SUMMARY

A total of eighteen thermophilic/thermotolerant fungi were isolated from various localities in and around the Raipur city. Samples were collected from bird's nest, compost, stable manure and mushroom compost material. One isolate from bird's nest, thirteen from compost material, three from stable manure and one from mushroom compost material were recorded. *Absidia corymbifera* were isolated from bird's nest. Among the thirteen isolates from compost, three were of *Aspergillus* sp., one of *Chrysosporium* sp., one of *Humicola* sp., three of *Malbranchea* sp. and five of *Mucor* sps. Similarly, from the stable manure three isolates were of *Sporotrichum* sp. and one of *Scytalidium thermophilum* from the mushroom compost material.

All the eighteen isolates were screened for the extracellular alkaline protease activity. All the eighteen isolates of thermophilic/thermotolerant fungi were screened for industrially useful enzyme i.e. extracellular alkaline protease activity at 45°C. *Aspergillus fumigatus* exhibited the highest alkaline protease activity, the value being 43.59±0.26 whereas lowest activity of 10.11±0.03 µg tyro./mg protein/10 min. was shown by *Mucor* sp.4.

The biomass of eighteen isolates were determined at 20, 45, 50, 55, 60°C under the stationary culture conditions to understand the growth rate of the different species, which varied considerably at any single temperature. Thirteen fungal isolates exhibited maximum biomass at 45°C, four at 50°C and one at 55°C.

The percent coagulation of protein was determined at 50°C for assessing the thermostability of protein. A gradual increase in percent coagulation of soluble proteins was recorded in all the isolates.
with gradual increase of time of incubation at 50°C. The two isolates *Chrysosporium* sp. and *Scytalidium thermophilum* exhibited minimum percentage coagulation of proteins at the end of 10 minutes, the values being 19.92±0.49 and 18.99±0.33 as compared to other isolates, whereas *Aspergillus fumigatus* and *Aspergillus* sp. 2 recorded the higher coagulation of protein, the value being 47.79±0.63 and 43.66±0.635, respectively.

Out of eighteen, twelve fungal isolates were recorded as good producer of alkaline protease at 45°C. The activity level of alkaline protease of twelve fungal isolates was further evaluated at higher temperatures i.e. 37, 45, 55, 65 and 75°C. A variable response of the increase of temperature from 37 to 75°C was recorded on the level of alkaline protease activity of different fungal isolates. Seven fungi i.e. *Absidia corymbifera*, *Aspergillus fumigatus*, *Malbranchea pulchella*, *Sporotrichum thermophile*, *Sporotrichum* sp. 1 and 2, and *Scytalidium thermophilum*, exhibiting the higher level of alkaline protease activity at high temperature, were selected for further study.

The effect of pH on the alkaline protease of selected seven fungi was assessed. It was found that *Aspergillus fumigatus*, *Malbranchea pulchella*, *Sporotrichum thermophile* and *Scytalidium thermophilum* showed higher activity of alkaline protease at the pH of 7.5, 11.5, 12.5, and 9.5, respectively.

After observing the effect of pH and temperature on the protease activity, four fungi were selected and subjected to different cultural amendments for optimum production of extracellular alkaline protease. The different carbon sources were sucrose, maltose, lactose, fructose, glucose, mannitol and nitrogen sources were yeast extract, soymeal, proteose peptone, gelatin, casein, biopeptone, tryptone as well as different concentrations of carbon and nitrogen sources were included in this study. Sucrose was recorded as the best carbon source for the production of extracellular alkaline protease by *Aspergillus*
fumigatus and Sporotrichum thermophile whereas, Malbranchea pulchella and Scytalidium thermophilum exhibited higher level of alkaline protease activity with maltose.

The highest level of extracellular alkaline protease activity was noted in culture filtrate of Aspergillus fumigatus and Malbranchea pulchella in presence of soymeal and proteose peptone, whereas yeast extract was the best nitrogen source for Sporotrichum thermophile, and Scytalidium thermophilum.

Two fungi, Malbranchea pulchella and Sporotrichum thermophile were recorded as good producer of extracellular alkaline protease. They were further grown in different concentrations of carbon i.e. 0.5, 1, 4, 5, 7, 10 g/l and nitrogen i.e. 0.4, 0.8, 0.9, 1.0, 1.1, 1.2 g/l to findout the best concentration of carbon and nitrogen for higher production of alkaline protease. The highest level of extracellular alkaline protease activity was recorded with 5.0 g/l maltose and 1.0 g/l of proteose peptone in the culture filtrate of Malbranchea pulchella whereas Sporotrichum thermophile yielded maximum alkaline protease activity in presence of 5.0 g/l sucrose and 0.9 g/l yeast extract as the suitable concentration of carbon and nitrogen sources, respectively.

The effect of temperature on the production of extracellular alkaline protease in Malbranchea pulchella and Sporotrichum thermophile was evaluated for higher production of alkaline protease. The highest level of alkaline protease activity was recorded at the growth temperature of 45°C in both the fungi.

Malbranchea pulchella was cultured at optimized culture conditions. First, a precipitation test was conducted using 80% ammonium sulphate and organic solvent. i.e. acetone in the ratio of 1:1, 1:2, 1:3. It was observed that 1:2 acetone precipitation resulted in the precipitation of maximum protein of our interest. Thus, the further purification was carried out with 1:2 acetone.
The precipitated sample was dissolved in 0.1 M tris HCl buffer pH 7.5 and dialysed against same buffer for 24 hours. The dialysate was fractionated by ion exchange chromatography using CM-Sepharose-CL-6B column. The fraction showing alkaline protease activity was pooled and concentrated with polyethylene glycol 35,000. The concentrated sample was subjected to gel filtration using Sephadex G-75 column and the protein was eluted with 0.1 M tris HCl buffer pH 7.5 resulting in 18.43 fold purification with a yield of 4 %. The specific activity of the enzyme increased from an initial value of 0.41 U/mg protein to the final level of 7.56 U/mg protein. When the purified enzyme was subjected to SDS-PAGE, a single band was recorded.

The enzyme was biochemically characterized in the following respects.

1. The Km and Vmax of the enzyme of *Malbranchea pulchella* with haemoglobin as substrate were 0.11 mM and 0.526 µg tyrosine released per 10 min per mg of enzyme, respectively.

2. The molecular weight of alkaline protease was approximately 33,000 Da as obtained from SDS polyacrylamide gel electrophoresis using standard protein markers i.e. myosin (rabbit muscle), phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme with their molecular weights 205,000; 97,400; 68,000; 43,000; 29,000; 20,000; 14,300 dalton, respectively.

3. Temperature and pH optima for alkaline protease activity was 75°C and 11.5, respectively.

4. Nine different metal ions in form of their chloride salts were investigated for their effects on the alkaline protease activity of *Malbranchea pulchella* at 1 mM, 5 mM and 10 mM concentrations. The four metal ions i.e. Mn**, Cu**, Fe**, Ca**, were found to be good activator for the enzyme activity and one
metal ion (MnCl₂) have no effect, whereas Ba++, Sn++, Zn++, Hg++, strongly inhibited the enzyme activity.

5. The extracellular alkaline protease activity of *Malbranchea pulchella* was totally inhibited by PMSF at 1, 5, and 10 mM concentrations. However, the activity of protease was also strongly inhibited by BME, DNBA, Urea, H₂O₂, I₂, EDTA.

Thus, it can be concluded that alkaline protease of *Malbranchea pulchella* is a serine protease. The temperature and pH optima for the enzyme was 75°C and 11.5, respectively. It appears that *Malbranchea pulchella* can be a potential source for exploiting as an industrial organism for production of extracellular alkaline protease.