Chapter 5

DISCUSSION
5.1 Sourcing of entomopathogenic fungi and taxonomy:

In the present study it was planned to have an insight into the sourcing of entomopathogenic fungi from nature. Entomopathogenic fungi can be found infecting living as well as dead insects in a variety of habitats. So an exercise was carried out to collect samples from aquatic and terrestrial habitats, laboratory colonies of mosquitoes and agro-ecosystems. As shown in Table 7 entomopathogenic fungi could be sourced from different localities of Goa. The easy availability of these fungi indicates that Goa being humid with tropical climate has rich bio-diversity of entomopathogenic fungi. An earlier solitary effort in this direction had yielded a rich haul of 40 mosquito-pathogenic fungal isolates from this region (Keshava Prasad et al., 2000). There exists definitely a great scope to tap this potential further from this and similar regions in the country. In the present study, we have deployed different techniques for the recognition of potential sources and isolation of mosquitopathogenic fungi. For example, the diseased insects were recognized on the basis of changes in their natural colouration, visible growth of the fungus on the outside of their cadavers, melanized areas on the cuticle, fragility, hardening of the cuticle, peculiar behaviour like lack of feed intake and tremors or curling showing irritability or mummification as has been earlier reported (Lacey & Brooks, 1997).

Sourcing of fungi was also done from Goa University Fungal Culture Collection. Seven fungal isolates which had previously shown promising larvicidal activity (i.e. larval mortality of 50% or above) on screening against 2nd instar larvae of Cx. quinquefasciatus were chosen for the present study. They were then screened for their activity against higher instar larvae especially the third instar larvae of the disease vectors viz., Cx. quinquefasciatus and An. stephensi. We found that the traditional taxonomic tools such as keys based on variations in morphological characters to be
5.2 Analysis of larvicidal potential and bio-efficacy of fungal isolates:

Preliminary testing of fungi for larvicidal potential

All the three isolates viz., Gliocladium, Penicillium and Trichoderma were effective in killing 3\textsuperscript{rd} instar Cx. quinquefasciatus larvae at a dose range of $10^5$-$10^6$ conidia/ml. In a previous study Keshava prasad et al. (2000) had screened 14-day-old culture of these fungi for larvicidal activity against Cx. quinquefasciatus 2\textsuperscript{nd} instar larvae using a dose range of $10^4$-$10^5$ conidia/ml. Hence the results of this and the previous study were more or less comparable in this regard. Both 2\textsuperscript{nd} and 3\textsuperscript{rd} instar larvae showed complete susceptibility to Gliocladium sp. isolate GUFCC 5044 on 24 h exposure and P. citrinum isolate on 48 h exposure at the same dosage implying thereby that these are indeed promising mosquitopathogens which could be suitably formulated and exploited for vector control. Upon repeated sub culturing, some of these isolates tended to loose some virulence. For example, G. roseum isolate GUFCC 5040 showed reduced virulence as larval mortality reduced from 100% to 72%, while in case of Trichoderma sp. isolate GUFCC 5088 drastic decrease in activity from 100% to 16% was observed in spite of the fact that much higher doses of $6.9 \times 10^6$ spores/ml and $14.19 \times 10^6$ spores/ml respectively were used in our study as compared to an earlier study in which doses between $10^5$-$10^6$ spores/ml were used. On the contrary, in case of T. atroviride isolate, a marginal increase in larvicidal activity from 65% to 70% mortality was observed at the same dose which showed that virulence was maintained in this isolate in spite of subculturing. However, this mortality was achieved on 72 h exposure in the present experiment as compared to 120 h exposure in the previous
study indicating that the 3\textsuperscript{rd} instar larvae were more susceptible than the 2\textsuperscript{nd} instar that were used in the previous study. Hence the exposure time to affect mortality was 48 hours shorter than in the previous experiment. In an earlier study Mohanty and Prakash (2002) have also observed that compared to 1\textsuperscript{st} and 2\textsuperscript{nd} instars of \textit{An. stephensi} larvae the 3\textsuperscript{rd} instar were more susceptible when exposed to different conidial concentrations of \textit{Chrysosporium lobatum}. The age of larva seems to be a deciding factor for the virulent activity of the mosquito-pathogenic fungi.

We found that \textit{T. atroviride} conidial suspension was much less effective against \textit{An. stephensi} 3\textsuperscript{rd} instar larvae as it resulted in only 25\% mortality in spite of the fact that almost two-fold higher dose was used against \textit{An. stephensi} as compared to \textit{Cx. quinquefasciatus}. This could be attributed to the manner in which the spores attach to the host and also due to host immune system which might be suppressed differently in different species (Chandler \textit{et al.} 1993). Similarly in bioassays conducted by Serit & Yap (1984), the 3\textsuperscript{rd} instar larvae differed in their susceptibilities to \textit{Tolypocladium cylindrosporum} as \textit{Mansonia uniformis} larvae were the most susceptible followed by \textit{Culex quinquefasciatus}, \textit{Anopheles balabacensis} and \textit{Aedes aegypti} in decreasing order. Therefore mosquito-pathogenic fungi seem to be quite selective in their targets and mosquito species show variation in vulnerability.

Our study shows that age of the culture of fungi seems to matter with regard to their pathogenicity to vector species. For example, spore suspension obtained from 21 day old culture at a 1.7 times higher dose (18 x 10\textsuperscript{6} spores/ml) of \textit{G. roseum} isolate GUFC 5040 resulted in similar mortality (47.4\%) in \textit{Cx. quinquefasciatus} 3\textsuperscript{rd} instar larvae on 48 h exposure while 46.7\% mortality was observed on 24 h exposure to 14 day old culture at a dose of (10.52 x 10\textsuperscript{6} spores/ml). Thus conidial suspension obtained from 14 d old culture of \textit{G. roseum} had rapid action as compared to 21 d old
culture. The pace of action on target vector is one of the important criteria for adoption of any microbial agent in the vector control programmes.

As the surviving larvae of vector mosquitoes exposed to *G. roseum* did not develop further into pupae and adults did not emerge from them, it appeared that conidia from 21 d had not only larvicidal but also growth inhibitory activity against *Culex* 3rd instar larvae quite similar to insect growth regulator (IGR) compounds. Hence it will be of interest to reconfirm whether this fungus mimics and can be used as an IGR by conducting an in-depth study. Further studies are also warranted on the surviving larvae as they could provide an insight into the sub-lethal effects of this fungus e.g. on fecundity of F₁ adults, fertility, egg laying, etc. in the susceptible vectors. When mature larvae and pupae of *Chilo suppressalis* were treated with sub-lethal doses of *B. bassiana*, the number of eggs laid by the adults formed from them were in an inverse ratio to the spore dosage to which they were exposed (Baye & Doye, 1976). This clearly indicates that fecundity of the next generation of target population exposed earlier to fungi at immature stages is affected and similar phenomenon needs to be explored in detail in mosquitoes exposed to various mosquitopathogenic fungi.

5.3 Mode of invasion of active fungi in mosquito larvae:

Mosquito-pathogenic fungi invade mosquito larvae through cuticle, spiracles or by ingestion depending upon which the development may take place in the gut, head or anal tissues, etc. (Lacey *et al.*, 1988; Sweeney, 1975; Sweeney *et al.*, 1983). Larval death may be attributed to histolysis, mechanical interference with spiracular functions, production of potent mycotoxins or physiological starvation (Ciegler, 1976; Lacey *et al.*, 1988).
In the present study, *Cx. quinquefasciatus* larvae exposed to *Gliocladium* sp. isolate GUFCC 5044 showed profuse mycelial growth on their cuticle. The haemocoel of the larvae was ramified with fungal mycelia and the organ tissues were disrupted at 24 h exposure. Also melanization was seen around invading hyphae in some areas of the midgut. These observations are similar to the ones made by Goettel (1988a) in the pathogenesis of *Tolypocladium cylindrosporum* in *Ae. aegypti* larvae.

When exposed to *T. atroviride*, the larval gut of *Cx. quinquefasciatus* and *An. stephensi* appeared packed with green coloured matter on exposure to green spores. Greenish faecal pellets were observed showing that the spores were ingested as well as egested. In *Anopheles* larvae, the conidia from the faecal pellets plated on antibiotic loaded CMA germinated implying thereby that the viable spores that germinate remained unaffected during their passage through larval gut and they escaped the action of gut enzymes or showed resistance to them. Also since the egested spores are viable, they will ensure recycling of fungus. It was observed by Sweeney (1975) and Sweeney *et al.* (1983) in *Cx. fatigans* larvae that the normal route of infection by *Culicinomyces clavisporus* was the digestive tract. *B. bassiana* (Miranpuri & Khachatourians, 1991) and *M. anisopliae* (Al-Aidroos & Roberts, 1978) infect *Ae. aegypti* larvae through the gut confirming that mosquitoes are very much vulnerable to this mode of attack. Reports on insect mortality due to the toxic activities of large number of ingested but ungerminated conidia in the mosquito larvae are also available (Serit & Yap, 1984).

Our studies on the fate of the conidia of *T. atroviride* after being held for more than two hours in the gut of *Cx. quinquefasciatus* larvae, showed that they were still viable. Prior to the exposure to inoculum, the gut contents were purged with sterile India ink to rule out any effects that the gut flora could have on the viability of the conidia.
These results are in accord with those of Goettel et al. (1988b) who concluded that the viability of *Tolypocladium cylindrosporum* spores was not reduced after a 2.5 h passage through the gut of the 2nd instar larvae of *Ae. aegypti*. This provides advantage to the fungal species as far as recycling and residual efficacy is concerned. Whether this passage through the host has negative or positive effect on the virulence of pathogen in the vector needs investigation?

The fate of fungal spores in different larvae after ingestion differs widely as reported by different authors. In the gut of *Cx. quinquefasciatus* larvae apparent digestion of *M. anisopliae* conidia was observed by Lacey et al. (1988). Disruption of ingested spores of *T. cylindrosporum* in the gut of *Aedes albopictus* was reported by Ravallec et al. (1989).

In the present study, the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* rapidly ingested green conidia of *P. citrinum* and within 2 hours, the larval gut was filled with spores which made the gut appear like a green tube under a microscope. Hence these fungi upon indiscriminate ingestion can begin their rapid lethal action soon after they are ingested. It is assumed that the suitable formulation of these fungi if deployed for vector control will be able to begin killing action on larvae almost instantaneously.

Since the larvae of *An. stephensi* show blackening of middle part of their head and thorax, one can easily observe under dissecting microscope that process of melanization also begins soon after ingestion. Melanization is a process of host cellular defense response to a pathogenic fungus as has been earlier reported (Ferron, 1978). Most of the larvae showed this feature after exposure and eventually the whole body of the larvae became black accompanied by mortality showing thereby that the fungal pathogen evokes similar response in majority of the exposed larvae. However
we observed that a few melanized larvae escaped death. Melanin is a polymer which strengthens the cuticle and barricades the entry of parasites and pathogens through this route (St. Leger et al., 1988b). This protective phenomenon has been previously reported and when pathogenic fungus succeeds in penetrating cuticle, the plasmatocytes which are present in the haemolymph accumulate around the fungus and give rise to melanization. Melanin is toxic and antimicrobial (Ourth & Renis, 1993) in nature by virtue of its capacity to bind proteins (Doering et al., 1999) and inhibit microbial lytic enzymes (Bull, 1970). In Manduca sexta larval cuticles on induction to melanize exhibited resistance to fungal penetration for duration of 72 h whereas unmelanized cuticles succumbed after 40 h (St. Leger et al., 1988b). It was stated by Golkar et al. (1993) that in the larvae of An. gambiae, the germ tube penetration by zoospores of Lagenidium giganteum provoked an intense melanization in the cuticle which protected 56% of larvae from death. They also observed variations in the host defense reaction with fast and intense melanization in An. gambiae as compared to Ae. aegypti and Cx. pipiens larvae. In the present study, in An. stephensi larvae despite the melanization, the highest dose of P. citrinum (20.02 x 10^6 spores/ml) yielded a larval mortality of 84% in 48 h. The degree of melanization differed in the exposed larvae. We observed that the immune response in An. stephensi larvae was faster compared to Cx. quinquefasciatus and Ae. aegypti larvae. Similar to the earlier studies by Lacey et al. (1988), the larval death could be due to physiological starvation and/or by production of mycotoxins by spores in the gut. The recovery of fungus on plating the gut of exposed larva proved that the green mass choking the gut was indeed comprised of ingested spores.

In appearance, finally the dead Culex larvae had turned pale and shriveled whereas Ae. aegypti larvae became transparent. In Cx. quinquefasciatus larvae, extensive
mycosis accompanied by emergence of hyphae through the cuticle was observed from the head, thorax, abdomen, anal siphon traversing the gut and haemocoel was observed which showed that once established fungus could extensively grow in larvae bringing in its wake death of the host. Not all the larvae however showed such luxurious mycelial growth as 50-60% of the dead larvae were without any apparent infection. This suggests that mortality in larvae may precede hyphal growth and proliferation of fungus in the host. Similar observations have been made by Goettel (1988a) in *Ae. aegypti* which were exposed to *T. cylindrosporum*. In that case 10-50% of exposed immatures died without any signs of fungal colonization inside the haemocoel and in a few of the exposed larvae, extensive mycelial growth occurred in the foregut, midgut, the colon or rectum but without any penetration of the host tissues. In the present study, no mycelial growth of *P. citrinum* was observed in the case of dead larvae of *Ae. aegypti* and *An. stephensi*.

The SEM studies confirmed that *Cx. quinquefasciatus* larvae exposed to *P. citrinum* had conidial attachment in abundance on to the surface of respiratory siphon and anal lobes, slightly lesser in the thoracic region and in patches on rest of the body. On the other hand, the SEM of the dissected gut of *Cx. quinquefasciatus* showed abundant conidia of *P. citrinum* and of these, a few were germinating as suggested by appressorial formation thus substantiating that the primary route of invasion and infection was through gut.

In case of exposed *Ae. aegypti* larvae, *P. citrinum* conidia were present sparsely on the surface confirming that mode of action was by internalization rather than exogenous growth in this species too.

Further the confirmation of above phenomenon was provided by the SEM of the faecal pellet of *Ae. aegypti* and *Cx. quinquefasciatus* larvae which clearly showed that
intact egested *P. citrinum* conidia were enveloped in the peritrophic membrane. Galli-Vallerio & Jongh (1906) had observed that the larvae of anopheline and culicine mosquitoes exposed to *Aspergillus niger* and *A. glaucus* produced long tails of type II peritrophic membrane. The midgut of mosquito larvae has type II peritrophic membrane which forms a continuous sleeve (or sleeves) that is always present and thought to protect the midgut epithelium from mechanical injury and pathogens (Lehane, 1997). Abedi & Brown (1961) had reported that the resistant and susceptible strains of *Ae. aegypti* larvae voided a visible peritrophic membrane in the faeces using it as a vehicle to excrete DDT and DDE when challenged with the insecticides. The resistant strain produced peritrophic membrane 9 times more than the susceptible strain and contained 3 times more DDE and 5-15 times as much DDT as produced by the susceptible strain.

Soares (1982) observed that in larvae of *Ae. sierrensis*, the route of invasion of *T. cylindrosporum* was through the cuticle and alimentary canal. Similar observations were made in our study on mode of action of *P. citrinum* in *Cx. quinquefasciatus* larvae with SEM showing the presence of conidia on the cuticle and inside of the gut. The gut seems to be the major route of invasion as germinating conidia were also seen in the gut in SEM and mycosis was also observed in some of the larvae with light microscopy. There is probability that toxins secreted by the conidia are also contributing to the mortality as the metabolic extracts of *P. citrinum* showed larvicidal activity.
5.4 Subculturing of the three isolates and loss of activity in case of *Gliocladium* sp. (isolate GUFCC 5044) and *Trichoderma atroviride*:

On successive subculturing in artificial media, fungi are known to lose virulence or undergo a change in the morphological characters. Attenuation of virulence is seen in major taxa of entomogenous fungi (Butt *et al.*, 2006). The decline in virulence occurs at different rates in different strains. *V. lecanii* showed loss of virulence after 2-3 subcultures (Nagaich, 1964) others after 10-12 times of subculturing (Hajek *et al.*, 1990), no decline in virulence on subculturing more than 12 times was reported by many (Hall, 1980; Ignoffo *et al.*, 1982; Vandenberg & Cantone, 2004). Aberrant morphology was reported in *Entomophaga maimaiga* after subculturing 50 times in liquid media (Hajek *et al.*, 1990).

This phenotypic degeneration encompasses a change in colour, growth form and reduced sporulation of the fungus. In our study, as discussed earlier, three promising isolates *Gliocladium* sp. isolate GUFCC 5044, *T. atroviride* and *P. citrinum* were chosen for studying mode of action and their bio-efficacy on mosquito larvae and they were repeatedly subcultured. *Gliocladium* sp. isolate GUFCC 5044 showed change in morphology, *T. atroviride* showed reduction in virulence while *P. citrinum* was the most stable and showed neither morphological change nor loss of virulence over a period of three years of repeated sub culturing in the artificial media. Hence this species was chosen for further evaluation of its bio-efficacy, metabolites, safety status to NTO and enzyme production.

The pathogenicity of *Culicinomyces clavisporus* to mosquitoes did not decrease apparently on repeated subculturing on artificial media (Cooper & Sweeney, 1982) in our study too there was no decline in the pathogenicity of *P. citrinum* on prolonged subculturing on artificial media. On the other hand, after several weeks of storage at
25°C aqueous suspensions of conidia lost their infectivity to mosquito larvae (Sweeney, 1981). In contrast in the present study however, the *P. citrinum* conidial suspension stored in the refrigerator was active after storing for a period of one year. Therefore this species has number of qualities that merit its further scientific exploration for development as mosquitocidal mycoinsecticide.

5.5 Analysis of bio-efficacy of promising isolate:

Out of the three main bioassays carried to assess the bio-efficacy of *Penicillium citrinum* against the 3rd instar larvae of three test vector species, two assays i.e. against *An. stephensi* and *Ae. aegypti* were amenable to probit analysis while the one against *Cx. quinquefasciatus* larvae was not. In a bioassay, differences in batches of insects can be a source of potential variation (Bucher & Morse, 1963; Burges & Thomson, 1971).

Balaraman et al. (1979) obtained 68.5 fold lower LD$_{50}$ values (2 x 10$^5$ spores/ml) in case of *M. anisopliae* against second instar *An. stephensi* larvae compared with LD$_{50}$ values of *P. citrinum* against the 3rd instar larvae observed in the present study. Serit & Yap (1984) reported in 3rd instar larvae of *An. balabacensis* the LD$_{50}$ value of 4.76 x 10$^5$ spores/ml on 96 h exposure to *T. cylindrosporum*. Although this concentration was 36 fold lower than *P. citrinum* (LD$_{50}$ = 17.13 x 10$^6$ spores/ml) against *An. stephensi* in the current study yet in the latter case, the LD$_{50}$ was achieved in 24 h and hence produced rapid mortality in larvae which is crucial in vector control operations. Further by increasing spore concentration of *P. citrinum* by 1.2 folds, i.e. 20.02 x 10$^6$ spores/ml, the mortality was increased to 84% in 48 h. Similarly, *Lagenidium giganteum* at a higher concentration of 3 x 10$^6$ spores/ml caused above 90% mortality in the 3rd instar larvae of *An. punctipennis* (Kramer, 1990).
In his study on the effect of different isolates of *T. cylindrosporum* against *Ae. aegypti*, second instar larvae, after ten days of continuous exposure, Goettel (1987c) obtained LD$_{50}$ values in the range of 0.7-67.6 x 10$^4$ conidia/ml. The LD$_{50}$ value obtained in the present study on continuous exposure of *Ae. aegypti* 3rd instar larvae to *P. citrinum*, was comparatively 100-fold higher with LD$_{50}$ value of 66.03 x 10$^6$ spores/ml at 48 h exposure but the time required to achieve 50% mortality was 5 times lesser with this species of fungus. Hence higher concentration of conidia can offset the cost of prolonged exposure which is crucial in tropical conditions of high temperature and shorter developmental span of mosquito immatures. da Costa *et al.* (1998) have reported mortality rates from 0 to 6.6% when *Penicillium corylophilum*, *P. fellutanum*, *P. implicatum*, *P. janthinellum*, *P. viridicatum* and *P. waksmanii* species were tested on second instar *Ae. aegypti* larvae and at different concentrations. In comparison, in the present study, the third instar larvae of *Ae. aegypti* showed a higher degree of susceptibility to *P. citrinum* with average percent mortality of 48%, 61.33% and 62.7% on 24 h, 48 h and 72 h exposure respectively.

In our study on 48 h exposure to *P. citrinum* conidial suspensions, the Cx. *quinquefasciatus* larvae were susceptible showing 88% mortality at a dose of 10 x 10$^6$ spores/ml. However in natural habitats, the spores will quickly settle down out of the feeding zone to less accessible substrata (Lacey *et al.*, 1988). But with proper formulation the spore suspension can be used effectively in the field. Oil formulations diminish effect of UV radiation on spores and are effective in increasing spore survival duration and fungal efficacy against insects (Hong *et al.* 2005, Inyang *et al.* 2000).

The two factor ANOVA also showed that the difference in mortalities was highly significant between different doses in case of *An. stephensi* larvae and *Ae. aegypti*...
larvae while it was not significant in case of *Cx. quinquefasciatus* larvae. The effect of time was significant in case of *An. stephensi* while highly significant in case of *Ae. aegypti* larvae and not significant in *Cx. quinquefasciatus* larvae. *Cx. quinquefasciatus* larvae were highly susceptible at very low doses compared to *An. stephensi* and *Ae. aegypti*, the latter requiring a much higher dose. Hence prolonged continuous exposure for a period of 72 h caused a greater mortality in *Ae. aegypti* compared to the other two.

5.6 Assessment of metabolites:

In their studies Vijayan & Balaraman (1991) obtained the LC50 values of fungal metabolites of 17 species in the range of 3-24 µl/ml against *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure, whereas in the present study, LC50 value was achieved on 24 h exposure with *Trichoderma atroviride* at a slightly higher dose of 26.36 µl/ml. The LC50 value of *Chrysosporium tropicum* evaluated against 3rd instar *Cx. quinquefasciatus* larvae was 79 µl/ml (Priyanka & Prakash, 2003), which is three-fold higher than *T. atroviride* observed in the present study. A crude extract of tolypin caused 100% mortality in the larvae of *Cx. pipiens* and *An. maculipennis* at a concentration of 100 µl/ml (Weiser & Matha, 1988b).

*Penicillium* species seem to be a never ending source of novel bioactive metabolites world-wide (Larsen *et al.*, 2007; Ge *et al.*, 2008; Takahashi & Lucas, 2008). They produce diverse secondary metabolites ranging from acetyl cholinesterase inhibitors (Kim *et al.*, 2001), immunosuppressants, cholesterol-lowering compounds (Kwon *et al.* 2002), mycotoxins (Frisvad & Samson, 2004), antibacterial (Lucas *et al.* 2007) and antifungal (Nicoletti *et al.*, 2007) agents.
In the present analysis *P. citrinum* metabolites extracted in methanol were highly larvicidal with 100% mortality in *Cx. quinquefasciatus* on 48 h exposure while *An. stephensi* larvae were slightly less susceptible with 94.1% mortality. Hence *Cx. quinquefasciatus* larvae were chosen as standard species to test activity of separated fractions. The LC₅₀ value of metabolites extracted from 14 d culture of *P. citrinum* was 4.429 µl/ml against *An. stephensi* 3rd instar larvae on 24 h exposure which indicates the extract is not only highly effective but also the mortality can be achieved in short time. The results of bioassays with metabolites from submerged cultures of *T. atroviride* and *P. citrinum* clearly suggest the production of larvicidal toxins. It is thus likely that the larval mortality observed during our studies, in the absence of fungal hyphae, could actually be due to a similar toxin/s produced by ingested conidia in the gut.

It was interesting to observe that *P. citrinum* metabolites extracted in methanol from 14 d culture caused 73% mortality in *An. stephensi* while the metabolites extracted from 20 d culture resulted in 60% mortality. The age of the conidia therefore seems to play an important role in the effectiveness of toxic metabolites. Hence for the separation of active fractions of *P. citrinum* metabolites 14 d culture would be of greater value. Our study has shown that all the three test vector species were susceptible to *P. citrinum* metabolites in the increasing order of *Ae. aegypti* → *An. stephensi* → *Cx. quinquefasciatus*. *Ae. aegypti* larvae showed least susceptibility requiring much higher doses and exposure time was not significant whereas in *An. stephensi* and *Cx. quinquefasciatus* larvae were far more susceptible and the duration of exposure was significant. The effectiveness of metabolites of *P. citrinum* did not diminish much after storage in the refrigerator for a period of thirteen months as
bioassays carried out with methanol extracts against *Cx. quinquefasciatus* showed a marginal reduction in mortality from 83.3% to 73%.

5.7 Larvicidal activity of partially purified metabolites:

Before running the bioassays to test the activity of partially purified metabolites, an assay was carried out by us to assess the effect of different solvents on the control larvae. The solvents Ethyl acetate, Pet ether, chloroform and n-butanol caused mortality in the *Cx. quinquefasciatus* larvae exposed but no mortality was seen with methanol. Hence we dried each separated fraction and then re-dissolved them in methanol or DMSO. The ethyl acetate and chloroform fractions which could not be redissolved in methanol were dissolved in DMSO. No mortality in *Cx. quinquefasciatus* larvae with DMSO was observed as was expected because DMSO is an inert material. The yellow amorphous granular concentrate of Pet ether fraction was re-dissolved in methanol because its solubility was good.

Bioassay of dichloromethane extract from mycelium of *B. bassiana* at 100 ppm showed activity against *Ae. aegypti* larvae (Gupta *et al*., 1995). The extract had Beauvericin and two analogues (Beauvericin A and B). Grove & Pople (1980) observed 86% mortality with beauvericin in *Ae. aegypti* larvae after 48 h exposure at 20 g ml\(^{-1}\), but only 39% when half the dose was used (10 g ml\(^{-1}\)). With LD\(_{50}\) values of 10 to 100 ppm a mixture of 70% destruxin A and 30% B from *M. anisopliae* was shown to be toxic to mosquito larvae (Roberts, 1974).

In a study by Abe *et al.* (2005), the butanol extract from 12-day solid state fermentation of *Penicillium citrinum* F 1539 exhibited insecticidal activity against adult Green peach aphids (*Myzus persicae*). They observed 100% mortality at 1000 ppm on 4-day exposure. In the present study the Pet ether fraction of methanol extract of *P. citrinum* GUFCC 5072 from 14-day submerged culture showed 98% mortality
in *Cx. quinquefasciatus* 3\(^{rd}\) instar larvae on 2-day exposure at 730 ppm i.e. at a dose of 0.73 mg ml\(^{-1}\). Comparatively these doses are much lower and hence highly potent. The highly larvicidal Pet ether fraction was of great interest and an attempt was made to further analyse the active fraction. Also it was observed that the chloroform fraction caused 38.3% and 43% mortality on exposure of 24 and 48 h respectively at a dose of 0.87 mg ml\(^{-1}\). As the mortality was below 50%, it was not investigated further. But as the surviving larvae exposed to this fraction did not pupate, we suspected that this fraction has growth inhibitory activity similar to Insect growth regulators which warrants further investigation.

In Russia from the culture broth of *P. citrinum* VKM FW-800 quinocitrinines A and B were obtained which demonstrated antifungal, antibacterial and antiproliferative activities (Kozlovsky *et al.*, 2003). Abe *et al.* (2005) isolated a new tetracyclic quinolone compound quinolactacide from the butanol extract of *P. citrinum* which showed 88% insecticidal activity at 250 ppm against aphids. They have attributed the lower mortality with pure compound to the poor solubility in methanol that was used to dissolve the sample for the bioassay.

The purification of the active fractions carried by TLC in the present work revealed multiple spots using 30% ethyl acetate in Pet Ether as the solvent. In the second solvent system i.e. 25% ethyl acetate in Pet Ether, each fraction loaded showed two spots each. Due to very less quantity of the active fractions obtained after separation, the larvicidal activity of each spot could not be checked. But it was enough in quantity to carry out NMR and IR study.
5.8 NMR (Nuclear Magnetic Resonance) and IR (Infra Red) of Active Fraction:

The spectral pattern of the initial NMR of unprocessed Pet ether fraction and the NMR of organic layer obtained after processing of Pet ether fraction corresponded. IR spectra of the aqueous layer clearly showed the absence of a prominent –OH group pointing that the active compound is not an acid.

The amount of compound obtained after processing was not enough to carry further work. Elucidation of the structure of the active compound needs to be carried out and structure-activity relationships studied. Not much work has been done on study of structure of metabolites with mosquitolarvicidal activity.

5.9 Production and estimation of enzymes of active fungal isolates:

Extracellular enzymes degrading cuticle are associated with virulence of entomopathogenic fungi (Jackson et al., 1985). Conversely no correlation between enzyme activity and pathogenicity was observed in other studies (Rosato et al., 1981). However cuticle degrading enzymes have played a major role in the invasive process of entomopathogenic fungi. Production of a range of extracellular cuticle-degrading enzymes in M. anisopliae when grown on locust cuticle in liquid medium was observed by St Leger et al. (1986a). The enzymes were produced sequentially corresponding to cuticular components of insects i.e. protein, chitin and lipids with esterases and proteolytic enzymes appearing first followed by N-acetylglucoaminidase (NAGase) while chitinase and lipase were produced last i.e. 3-5 days later. The activity of endoprotease Prl appeared to be pathogenicity-determinant as it was produced in large amount during infection and had high cuticle degrading ability (St Leger et al., 1987a, b). Prl inhibitors reduced mortality, reduced browning,
invasion of haemolymph and curtailed insect growth rate showing that Pr1 is important for penetration. *Beauveria bassiana, Nomuraea rileyi, Verticillium lecanii* and *Aschersonia aleyrodis* culture filtrates have shown Pr 1-like enzymes (St. Leger *et al.*, 1987b; El-Sayed *et al.*, 1993; Chrzanowska *et al.*, 2001).

The processing of microbial proteases is simpler compared to animal and plant proteases as they are generally extracellular in nature (Gupta *et al.*, 2002). In the present work protease activity assay carried out in the mosquito-pathogenic *P. citrinum* showed highest protease activity (7.78 U ml⁻¹ min⁻¹) at 168 h of incubation and it was comparatively lower and slower than *P. chrysogenum* which showed highest protease activity (12 U ml⁻¹ min⁻¹) after 72 h of incubation in a study by Haq *et al.*, 2006. *G. roseum* isolate GUFCC 5039 showed a still lower activity (4.82 U ml⁻¹ min⁻¹) at 168 h of incubation. The reduced activity after this period of incubation may be associated with depletion of available nutrients (Romero *et al.*, 1998).

Protease activity was detected on second day of incubation in *P. citrinum* in the present study. Similar observations were made in case of *Aspergillus terreus* by Wu *et al.* 2006. The onset of protease production on the second day was correlated by these workers to the increase in pH of the medium above 6.5.

Chitinase is an inducible enzyme (Smith & Grula, 1983) and chitin (St. Leger *et al.*, 1986c) is masked by the proteins, hence only after degradation of cuticular proteins the chitin becomes available which is the reason why chitinase appears late. Inhibitors in the cuticle were thought to be responsible for the absence of chitinase activity during penetration (St Leger *et al.*, 1986b). In our study on *P. citrinum*, chitinase assay carried out showed highest activity (0.012 U ml⁻¹ min⁻¹) at 72 h of incubation. In *Trichoderma harzianum* isolate Binod *et al.* (2007) have recorded chitinase activity (0.75 U ml⁻¹ min⁻¹) at 24 h of incubation with highest activity (10.2 U ml⁻¹ min⁻¹) at 96
h of incubation. Comparatively the chitinase activity in the current study was very low.

It was observed in ultra structural study of the wireworm cuticle, the wax layer on the cuticle hydrolyses just beneath the appressoria and germ tubes of *M. anisopliae* which is attributed to lipase or esterase activity (Zacharuk, 1970; Charnley & St Leger, 1991). Hegedus & Khachatourians (1988) suggested that *in vivo* lipase maybe important in the colonization of the haemolymph rather than the cuticle.

Protease activity was detected in *P. citrinum* at 48 h of fermentation whereas chitinase activity was detected at 24 h of fermentation. Studies on lipase activity also need to be carried out in *P. citrinum*.

### 5.10 Evaluation of bio-safety of *Penicillium citrinum* to non-target organisms:

Fungi have a very narrow to wide host range, which differs from species to species. *Pandora neoaphidis* which is strictly pathogenic to aphids, does not pose threat to other non-target organisms unlike *B. bassiana*, that infects over 700 arthropod species (Li, 1988) and poses a much bigger risk. A fungus with narrow range is usually an obligate parasite and is seen to cause epizootics, where as a fungus with broad spectrum of hosts is facultative in nature. Testing of infectivity to invertebrate Non-Target Organisms (NTOs) is an important criterion for registration of entomopathogenic fungi as biological control agents (Hall *et al.*, 1982). Bio-safety assessments are predominantly lab based. Entomopathogenic fungi have an edge over the chemical pesticides in the risk posed by them in the environment. Integrated use of fungal control agents is advocated with other control strategies to ensure minimal risk to the NTO (Falcon, 1973; Fuxa, 1987; Goettel & Johnson, 1992). Goettel (1995) opines that it is doubtful whether the artificial augmentation of an indigenous fungus
with wide host range would cause permanent ecological damage as these fungi are ubiquitous, are not very virulent and seldom cause epizootics.

In the present lab based study, biocontrol was achieved by using entomopathogenic fungi targeting an aquatic stage of mosquito life cycle so it was necessary to evaluate the effect of the promising fungal isolate on a NTO that was aquatic in nature. Natural predator of the larvae *Aplocheilus blocki* a larvivorous fish was assessed as a vertebrate model to study the bio-safety of *P. citrinum* in the aquatic environment. Standard 7-day chronic exposure to *P. citrinum* at a high dose (34.76 x 10⁷ spores/ml) did not cause any effect on the external morphology and digestive system especially liver and gall bladder were apparently normal. The gill chamber had green bolus-like material enveloped in mucous but the gills appeared normal. A low mortality of 9% was observed in the experimental aquaria.

The invertebrate NTO, a semi-aquatic insect *L. fossarum fossarum* subjected to standard 4-day chronic exposure of *P. citrinum* conidial suspension at a dose of 3 x 10⁸ spores/ml and observed for ten days did not show any external growth of fungus or sluggishness in the host but a low grade mortality of 4% (1/25) was observed. However whether it was due to fungus was not clear. The bio-safety of crude metabolites of *P. citrinum* (40 µl/ml) tested by acute 20 sec exposure of *L. fossarum fossarum* to the metabolites did not cause sluggishness or mortality for 48 h post exposure though the dose was ten-fold higher than that used against mosquito larvae. The above findings indicate that *P. citrinum* and its metabolites are relatively safe to NTOs but further studies on a wider range of NTO’s are needed to arrive at a conclusion.

The prime choice for the control of mosquito vectors the world over is synthetic insecticides but due to the impact of chemical insecticides on environment and the
ever developing resistance in the insects, the use of biological control agents has
gained attention. In vector control programmes in India, larvivorous fishes and the
bacilli *Bacillus thuringiensis* var. *israelensis* (*Bti*) have been used successfully in
large scale. Fungal biocontrol agents are an attractive option and as shown in Table 3
there is an increasing interest in their commercial production for insect control.

Entomopathogenic fungi are now being explored to increase arsenal in the fight
against vector borne diseases.

In conclusion, this thesis documents the results of an extensive and elaborate work
which was accomplished at National Institute of Malaria Research (NIMR), Campal,
Panaji for the screening of fungi to test larvicidal activity, effect on NTO and
metabolite testing and at Mycolab of Botany Dept. of Goa University Taleigao, Panaji
for the isolation, culturing and harvesting of fungi and enzyme studies. At National
Institute of Oceanography (NIO), Dona Paula, Panaji work on partial purification and
separation of metabolites was carried out. Our studies though are “a drop in the
ocean” and of basic nature they provide an insight into the mode of action and bio-
efficacy of the indigenously isolated fungi against the vector larvae. This study
demonstrates that local strains of *Gliocladium, Penicillium, Trichoderma* spp. have
the potential to kill larvae of vectors like *Anopheles, Culex* and *Aedes*. Lab studies of
*P. citrinum* against NTO are encouraging and need to be analyzed further in the field.

These mosquito-pathogenic fungi need to be studied further extensively with respect
to large-scale production of conidia, the stability of these formulations under
laboratory and field conditions needs to be evaluated. Secondary metabolites have
shown promising larvicidal activity, the active fraction needs to be studied in detail
and has a potential of commercial exploitation.