Diabetes is an increasing global threat. Each year, over three million deaths world wide are attributable to diabetes related causes. The International Diabetes Federation (IDF) estimates that currently 194 million people world wide, have diabetes and this figure will rise to 333 million by 2025 (International Diabetes Federation 2004). This rise is proportional with the diabetes complications such as risk of acidosis, hypoglycemia or worse post-operative prognosis, higher predisposition to infections, prolonged wound healing and recovery. The economic burden of diabetes is substantial, as it currently account for an average of around 8% of total heath care budgets in developed countries. Diabetes complications not only reduce quality and length of life but are also responsible for enormous heath care costs. The war against diabetes through the development of new drugs is an ongoing continuous process. Unfortunately, the speed with which our knowledge of diabetes and its effects is expanding is not matched by the availability of new drugs. As human insulin, is more expensive than animal insulin, this is unaffordable by people in many developing countries. The drawback of animal & human insulin is development of antigenicity with its continuous use. As an alternative used of herbal medicine started to take over animal vaccines.

Plants have always been a good source for drugs and many of currently available drugs are derived directly or indirectly from them. Anti-diabetic activity has been shown by few plants (Saifi et al. 1971; Mukherjee et al. 1972; Coimbra et al. 1992; Ajit kar et al. 1999). The active principles of anti-diabetic plants are alkaloids, glycosides,
galactomannan, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions (Wang et al. 2005; Jung et al. 2006; Zhang et al. 2000).

For centuries, native peoples have harvested *S. platensis* from Chad Lake in Africa and Texcoco Lake in Mexico for use as a source of food (Vonshak 1997) because *Spirulina* possess wide range of essential nutrients of high nutritional values such as provitamins, minerals, proteins and polyunsaturated fatty acids such as gamma-linolenic acid (Miranda et al. 1998). Later *Spirulina* has been studied extensively due to of its therapeutic properties like- antioxidant (Estrada et al. 2001), anti-viral (Hayashi et al. 1996; Gustafson et al. 1989), anti-cancer (Babu 1995; Schwartz et al. 1988), hypocholesterolemic compound (Nakaya et al. 1988). Presently *Spirulina* is recognized as one of the biotechnological important microorganism. Among the cyanobacteria it ranks at first place in commercial ventures. The United Nations world food conference declared *Spirulina* as “the best for tomorrow”, and it is gaining popularity in recent years as a food supplement (Kapoor and Mehta. 1993). Therefore, present study *Spirulina* was selected a priority.

5.1 Screening of *Spirulina* for Insulin like Protein

During screening of 23 *Spirulina* strains for insulin like protein by ELISA technique utilizing anti-human insulin antibody, only 16 strains gave positive results. Their concentration values ranged from 2.373-33.935 µg g⁻¹ and maximum quantity of insulin like protein (33.9 µg g⁻¹) was found in *S. platensis* CFTRI, Mysore (Fig 4.1; Table 4.1). The quantitative comparision of the data of present study could not be made with any
bioresource due to lack of such informations in the available literature except one report, where it is quantified in the developing fruits of cowpea (*Vigna unguiculata*) by ELISA using a guinea pig anti-human insulin antibody and the reported concentration of insulin was 10.84±0.7 µg per fruit (Venancio et al. 2003).

After the discovery of the peptide hormone insulin was in 1921 from the pancreas of dogs (Banting and Best 1922) it was suggested that a hormone analogous to insulin must be present wherever glucose is metabolized, i.e., it might be present in plants (Best and Scott 1923). Since then, many scientists found insulin like antigen from plants by using different methods of identification like ELISA assay, immunolocalization method and hypoglycaemic activity in animal models. Collip (1923) who had developed the method for the extraction of insulin from pancreas, also obtained an active extract from green wheat leaves and ordinary lawn grass. Best and Scott (1923) also got success in reporting the presence of insulin-like materials from germinating potatoes and rice. In 1970s Khanna and collaborators (1974 & 1976) reported presence of insulin from the fruits of *Momordica charantia* (bitter gourd). In addition, materials resembling insulin were described in spinach, rye and *Lemna gibba*, which were recognized by broad spectrum anti-pork and anti-chicken insulin antibodies. (Collier et al. 1987).

Silva et al (2002) extended their work to lower plants also and reported insulin like proteins from aerial parts of mosses, whisk ferns (*Psilotum nudum*), *Selaginella, Equisetum, Spirulina maxima, Gracilariopsis*, gymnosperms, including monocots and dicots angiosperms using modified ELISA and Western Blotting.
5.2 Characterization of insulin like protein

5.2.1 SDS-PAGE

Identification of extracted insulin like protein was done by SDS-PAGE using bovine insulin as marker with a molecular mass of approximately 6 KDa. A protein band could be detected in each lane at the same position as a bovine insulin [Fig. 4.2.1 (a), & Fig. 4.2.1(b)]. Insulin like protein isolated from different strains of *Spirulina* showed electrophoretic mobility identical with other insulin sources as follow

1. **Animal Sources:**

   Human insulin (5.8 KDa), Bovine insulin (5.7 KDa)

2. **Higher Plant Sources:**

   *Phaseolus vulgaris* (5.7 KDa) (Silva et al. 2002),

   *Spinacea oleracea* and *Lemna gibba* (6 KDa) (Collier et al. 1987),

   *Vigna unguiculata* (6KDa) (Venancio et al. 2003),

   *Bauhinia variegate* (5.7 KDa) (Azevedo et al. 2006),


3. **Lower Plant Sources:**

   *Spirulina maxima* (5.7 KDa) (Silva et al. 2002),

   *Gracilariopsis sp* (5.7 KDa) (Silva et al. 2002),

   *Saccharomyces cerevisiae* (5.7 KDa) (Silva et al. 2002).
5.2.2 Western blotting

During western blotting, SDS-PAGE gels of all 16 strains that tested positive for insulin like protein were treated with the anti-insulin antibody, only in six lanes a band(s) was observed at the same position as bovine insulin [Fig. 4.2.2 (a) Lane no. 2, 3, 4 and 8; Fig. 4.2.2 (b) Lane no. 2 and 5]. These lanes correspond to S. platensis CFTRI, Mysore, Spirulina NCCU- 482, Spirulina NCCU- 483, S. platensis S5, Spirulina NCCU- 481 and A. maxima (SAE-49-88). In rest of the strain (lanes) bands could not be observed probably because of low concentration of insulin like protein.

Insulin extracts from Spirulina maxima, Saccharomyces cerevisiae, Gracilarioopsis sp and Phaseolus vulgaris also showed similar immuno reactivity (Silva et al. 2002). Similarly, when SDS band of Vigna unguiculata and Bauhinia variegata were transferred to nitrocellulose membrane and treated with the anti–insulin antibody, a band at the same position as insulin (6 kDa) was observed (Venancio et al. 2003; Azevedo et al. 2006).

5.2.3 RP-HPLC

Insulin isolated from S. platensis CFTRI, Mysore, S. platensis S5, Spirulina NCCU- 482, Spirulina NCCU- 483, A. platensis (Bellarpur), A. maxima (SAE-25780), A. maxima (SAE-49-88), Spirulina NCCU- 481, Spirulina NCCU- 477 and bovine insulin (Standard) during RP-HPLC characterization presented similar chromatographic behavior in terms of retention time [Fig.4.2.3 (a)-(i); Table 4.2]. Retention time of isolated insulin like protein ranged from 11.428 to 11.55 min where as bovine insulin standard showed 11.33min. In our HPLC findings peak(s) of Spirulina strains and
bovine insulin standard were at the same position. Our findings are similar to the other findings. Collier et al. (1987) showed similar retention time of *Spinach, Lemna gibba* G3 protein and swine insulin by HPLC (eluted on C-3 column). Venancio et al. (2003) isolated insulin-like material from cowpea fruits and compared with commercial bovine insulin by HPLC. These two also presented similar chromatographic behaviour, with retention times close to 31 minutes, at a C4 column. Richard and Anderson (2004) found similar HPLC peak as that of insulin in *Cinnamon*. Azevedo et al, (2006), showed that the insulin-like material isolated from cowpea (*Bauhinia variegate*) leaves and commercial bovine insulin have similar chromatographic behaviour at 280 nm.

**5.2.4 Circular Dichroism**

The highest amount of insulin like protein was found in *S.platensis* CFTRI, Mysore and thus it was selected for Circular Dichroism (CD) spectra based qualitative analysis. For this CD spectra of *S.platensis* CFTRI, Mysore insulin like protein was taken on JASCO J-715 Spectropolarimeter in the far UV region maintained at pH 7 and 25 °C and compared with standard bovine insulin. The CD spectrum of the both showed the dip (negative maxima) at 222 and 208nm, a characteristic of the α-helical protein (Fig.4.2.4). Submission of data to K2D software showed 33% α-helical content in bovine insulin and 30% α-helical content in *S.platensis* CFTRI, Mysore. To the best of our knowledge there is no report of qualitative characterization (by Circular Dichroism) of insulin like protein in plants, though it is a very frequently used technique for physical characterization of proteins.
5.2.5 MALDI-TOF

The amino acid sequence of isolated insulin like protein from *S. platensis* CFTRI showed only partial identity with bovine insulin. 3 residues (44 GER 46) of bovine insulin matched with 3 residues (126 GER 128) of *S. platensis* CFTRI which might be responsible for immunoactivity in ELISA and Western Blotting. Insulin like protein extracted from *Bauhinia variegata* leaves (cow’s foot) showed only partial amino acid sequences identical with bovine insulin (Azevedo 2003). Among the plant insulin protein so far, studied only *Canavalis ensiformis* (Jack bean) seed coat insulin and *Vigna unguiculata* (cow pea) showed sequence equal to that of bovine insulin (α and β chain) (Oliveira et al. 1999; Venancio et al. 2003). Silva et al. (2002) could not find any sequences related to vertebrate insulin from extracts of *Spirulina maxima*, *Saccharomyces cerevisiae*, *Gracilariopsis* and *Phaseolus vulgaris* tested positive for insulin like protein in the plate assay and gave a positive result in western blot. *Momordica charantia* (which is reported to have hypoglycaemic effect) insulin like protein, but its sequences were not identical with bovine insulin (Khanna et al. 1981). The sequence of insect insulin like antigen bombyxinA2 proved that it was definitely an insulin-like peptide. It contained the A and B peptide chains crosslinked by invariant disulfide bonds found in all insulin-like molecules and exhibited 30% sequence identity to human insulin (Nagasawa et al. 1986).

5.3 *In-vivo* studies

Animals exhibiting a syndrome of insulin resistance and type 2 diabetes, with characteristics similar to humans, have been used extensively in diabetes research. Early
In the present investigation, Streptozotocin (STZ) was used to induce diabetes in experimental animals. It is a naturally occurring nitrosourea product of *Streptomyces achromogenes* and interferes with cellular metabolic oxidative mechanisms (Papaccio et al. 2000). Intraperitoneal administration of streptozotocin (45 mg kg\(^{-1}\)) effectively induced diabetes in normal rats, as reflected by hyperglycemia, polyphagia, polydipsia and body weight loss when compared with normal rats.

STZ-induced diabetic rats showed increased body weight after their treatment with *Spirulina* (crude extract, aqueous extract, ethanolic extract and insulin-like protein). As far as relative efficacy in increasing or maintaining body weight is concerned, crude extract and insulin-like protein of *Spirulina* seem to be more promising than their aqueous extract and ethanolic extract [Fig 4.3 a (i); Table 4.3 (a)]. However, the ethanolic extract was found to be slightly more effective than the aqueous extract which may be due to higher solvent extraction capacity of ethanol rather than water. Increase in body weight (8.41% - 16.41%) may be the reflection of the improved health of the *Spirulina*-treated diabetic animals. Increases in the body weight in *Spirulina maxima* treated diabetic rats was also reported by Layam et al. (2006) and Pandey et al. (2011).

From the results obtained, it is obvious that the *Spirulina* statistically decreased the blood glucose concentration significantly in STZ induced diabetic rats, which probably
function like insulin or stimulate the \(\beta\) cells of islets of Langerhans to increase the output of insulin that result in lowering of blood sugar level. Oral administration of crude extract and *Spirulina’s* insulin-like protein reversed the diabetic effects and their glucose level decreased from 260.450 ± 3.394 to 126.367±2.736, 260.450±3.394 to 131.567±1.584 \((p<0.0001)\) [Fig. 4.3 a (ii); Table 4.3 (a)]. In another study the oral administration of 15 mg kg\(^{-1}\) *Spirulina* for 45 days to diabetic rats significantly reduced the blood glucose level (Layam et al. 2006). Not only this, 2 g/day *Spirulina* supplementation to human (patients) for 21 days also showed a significant decrease in their fasting blood sugar level (Mani et al. 2000).

The possible mechanism by which *Spirulina* brings about its antihyperglycemic action may be through potentiation of the pancreatic secretion of insulin from islet \(\beta\)-cell or due to enhanced transport of blood glucose to the peripheral tissue. Generation of peptides and polypeptides by the digestion of *Spirulina* proteins or due to high fibre content in *Spirulina* that interfered with the absorption of glucose (Mani et al. 2002).

It was reported that during diabetes, the excess blood glucose reacts with hemoglobin to form glycosylated hemoglobin (HbA\(_{1C}\)) (Koening et al. 1976). Therefore, the level of HbA\(_{1C}\) is always monitored as a reliable index of glycemic control in diabetes (Gabbay 1976). Administration of crude, aqueous extract, ethanolic extract and insulin-like protein of *Spirulina* brought back the elevated HbA\(_{1C}\) levels (1.065 to 0.663 mg g\(^{-1}\)) to near normal levels (0.573 mg g\(^{-1}\)) \((p<0.0001)\) [Fig. 4.3 a (iii); Table 4.3 (a)]. Decrease in HbA\(_{1C}\) it suggested due to its (*Spirulina*) being a good source of iron.
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Diabetes mellitus is associated with increased activity of Liver Functional Test (LFT) related enzymes. In present study also STZ-induced diabetic rats showed increased activity of SGOT (28.522 to 108.32 UL\(^{-1}\)), SGPT (54.342 to 90.692 UL\(^{-1}\)), ALP (109.583 to 240.963 UL\(^{-1}\)) and Bilirubin total (0.773 to 3.4 mg dL\(^{-1}\)) [Fig. 4.3 b (i), (ii), (iii), (iv); Table 4.3 (b)]. *Spirulina* treated (crude extract and insulin-like protein) diabetic rats resulted in decrease of SGOT, SGPT, ALP and Bilirubin total levels (\(p<0.0001\)). The restoration of SGOT and SGPT activities to their respective normal levels after administration of *Spirulina* showed hepatoprotective effect of this alga. The presence of antioxidants eg. \(\beta\)-carotene, phycocyanins in *Spirulina*, helps in boosting the hepatoprotection.(Becker et al. 1986).

In the present study, diabetic rats also show renal dysfunction, i.e. increased serum creatinine (1.132 to 2.782 mg dL\(^{-1}\)), uric acid (5.777 to 10.307 mg dL\(^{-1}\)and blood urea (16.983 to 27.195 mg dL\(^{-1}\)) reflecting a decline in the glomerular filtration rate. In diabetic rats *Spirulina* treatment resulted in decreased level of serum creatinine (2.782 to 1.380 mg dL\(^{-1}\) with crude extract and 2.782 to 1.338 mg dL\(^{-1}\) with insulin like protein), uric acid (10.307 to 6.582 mg dL\(^{-1}\) with crude extract and 10.307 to 6.343 mg dL\(^{-1}\) with insulin like protein) and blood urea (27.195 to 19.708 mg dL\(^{-1}\) and 27.195 to 19.047 mg dL\(^{-1}\) with insulin like protein) [Fig. 4.3 c (i), (ii), (iii); Table 4.3 (c)]. It may be possible, that *Spirulina*, due to its potential antioxidant properties, improved renal function via attenuating oxidative stress- mediated decline in kidney function.

**5.4 Growth phase and Insulin content**

In order to find out which phase of the growth is best for insulin yield, the growth was measured in terms of protein every 3\(^{rd}\) day until 18 days, along with insulin
determination. A direct correlation between growth and insulin content of *Spirulina* was observed until 12 days [Table 4.4 (a), (b)], then both declined suggesting that late log phase is the ideal condition for insulin isolation from *Spirulina*.

5.5 Culture condition manipulation

5.5.1 Nitrogen (NaNO₃)

In general sodium nitrate enhanced the growth (as protein) and insulin like protein in concentration dependant manner. Highest growth (651 mg g⁻¹) and insulin like protein (41.44 µg.g⁻¹) were observed on 12th day in presence of 55mM sodium nitrate. This suggested that nitrogen play important role in growth as well as production of insulin like protein in *S.platensis* CFTRI Mysore [Fig 4.5.1 (a), (b); Table 4.5.1 (a), (b)]. Nitrogen is required for synthesis of the amino acid, which make up proteins and other value added substances (Raven et al. 1992, D’Souza et al. 2000).

5.5.2 Phosphorus (K₂HPO₄)

The increase in growth (641.79 mg g⁻¹) and insulin like protein (35.20 µg.g⁻¹) were observed under different concentration of phosphorus [Fig. 4.5.2 (a), (b); Table 4.5.2 (a), (b)]. As phosphorus is a major nutrient required for the growth of alga and determines its primary productivity. Mostert and Grobbelaar (1987) have indicated the essential role of phosphorus in maintaining high production rates of microalgae mass cultures. The major form in which algae acquire phosphorus is as inorganic phosphate, either as H₂PO₄ or HPO₄⁻ (Faintuch et al.1991).
5.5.3 Bicarbonate (NaHCO₃)

At 180 mM concentration of NaHCO₃ increase in growth (651.43 mg g⁻¹) and insulin like protein (35.90 µg.g⁻¹) were observed [Fig. 4.5.3 (a), (b); Table 4.5.3 (a), (b)]. *Spirulina* has a high bicarbonate requirement, which acts not only as a carbon source but helps to maintain alkaline conditions. During *Spirulina platensis* cultivation addition of bicarbonate to the culture medium gradually produces CO₂ that increase the pH (Richmond and Grobbelaar 1986). However, beyond optimum condition increase in bicarbonate/pH acts as an autoinhibitor of cell growth (Richmond 2000).

5.5.4 Sulphur (K₂SO₄, MgSO₄ and FeSO₄)

All sulphur sources enhanced the growth (as protein) and insulin like protein in concentration dependant manner. Highest growth (657 mg g⁻¹) and insulin like protein (42.77 µg.g⁻¹) were observed on 12th day in presence of K₂SO₄ (8.5 mM) + MgSO₄ (1.05 mM) +and FeSO₄ (0.055 mM) [Fig. 4.5.4 (a), (b); Table 4.5.4 (a), (b)]. Sulphur is an essential constituent of certain essential amino acids, vitamins and sulpholipids, hence, is also essential for growth of *Spirulina*.

5.5.5 Calcium (CaCl₂)

In general CaCl₂ enhanced the growth (as protein) and insulin like protein in concentration dependant manner. Highest growth (642.67 mg g⁻¹) and insulin like protein (36.00 µg.g⁻¹) were observed on 12th day in presence of 1.2 mM CaCl₂ [Fig. 4.5.5 (a), (b); Table 4.5.5 (a), (b)]. Calcium is involved in various metabolic functions and is a component of many cellular components. Calcium induces has also been reported in human beings where it triggers INS gene promototers which response
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towards glucose increment in blood and is also involved in degranulation of granules possessing insulin in β-cells of pancreases.

5.6 Optimization for growth (as protein) and insulin like protein

5.6.1 Glucose (Anhydrous dextrose)

In general growth and insulin like protein were not much attend in presence of glucose. At 10mM of glucose, growth (644 mg g⁻¹) and insulin like protein (35.66 µg g⁻¹) were enhanced till 12th day. [Fig. 4.6.1 (a), (b); Table 4.6.1 (a), (b)]. Vonshak et al. (2000) also reported that *Spirulina platensis* grows faster and achieved a higher biomass concentration with the need for less light in mixotrophic culture than in photoautotrophic culture. Although, most microalgae are photoautotrophs, some microalgae can use organic carbon substances as the sources of energy and carbon for cell growth. Mixotrophy is growth in which organic carbon is assimilated in the light simultaneously with carbon dioxide fixation. A considerable number of algae, for example *Chlamydomonas* (Lalibertè and de la Noüé 1993), *Spirulina* (Marquez et al. 1993), *Chlorella* (Endo et al. 1977), *Galdieria* (Gross and Schnarrenberger 1995), *Scenedesmus* (Abeliovich and Weisman 1978), and *Micractinium* (Bouarab et al. 2004); can grow mixotrophically and heterotrophically in the presence of organic matter such as carbohydrates.

5.6.2 Selenium (Na₂Se₂O₃)

On 12th day though was not much affected till 0.3mM selenium but the insulin was increased (8.94%) [Fig. 4.6.2 (a), (b); Table 4.6.2 (a), (b)], this may be due to selenium induced oxidative stress. Selenium is also a part of many Selenium-dependent enzymes
such as Glu-peroxidase. High Selenium depend inhibition of the biomass and growth rate was also reported by Chen et al. (2005).

5.6.3 pH

During present study effect of pH (8, 10, 12) was observed on growth (as protein) and insulin like protein. Maximum growth and insulin like protein were observed at pH 10 as compared to control. [Fig. 4.6.3 (a), (b); Table 4.6.3 (a), (b)]. Belkin and Boussiba (1971) also demonstrated that optimum pH (9 to 9.5) for growth of *Spirulina platensis*. 