Flavobacterium sp. and A. niger were partially purified by ammonium sulphate precipitation followed by ultracentrifugations and dialysis against phosphate buffer and filter sterilization. The xylanase assay of the partially purified enzyme fractions revealed that although per cent recovery of enzyme from both organisms was low yet there was significant increase in their specific activities (15.83 U and 53.63 U). The overall increase in specific activities of the xylanases from Flavobacterium sp. and A. niger was 4.11 and 3.31 times respectively in comparison to the enzyme activity in culture supernatants.

The activity of the partially purified xylanases from Flavobacterium sp. and A. niger was assayed at different pH (3.0-7.0), incubation temperatures (30-90° C) and varied concentrations of substrate (0.25 per cent to 3.0 per cent xylan) and following results were obtained.

i) Maximum activities of xylanases from Flavobacterium sp. (16.21 U) and A. niger (53.63 U) were observed at pH 4.5 with optimum pH range of 4.0-5.0 for both enzyme fractions.

ii) A continuous increase in the specific activities of enzyme from the bacterium and mold was observed with increasing concentration of xylan from 0.25 per cent to 3.0 per cent with maximum specific activity of both at 3 per cent concentration of xylan (33.36 U and 98.84 U/mg proteins).
iii) The xylanases from *Flavobacterium* sp and *A. niger* showed maximum activity at 50 °C and 45 °C with optimum temperature range of 40-50 °C and 45-65 °C respectively.

8. Saccharification of lignocelluloses by partially purified xylanases:

All biomass samples (untreated as well as pretreated) were subjected to enzymatic saccharification with partially purified xylanases from both *Flavobacterium* sp. and *A. niger*. The results obtained indicated that the level of reducing sugars released by the action of partially purified xylanases was higher in comparison to the sugars released by the hydrolytic action of the organisms alone. In addition, the enzymatic saccharification was appreciably affected by pretreatments of the biomass. The gamma irradiation pretreatment was most suitable followed by H₂O₂-Mn⁺⁺ pretreatments. In all, the pretreated woods and barks of *Quercus incana* and *Robinia pseudoacacia* and needles of *C. deodara*, *P. roxburghii* and *P. wallichiana* not only supported growth of xylanolytic organisms but also responded well to the hydrolytic action of the partially purified xylanases from both organisms and released maximum sugars. The xylanolytic microorganisms (*Flavobacterium* sp. and *Aspergillus niger*) isolated from forest soil have emerged as potent producers of xylanases. Xylanase production by these microbial isolates can be increased by optimization of some of the culture conditions. The level of SCP production by these organisms alone or in combination with yeasts (from lignocelluloses) is quite satisfactory. Moreover, the sugars produced by the enzymatic saccharification of lignocelluloses can efficiently be fermented to ethanol by using ethanogenic yeasts. In addition, the partially purified xylanases efficiently hydrolysed the lignocelluloses. Further studies with these organisms may help us in using them as major tools in the field of biotechnology.
Despite our knowledge of microbial xylanolytic systems in past few years, further studies are needed for isolation and selection of hyper xylanase producing strains and to achieve a complete understanding of microbial degradation and proper utilization of xylan for elaborating economically feasible biotechnological applications for commercializing the xylanolytic systems. The work reported in the present thesis therefore represents part of our efforts to strengthen the overall economics of the processing of lignocellulosic forest biomass for the production of energy, and SCP.