Chapter 7

SUMMARY AND CONCLUSION
Summary

This thesis focuses on the production of gamma linolenic acid (GLA; 18:3), a ω-6 polyunsaturated fatty acid with applications in foods and pharmaceuticals, by Mortierella rammanniana. This eukaryotic heterotrophic oleaginous fungus has been studied since the end of the nineteenth century and has been identified as a good producer of GLA. M. rammanniana can accumulate lipid to over 37% of its biomass dry weight, with a high content of GLA (over 10% of the total lipid). Other polyunsaturated fatty acids represent less than 1% of the M. rammanniana derived oil. Conventionally, it is produced from plant sources such as seeds of evening primrose, borage and blackcurrant. However, to meet the increasing demand, extensive research is being carried out for its production from microbial sources so as not to rely only on plant sources.

Production of GLA from fungal sources is an alternative to production from plant seeds. Many reports have shown the ability of a few fungal species of storing high lipid profile composed of unusual fatty acids. Fungal species have numerous advantages, which make them interesting candidates for alternate GLA sources. Unlike other plants, they do not require agricultural land and their resulting fatty acid profiles could be manipulated and changed substantially by simply varying their growth conditions rather than having to resort to genetic manipulation or extensive breeding efforts.

Recent research suggests that γ-linolenic acid is unique among the n-6 polyunsaturated fatty acid (PUFA) family members as it ameliorates many health problems. There are many evidences to suggest GLA for pharmacological and dietetic purposes. GLA administered orally is capable of suppressing human T-cell proliferation. It suppresses activation of T-cell, it is effective against rheumatoid arthritis, decreases cardiovascular risk factors,
normalizes nerve conduction velocity and sciatic endoneurial blood flow. The major demand for GLA is at present in the cosmetic formula market and is expected to be 100 to 200 tons of GLA. The commercial benefits of GLA are self-evident.

The aim of the research described in this thesis was to identify relevant process parameters for the large-scale production of gamma linolenic acid (GLA) with \textit{M. rammanniana}. Several cultivation protocols for \textit{M. rammanniana} were developed, analyzed and optimized with respect to the production of biomass, lipid and GLA and solutions were sought for newly identified bottlenecks for industrial GLA production.

Soil and water samples were collected from the virgin areas of North Western Himalayas. 24 pure bacteria and 30 fungi belonging to order mucorales were isolated. All the isolated microorganisms were checked for oleaogenecity. Out of all organisms' one fungus identified and characterized as \textit{Mortierella rammanniana} was selected for further studies. Microscopy including phase contrast and scanning electron micrographs of the spores of \textit{M. rammanniana} were taken. The fungal mycelium was tested for lipase production and even for anticancer activity against human cancer cell lines. The culture produced lipase but it was not specific and the amount was also quite less to be used industrially. Where as no anticancer activity was observed in comparison to the standard drugs being already used in the market.

Various fermentation parameters for the growth, lipid and GLA production by \textit{M. rammanniana} were optimized at the shake flask. The culture was grown in four different media with glucose; peptone and yeast extract as constituents was selected for further studies. Eight different carbon sources including corn steep liquor and molasses were tested at three different concentrations of 3.5 and 10%. Glucose at 100g/L was the optimized
concentration. As on increasing the concentration the cells started bursting. The process was also run as a fed batch in order to increase the carbon intake capacity of *M. ramanniana*. The best nitrogen source among all the tested sources was peptone and the optimized C:N ratio was 23:1. There is huge difference in the carbon and nitrogen ratio in the media because as soon as the nitrogen exhaust from the media cell stops proliferation and excess of carbon is stored as lipids.

The fungal culture was grown at 12 to 30°C and the temperature which best suited the growth conditions were 28°C. Five different metal ions (CuSO₄, ZnSO₄, FeCl₂, MgSO₄ and MnSO₄) at three concentrations (5, 50, and 500 mg/L) were added to the media. It was observed that addition of metal ions didn’t accelerate the production of GLA but on the other hand they could have added to the cost of the process. Effect of three surfactants (Tween 20, Tween 80 and Triton X) was studied. The pH was studied in the range of 5.0-9.0. Optimum pH of the media was 6.5 and at the time of production of GLA it comes down to 4.0. Effect of inoculum age and inoculum percentage was studied. For GLA production the optimum inoculum age was 24 h and inoculum percentage was 8%. It was observed that the growth of culture was best when grown at 200 rpm where as negligible growth was observed at static conditions proving that culture required oxygen for growth. There was no effect of disrupted mycelia on GLA production. Different desaturase inhibitors were tested to stop the chain elongation in *M. ramanniana*. Among the tested inhibitors were curcumin, DHA, ARA and acrylic acid. The media was supplemented with vegetable oils and also sodium acetate. It was observed that there was 2-3 fold increases in lipid and biomass production but no GLA was there. A complete cessation occurred. This could be due to the inactivation of delta 6 desaturases present in the system.
Scale up of the process was done in 13L bioreactor with all the conditions optimized at the shake flask level. In the bioreactor the productivity of GLA increased and the production time decreased by 24h. The maximum yield of GLA was 791 mg/l in 144h in shake flask, whereas 960 mg/l was recorded in 120 h in a bioreactor.

Three different extraction solvents were used to check their effect on GLA recovery in the process. The solvent system used were chloroform: methanol (2:1), hexane: isopropanol (3:2) and ethyl acetate. The total fatty acid composition varied in fungal cultures depending on the extraction conditions of the three methods chloroform: methanol (2:1) was found to be the best for extraction of lipid and fatty acids from *M. ramanniana*. In this chapter Enrichment of GLA from the fungal lipids by a urea inclusion method was also studied. Most of the saturated and monosaturated, 93.0% and 84.6%, respectively were removed by forming urea inclusion compounds. GLA was concentrated after urea inclusion. Its content in total fatty acids increased 6.2 folds and recovery of reached 57.1% with a recovery of 90%. The pure GLA obtained was identified and confirmed by coupled gas chromatography and mass spectroscopy.
Conclusion

- Various bacteria and fungi were isolated and screened for the production of GLA from the soils collected from virgin areas of North western himalayas. Molecular identification and morphological studies of the selected strain was done. The selected strain out of the isolated lot was identified as *Mortierella rammamiana*.

- The optimised conditions for GLA production at shake flask level. To optimize the production of gamma linolenic acid by *M. rammamiana* effect of carbon source, nitrogen source, inoculum age, inoculum percentage initial pH, temperature, metal ion concentration, fed batch culture, non ionic surfactants, desaturase inhibitors and oil supplementation was studied. The production of gamma linolenic acid reached 920 mg l\(^{-1}\) when the production media composed of 10% glucose, 1% peptone and 0.1% yeast extract pH 6.5 and incubated at 30\(^{\circ}\)C for 144 h. Carbon and nitrogen sources showed major effect on the increase in gamma linolenic acid production. Repression in \(\Delta 6\)-desaturase activities was observed in the presence of supplemented oil. This study highlights conditions for increasing gamma linolenic acid production by *M. rammamiana* and an insight into rapidly gaining high production of polyunsaturated fatty acids.

- The valuable polyunsaturated fatty acid gamma linolenic acid, with applications in foods and pharmaceuticals, can be produced at a high overall volumetric rate by cultivation of *M. rammamiana* in bioreactor. GLA obtained was identified by gas liquid chromatography and spectroscopic studies. No GLA was detected at zero time cultures or in un inoculated culture broth. The maximum yield of GLA was 791 mg l\(^{-1}\).
in 144h in shake flask, whereas 960 mg/l was recorded in 120 h in a bioreactor.

- The efficacy of three extraction methods for determining the lipid and fatty acid composition of *M. rammanniana* was studied. The extraction methods were chloroform: methanol (2:1), hexane: isopropanol (3:2) and ethyl acetate. The total fatty acid composition varied in fungal cultures depending on the extraction conditions of the three methods chloroform: methanol (2:1) was found to be the best for extraction of lipid and fatty acids from *M. rammanniana*. In this chapter Enrichment of GLA from the fungal lipids by a urea inclusion method was also studied. Most of the saturated and monosaturated, 93.0% and 84.6%, respectively were removed by forming urea inclusion compounds. GLA was concentrated after urea inclusion. Its content in total fatty acids increased 6.2 folds and recovery of reached 57.1% with a recovery of 90%.