Chapter V

Discussion

The market demand for HSA is more than 500 tons per year worldwide (He et al., 2011). Currently, commercial production of HSA is primarily based on collected human plasma (pHSA), which is limited in supply but of high clinical demand. As the source of the blood can vary, there is the potential risk of HSA contamination by blood-derived pathogens. Thus, the development of a low-cost method for the production of recombinant HSA (rHSA) is essential as a safer and potentially unlimited alternative to pHSA (Kobayashi, 2006). Though different expression systems are available for the production of rHSA, they all come up with a high price tag due to high cost of capitalization and production. It has been estimated that costs for recombinant protein production in mammalian cell culture is approximately US$150 per gram, whereas production in plant systems cost approximately US$0.05 per gram (Dove, 2002). The expression of biologics in the green microalga Chlamydomonas reinhardtii offers an attractive alternative to traditional expression systems as stable transgenic lines can be generated in a relatively short period of time and the production of proteins in algae has an inherently low cost due to highly reduced media charges (US$0.002 per litre) (Mayfield et al., 2003; Franklin and Mayfield, 2005). So the production of rHSA in C. reinhardtii is expected to be an alternate cost effective method.

V. 1. Culture conditions

The constituents of the growth media are critical variables in defining not only growth rate, but final cell density of algal cultures. Media formulations, such as TAP and HS containing relatively high carbon content are better able to support rapid and dense algal cell growth than carbon poor media formulations such as BG11. Likewise, carbon rich growth conditions that lack other key elements such as nitrogen or sulfur also suffer from poor growth. These
nutritional restrictions often result in decreases in protein or DNA synthesis as a result of the lack of key building block components (Deng et al., 2011). In the present study, two different media were tested for culturing C. reinhardtii; TAP and HS media. Even though C. reinhardtii was successfully grown in both media, TAP was found to be more efficient in terms of relative growth and cell count. Another important factor is that the selected medium should support the growth of both Agrobacterium and Chlamydomonas. The TAP medium allows simultaneous growth of both algae and bacteria. TAP medium was used for the Agrobacterium mediated transformation of micro algae Chlamydomonas reinhardtii, Haematococcus pluvialis and Dunaliella bardawil in earlier studies (Kumar et al., 2004; Kathiresan et al., 2009; Anila et al., 2011).

**V. 2. Vector construction**

The binary vector pLGMR.HSA harbors human serum albumin gene (HSA) under the control of CaMV 35S promoter and neomycin phosphotransferase II (nptII) gene as marker gene (Arokiaraj et al., 2002). The basal vector used for the construction of pLGMR.HSA was pBIN 19. In pLGMR.HSA, the nptII marker gene is situated in the right border region of T-DNA and HSA is situated near left border. T-DNA transfer starts at the right border and proceeds to the left border. Therefore it is advisable to have the selectable marker at the left border and the gene of interest at the right border so that the gene of interest is transferred first and the selectable marker second. This means that selected transformed plants have a greater chance of containing the gene of interest. pBin19 has been dominating the landscape of binary plasmids for several decades (Komori et al., 2007). This plasmid is the first to be commercialized for use in plant genetic transformation (Dafny-Yelin, 2007). In a previous study, this vector was modified to create a new vector pBINPLUS with plant selectable marker located near the T-DNA’s left border, a feature that ensures complete integration of the T-DNA sequences into the plant genome (Van Engelen et al., 1995). The binary vector pART27 was designed by Gleave
so as to have the \textit{nptII} gene close to the left border to ensure that the selection marker is the last to be transferred into plants. This strategy ensures that all plants selected on kanamycin will have a complete T-DNA inclusive of the gene of interest (Gleave, 1992).

There were reports that in \textit{Chlamydomonas}, the selection based on neomycin phosphotransferase II (\textit{nptII}) gene is found to be ineffective and there are lots of non transformants (Bateman and Purton, 2000). Hygromycin phosphotransferase (\textit{hptII}) gene was found to be an efficient marker gene in microalgae (Ladygin and Boutanaev, 2002; Kumar \textit{et al.}, 2004; Kathiresan \textit{et al.}, 2009; Anila \textit{et al.}, 2011).

So in order to make the transformation efficient and effective, we have to construct a new vector which possess \textit{hptII} gene as the selectable marker and harbors gene of interest HSA at T-DNA right border. pCAMBIA 1301 T-DNA binary vector was selected for the present study which harbors hygromycin phosphotransferase II (\textit{hptII}) as marker gene near T-DNA left border. pCAMBIA vectors were used earlier for the successful transformation of microalgae (Kumar \textit{et al.}, 2004; Kathiresan \textit{et al.}, 2009; Anila \textit{et al.}, 2011; Cha \textit{et al.}, 2011; Cha \textit{et al.}, 2012; Cheng \textit{et al.}, 2012).

The pCAMBIA vector backbone is derived from the pPZP vectors (Hajdukiewicz \textit{et al.}, 1994). Plant selection genes in the pCAMBIA vectors are driven by a double-enhancer version of the CaMV 35S promoter and terminated by the CaMV 35S polyA signal. pCAMBIA 1301 plasmid is suitable for insertion of other genes of interest by excising the \textit{gusA} reporter gene located near the right border. Reporter gene features a hexa-Histidine tag at the C-terminus. The sequence for this tag occurs between the \textit{NheI} site and \textit{PmlI} site. Insertion without a stop codon and in frame at the \textit{NheI} site will append a hexa-Histidine tag to the protein of interest which enables simple purification on immobilised metal affinity chromatography resins.
Chapter V

The reporter gene in pCAMBIA 1301 is driven by CaMV 35S promoter. Although the cauliflower mosaic virus 35S (CaMV 35S) promoter drives strong and constitutive expression in most dicotyledonous and some monocotyledonous plants (Benfey et al., 1990), it was not considered to be a useful promoter in algae. Rather, in algal transformation, the most effective promoters have been derived from highly expressed algal genes. But studies in Chlamydomonas by Kumar and team in 2004 describe the functionality of CaMV 35S promoter using Agrobacterium mediated transformation methodology. They used pCAMBIA 1304 binary vector which harbours hygromycin antibiotic selection gene (hptII) driven by CaMV 35S promoter and gfp:GUS fusion as reporter gene under the control of CaMV 35S promoter for Agrobacterium mediated genetic transformation. The detection of hpt transcripts from a CaMV 35S promoter cassette is contradictory with an early report (Day et al., 1990), where the promoter could not drive expression of transgene in Chlamydomonas. This can be attributed to the possible advantages of Agrobacterium T-DNA to be targeted to and integrated at potentially transcribable regions of the genome (Hiei et al., 1994). Moreover, in the earlier report (Day et al., 1990) where the functionality of the promoter was tested, the transformant lines were not the result of a direct selection on the corresponding antibiotic. The data by Kumar and his team (Kumar et al., 2004) is the first documentation of the functionality of the CaMV 35S promoter in an algal system using transformation methodology. Moreover, the detection of reporter gene activities in the transformed lines as well as the detection of the GFP: GUS protein by immunoblotting provided further evidences for the efficient expression of the transgenes by CaMV 35S promoter. Successful Agrobacterium mediated transformation of green algae Haematococcus pluvialis (Kathiresan et al., 2009) and Dunaliella bardawil (Anila et al., 2011) were observed with CaMV 35S promoter drives cassettes. So CaMV 35S promoter was selected to drive the gene of interest HSA in the present study. The 1.8 kb HSA cDNA was successfully PCR amplified from the binary vector pLGMR.HSA using restriction site anchored primers. The PCR
amplification was carried out using Platinum® Pfx DNA Polymerase enzyme. It possesses proofreading 3’ to 5’ exonuclease activity and is a highly processive enzyme with fast chain extension capability (Cline et al., 1996). Platinum® Pfx DNA Polymerase is ideal for demanding PCR applications such as PCR expression cloning. Amplification with this enzyme ensures that the PCR product is without PCR errors/mutations. The binary vector was double digested to release GUS gene and the PCR amplified HSA cDNA was inserted at its position so that it carries a hexa-Histidine tag at the C-terminus. The newly constructed vector was named pSBS.HSA and sequenced to check the orientation of the insert. Sequencing analysis confirmed that the inserted HSA cDNA is in right orientation.

V. 3. Transformation of *Nicotiana tabaccum*

*Nicotiana tabaccum* has served as model species for the plant sciences for more than 20 years (Wang, 2006a). Transformation experiments were conducted in tobacco plants to study the efficiency of the newly constructed plasmid vector pSBS.HSA. Tobacco plants were selected as the experimental material as they were highly amenable to transformation studies. As a model plant, tobacco benefits from well-established gene transfer and regeneration methodologies. Majority of the discoveries in the field of plant cell, tissue culture have originated from the experimentation with tobacco plants (Ganapathi et al., 2004). The co-cultivation of leaf discs with *Agrobacterium* can produce tobacco transformants with high quality and fertility. Considering of these advantages of *N. tabaccum* in comparison with other systems, efficiency of the newly constructed plasmid vector was tested in tobacco transgenic lines. In a study conducted by Cancino and co-workers (2004) in *passiflora mollissima* (Banana passion fruit), they used *N. tabacum* as a model plant and found that the *Agrobacterium* strain EHA 105 is highly effective in leaf explants of *N. tabacum*. The explants inoculated with strain EHA 105 showed gus gene expression without supplementation of naringenin (a vir gene inducer) in the co-cultivation medium, although when
added it further increased GUS activity. The *Agrobacterium* EHA 105 is a super virulent strain resulting in successful transformation of plants (Esuola *et al*., 2011; Tripathi *et al*., 2005; Bosque-Perez *et al*., 2000). Therefore, the *Agrobacterium* strain EHA 105 harboring plasmid vector pSBS.HSA was used for the transformation of tobacco plant. Successful transformation was observed with tobacco leaf discs and transgenic plants were generated. Presence of T-DNA region in transgenic plant was confirmed through molecular analysis. Hygromycin resistant transgenic tobacco plants were screened by PCR. When the PCR was carried out using primers specific for HSA gene sequence, a single band of 1.8 kb was amplified from the genomic DNA isolated from all the transformed plants and for *hptII* gene a single band of 610 bp was amplified. Presence of HSA transcripts in transgenic tobacco leaves were detected through RT-PCR analysis. These results indicate that the newly constructed vector is functional and able to express the gene of interest HSA.

**V. 4. *C. reinhardtii* transformation**

Early works on *C. reinhardtii* transformation with the *nptII* gene as a selectable marker had two major drawbacks, low (~10^1) transformation efficiency (Hasnain *et al*., 1985) and high rate of spontaneous mutations to kanamycin resistance (Hall *et al*., 1993; Harris *et al*., 1989). In view of this, the hygromycin phosphotransferase (*hpt*) gene was selected as marker. An earlier study shows that cells of the *C. reinhardtii* CW-15 mutant (which does not produce the cell wall) are sensitive to a low (10 mg/l) concentration of hygromycin and die in 4–5 days of culturing in its presence. Transgenic colonies were selected on 10 mg/l hygromycin and resistant clones were maintained for eight months (approximately 350 cell generations) by repetitive sub-culture on the nonselective hygromycin-free medium (Ladygin and Boutanaev, 2002). Kumar and co-workers (2004, 2007) used 10 mg/l hygromycin for the selection of transgenic *C. reinhardtii* transformed with *Agrobacterium* and the transgenic cells retained the hygromycin-resistant
phenotype for as long as 18 months, even when cells were maintained in hygromycin-free medium. In a recent study by Rasala and co-workers, transgenic \textit{C. reinhardtii} were selected on TAP/agar plates supplemented with 15 $\mu$g/ml hygromycin B and nuclear transformations of \textit{C. reinhardtii} based on the hygromycin B resistance yielded 10 to 100-fold more colonies than with bleomycin antibiotic resistance (\textit{ble}) gene (Rasala et al., 2012).

In the present study, 5 mg/l hygromycin was enough to completely inhibit the growth of untransformed \textit{Chlamydomonas} cells in TAP broth. But the same cells were able to tolerate higher concentrations of hygromycin up to 10 mg/l in TAP agar. Similar results were observed with the microalga \textit{H. pluvialis}, which were able to tolerate hygromycin up to a concentration of 2 mg/l in liquid medium and up to 10 mg/l in solid medium (Kathiresan et al., 2009). This tolerance may be due to differences in membrane permeability among cells grown in the two media. Higher inoculum cell densities allowed greater tolerance to hygromycin in liquid media, which may be due to quicker degradation of hygromycin to tolerable levels by higher inoculum load than by lower inoculum levels. Transformants of \textit{C. reinhardtii} grew slowly on selection medium, while their growth rates were normal and comparable when transferred to non-selection medium. So hygromycin concentrations of 10 mg/l in TAP agar and 5 mg/l in TAP broth were used for the selection of transformed cells. We were able to maintain the transformants stable for >2.5 years in hygromycin free media.

Colony formation and contamination in transformed plates by residual \textit{Agrobacterium} is a major problem faced in transformation studies. In an earlier study, cefotaxime 500 mg/l was found to be enough to prevent \textit{Agrobacterium} growth in \textit{C. reinhardtii} selection plate (Kumar et al., 2004). A concentration of 500 mg/l cefotaxime inhibited the growth of \textit{Agrobacterium} in \textit{H. pluvialis} transformation (Kathiresan et al., 2009). In order to reduce the growth of \textit{A. tumefaciens} from \textit{Dunaliella bardawil} selection plates, a combination of 500 mg/l cefotaxime and 300 mg/l potassium clavulanate was used (Anila et al.,
2011). Generally 500 mg/l cefotaxime is being used in transformation studies of plants for the removal of *Agrobacterium*. In the present study, different concentrations of cefotaxime were tested to find the toxicity of the antibiotic on *C. reinhardtii*. It was confirmed that the established concentration of 500 mg/l is not hindering the growth of *Chlamydomonas*. For the selection of transformants, *Chlamydomonas* cells co-cultivated with *Agrobacterium* were plated on TAP agar with 10 mg/l hygromycin and 500 mg/l cefotaxime. It was observed that a concentration of 500 mg/l cefotaxime completely inhibited the growth of *Agrobacterium* on selection plates. When streaked on LB medium, the *Chlamydomonas* colonies did not show any sign of bacterial growth. Hence a cefotaxime concentration of 500 mg/l was selected in the present study for the removal of residual *Agrobacterium*.

*Agrobacterium* has the ability to deliver DNA to plant cells and permanently alter the plant genome. Transformation of plants by *Agrobacterium*-mediated DNA transfer is the most commonly used phenomenon in accomplishing plant gene transfer. The discovery of this unique feature 30 years ago has provided plant scientists with a powerful tool to genetically transform plants for both basic research purposes and for agricultural development. *Agrobacterium*-mediated transformation offers remarkable advantages over direct gene transfer methodologies. It reduces the copy numbers of the transgene, potentially leading to fewer problems with transgene co-suppression and instability (Shibata and Liu, 2000). Protocols have been developed for efficient *Agrobacterium*-mediated transformation in both dicotyledonous and monocotyledonous plants, including a large number of crop species. Strains containing super virulent plasmids have facilitated transformation of some recalcitrant monocotyledonous plants.

Only a few species of microalgae were transformed using *Agrobacterium* till date - *Volvox carteri* (Schiedlmeier *et al.*, 1994), *Haematococcus pluvialis* (Kathiresan *et al.*, 2009), *Nannochloropsis sp* (Cha *et al.*, 2011), *Chlorella*
vulgaris (Cha et al., 2012), Schizochytrium sp. (Cheng et al., 2012), Dunaliella bardawil (Anila et al., 2011) and Chlamydomonas reinhardtii. Agrobacterium-mediated genetic transformation of C. reinhardtii was first reported in 2004 by Kumar and co-workers. Earlier studies in Agrobacterium-mediated transformation of Chlamydomonas were carried out using only two strains of Agrobacterium: LBA 4404 and A348. For other microalgae, Agrobacterium strains LBA 4404 and EHA 101 were used. Agrobacterium strain is an important factor determining transformation efficiency. Studies related to the efficiency of Agrobacterium strains in Chlamydomonas transformation were not reported so far. So an attempt was made to find out the transformation efficiency of commonly used strains of Agrobacterium tumefaciens in microalga C. reinhardtii. Three A. tumefaciens strains were used for the present study—LBA 4404, EHA 101 and EHA 105. In our experiments, the transformation efficiency of strain EHA 105 was greatly higher than EHA 101 and LBA 4404 based on the number of transgenic colonies observed. This may be due to higher compatibility of EHA105 with C. reinhardtii.

A lot of similar studies were reported in plants. Agrobacterium strains EHA 101 and EHA 105 were more effective than other common strains since both are derived from the supervirulent wild type strain A281 (Hood et al., 1993), whereas strain LBA 4404 was derived from the less virulent strain Ach5 (Hoekema et al., 1983). The transformation studies on plantain cultivar Agbagba by various strains indicated that EHA105 is the ideal strain for plantain (Tripathi et al., 2005). The A. tumefaciens strains EHA105, LBA4404, C58 and GV2260 were used in the study. Bosque-Perez and co-workers (2000) reported similarly that EHA105 is more efficient in the transformation of Grand Nain using meristem pieces. Strain EHA 105 proved significantly better than EHA 101 in transforming Phalaenopsis violacea orchid species (Subramaniam, 2010). Comparison of the Agrobacterium strains for genetic transformation of banana cultivar Williams suggested that EHA105 is more efficient in the transformation than GV2260 and C58 (Esuola et al., 2011).
In the present study also EHA 105 performed better than other two strains under study (EHA 101 and LBA 4404). Therefore, EHA105 can be suggested for use in Agrobacterium mediated transformation of C. reinhardtii and other similar algal species.

V. 4a. Agrobacterium mediated genetic transformation of Chlamydomonas

Agrobacterium-mediated genetic transformation of C. reinhardtii was carried out following the protocol of Rajam and Kumar (2006). Transgenic colonies were observed after 5 days of incubation on selection media containing 10 mg/l hygromycin and 500 mg/l cefotaxime. The transformation efficiencies for all three Agrobacterium strains under study were lower when the above mentioned protocol was applied. So in order to improve the transformation efficiency, the protocol was modified.

V. 4b. Modified protocol for Agrobacterium mediated genetic transformation of Chlamydomonas

A. tumefaciens genetically transforms plants and other organisms by transferring a portion of the T-DNA, from the bacterium to the host (Gelvin, 2000; Gelvin, 2003). Many transformation-associated events that occur within the bacterium are under tight genetic regulation. These events are directed by proteins encoded by the “vir regulon”, a group of genes that respond to phenolic molecules (Lai et al., 2006). Although numerous molecules that can stimulate vir gene induction have been described (including vanillin, methyl sinapate, methyl ferulate, methyl syringate, syringaldehyde, coniferyl alcohol, 2′,4′,4-trihydroxy-3-methoxychalcone, and 2′,4′,4-trihydroxy-3,5-dimethoxychalcone), acetylsyrongone (3,5-methoxy-4-hydroxyacetophenone [AS]) has become the favored molecule to use for inducing the vir genes of Agrobacterium (Song et al., 1991; Dye et al., 1997). Vir gene induction is maximal at acid pH (~5.2–6.0); there is little induction at neutral pH (Godwin et al., 1991). Therefore, it is useless to add AS to the “rich media” (pH 7.0) frequently utilized to grow Agrobacterium vegetatively. Rich medium, even when adjusted to pH 5.6, also is a poor vir induction medium (Liu et al., 1993). Conditions commonly used for vir induction
utilize a “minimal medium” base. The temperature optimum for vir gene induction (~25°C) is generally lower than that optimal for vegetative growth of Agrobacterium (28–30°C) (Alt-Moerbe et al., 1988; Turk et al., 1991). The medium used for the co-cultivation of C. reinhardtii and Agrobacterium is TAP (Tris acetate phosphate) medium with pH 7 (Rajam and Kumar, 2006).

Previous reports show that induction of the vir genes of Agrobacterium by incubation of the bacteria with acetosyringone prior to co-cultivation enhances transformation (Van Wordragen and Dons, 1992; Jacq et al., 1993). Vernade and co-workers established growth conditions for efficient induction of the vir genes of A. tumefaciens by acetosyringone and glycine betaine. Optimal induction was attained by inoculating freshly grown Agrobacterium to an induction medium at a pH below 5.2 containing an osmoprotectant glycine betaine. Proteins associated with the inducible state suggest that vir gene induction is linked to the adaptation of the cells to an unfavorable environment or shock (Vernade et al., 1988). A straightforward explanation for the pH effect would be a pH-dependent binding of acetosyringone to the virA gene product, which is believed to be the receptor (Stachel et al., 1986). Acetosyringone, as well as the osmoprotectants betaine phosphate and proline, have been reported to improve transformation efficiency in apple when added to the virulence induction medium (James et al., 1993). In another study, efficiency of transformation was increased by growing Agrobacterium cells in a vir gene-induction medium containing acetosyringone and glycine betaine at temperature between 24–27°C with gentle agitation for 4.5 hr (Norelli et al., 1996). Similar results were obtained in the genetic transformation of plant species like Malus and Hevea. (Wang, 2006b).

Taking into consideration the issues discussed above, we have made an attempt for the effective Agrobacterium vir gene induction that can enhance transformation efficiency in C. reinhardtii.

In the present study, 48 hr grown Agrobacterium cells were pelleted and resuspended in TAP induction medium containing 100 µM acetosyringone and
1 mM glycine betaine. The pH of the TAP induction medium was adjusted to 5.2. The flasks were incubated at 25°C for 4 hrs with 100 rpm for vir gene induction. 48 hr grown Chlamydomonas was inoculated into TAP induction medium (broth) containing Agrobacterium culture for infection and incubated at 25°C for 30 min with mild agitation. After induction, the Chlamydomonas+Agrobacterium culture from the TAP induction medium was incubated for co-cultivation. The pellet was finally plated onto TAP agar containing 500 mg/l cefotaxime and 10 mg/l hygromycin for selection. Transgenic colonies were appeared on selection plates after 5 days of incubation. The transformation efficiency was greatly improved by incubating Agrobacterium in induction medium with vir gene inducer acitosyringone, osmoprotectant glycine betaine and reduced pH. We have successfully developed a new method for the Agrobacterium mediated genetic transformation of C. reinhardtii for the high frequency of transformants. In this method also, EHA 105 showed highest transformation frequency than other strains. Hence EHA 105 was selected as the ideal Agrobacterium strain for Chlamydomonas transformation in the present study.

V. 5. Molecular confirmation of transformation

PCR analyses were used to confirm the presence and integration of T-DNA into the Chlamydomonas genome. PCR was carried out using HSA and hptII specific primers. All transgenic lines successfully amplified the 1.8 kb HSA fragment and 610 bp amplicon of hptII gene. To confirm that the amplified product from the cell lines is HSA itself, the fragment was cloned and sequenced. BLAST and ClustalW analyses of the nucleotide sequence data confirmed that the amplified fragment is HSA cDNA itself.

In order to check the presence of HSA gene transcripts in transgenic Chlamydomonas cell lines, RT-PCR was carried out. RT-PCR was done with the cDNA synthesized from RNA of transgenic as well as non-transgenic cell lines. The 1.8 kb amplicon of HSA cDNA was amplified when cDNA synthesized from
the RNA of transgenic cell lines was used as the template, indicating that HSA transgene integrated in the genome of transgenic *Chlamydomonas* is getting expressed. When cDNA from non-transgenic line was used as template, no amplification was observed.

**V. 6. Isolation, purification and quantification of rHSA**

Total protein was isolated from the transgenic lines of *C. reinhardtii* (CR-SBS105) following phenol extraction method. SDS-PAGE on 10% acrylamide gel showed that the isolated protein is of good quality without any degradation. The transgenic CR-SBS105 lines showed the presence of 66 kDa band of HSA protein whereas that band was absent in protein isolated from non transgenic line. Total protein estimation was done following Bradfords method using BSA as standard. When 1 g of pelleted algal cells was used for the isolation of protein, ~200 mg total protein could be isolated. From the total protein, rHSA was purified with the help of hexa his tag.

Isolation and purification of protein complexes can be an inefficient and time-consuming process. His tagged protein purification method is a quick, cost-effective, and generally applicable one-step approach to removing protein impurities, endotoxins, and detergent. In *C. reinhardtii*, the His6-tagged PS1 was purified with a yield of 80–90% from detergent-solubilized thylakoid membranes within 3 h in a single step using a Ni-nitrilotriacetic acid (Ni-NTA) column. His6-tagged PS1 reparation was highly pure and extremely low in uncoupled pigments. Moreover, the introduced tag appeared to have no adverse effect upon PS1 structure/function (Gulis *et al.*, 2008). His6-tag, as it has previously been used to good result with the photosynthetic reaction center of purple bacteria (Goldsmith and Boxer, 1996) and PS2 of *C. reinhardtii* (Sugiura *et al.*, 1998). In the present study also, His6-tag facilitated the purification of high quality rHSA. SDS PAGE analysis of the column purified sample was carried out to ensure that the sample is without any contamination. A clear single band of 66 kDa HSA on coomassie blue stained 10% gel indicates the purity of sample. Protein
quantification was done following Bradfords method using BSA as standard. When 1 g of total protein was used for the column purification process, we were able to recover ~5 mg of purified rHSA. The purity of the rHSA was verified using HPLC analysis. The analysis showed a single clear major peak confirming the purity of rHSA.

The identity of the His-tag purified protein was further confirmed through western blotting analysis. The His6-tagged protein run on 10% gel was conjugated with anti-his antibody and detected. The anti-his antibody was found to bind with all the transgenic samples analyzed indicating that these lines are expressing transgenic protein as this band was absent in the non transgenic control.

The highest amount of protein expression achieved through nuclear transformation of *C. reinhardtii* was 0.2% (Neupert et al., 2009). In that study, the glass bead transformed *Chlamydomonas* lines were subjected to UV mutation via irradiation to improve expression level. In present study, an expression level of 0.5% of TSP could be achieved though *Agrobacterium* mediated genetic transformation. This is the first report of expression, purification and characterization of a therapeutically important protein in *C. reinhardtii* through *Agrobacterium* mediated transformation.

Algal biomass can presently be produced at about $3/kg at commercial scale (Chisti, 2007), and as 25% of biomass is soluble protein, we can project that at 2% of soluble protein, we can presently make recombinant protein at about $0.6/g prior to purification. This is near the cost estimates for the least expensive protein expression systems presently available (Dove, 2002) and considerably cheaper than mammalian cell culture. With the expected improvements in expression levels above 2% TSP, and the continued reduction in algal biomass cost associated with the large-scale efforts to use algae for transgenic protein production, we anticipate at least a 10-fold reduction in these cost over the next few years, which should make algal protein production the least expensive
platform available (Rasala et al., 2010). This reduced cost of goods, coupled with an ability to rapidly scale production in inexpensive bioreactors, suggests that algae may become an economically superior platform for therapeutic protein production in the future.

V. 7. Characterization of HSA

FT-IR

FT-IR, a powerful technique for the study of hydrogen bonding, is a very popular technique for detecting structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments (Li et al., 2006). In the IR region, the frequencies of bands due to the amide I–III vibrations are sensitive to the secondary structure of proteins. The spectral shifting and intensity variations of protein amide I shows band at 1656–1655 cm\(^{-1}\) (mainly C=O stretch) and amide II shows band at 1547–1543 cm\(^{-1}\) (C–N stretching coupled with N-H bending modes) (Beaucemin et al., 2007; Belatik et al., 2012). In the present study, characteristic peaks of amide I (1644 cm\(^{-1}\)) and amide II (1545 cm\(^{-1}\)) were observed. Similar characteristic peaks were observed in earlier studies conducted with HSA protein (Liu, 2003; Wang, 2005). This observation confirms that intact secondary structure of HSA is maintained in the rHSA produced from *C. reinhardtii*.

MALDI-TOF MS

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) peptide mass fingerprinting (PMF) is the fastest and cheapest method of protein identification. In an effort to demonstrate bio-equivalence of rHSA produced by *Chlamydomonas* transgenic cells, MALDI-TOF MS techniques were applied for a highly purified preparation of rHSA.

The identity of rHSA to its natural counterpart was established with high precision using peptide mass fingerprinting of tryptic peptides. To obtain a good sensitivity, enough mass accuracy and access to the most of the data (i.e., to
cover the sequence), the protein must be digested (cut) by an endoprotease to generate multiple protein-specific fragments. If the endoprotease has sufficient specificity and if the protein is available in databases, a computed comparison of the experimental peptide masses (generated by the endoproteic digestion and detected by MALDI-TOF mass spectrometry) with the peptide masses of the in-silico digestion of all the proteins present in the database will allow the identification of protein candidates. The acquired mass spectral data were queried against the NCBI nonredundant protein database using the MASCOT search engine, a probability-based protein identification tool (Creasy and Cottrell, 2002; Perkins et al., 1999).

The results from peptide mass finger printing by MALDI–TOF-MS clearly demonstrate that the recombinant protein is HSA itself. The molecular mass of the intact protein was determined to be 66,416 Da in good agreement with that of pure HSA. The acquired m/z values for tryptic digest were transferred to MASCOT search engine. The MASCOT search assigned HSA protein as the highest ranked candidate in similarity. Thus it was confirmed that the purified recombinant protein expressed in transgenic C. reinhardtii in the present study is Human Serum Albumin protein itself.

V. 8. Cell cytotoxicity study of rHSA

The therapeutic and toxicological effects of a compound are important parameters in the verification of its applicability in pharmacology (Queiroz, et al., 2007). In the present study, we examined the cytotoxicity of rHSA produced from C. reinhardtii on human lymphocytes, assessing its effect by MTT reduction [(3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide].

The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present
during MTT exposure. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multi-well scanning spectrophotometer (ELISA reader) and show a high degree of precision (Mosmann, 1983).

Different concentrations of rHSA protein was incubated with human lymphocytes in presence of MTT for 72 hrs. The amount of MTT reduced by cells to its blue formazan derivative during 72 hrs of culture was quantified spectrophotometrically at 570 nm using an ELISA reader.

It was observed that rHSA produced by transgenic *C. reinhardtii* is not inhibiting the viability of lymphocytes at all the concentrations tested.