Summary & Conclusion
Food and energy security are the two major challenges being extensively discussed globally at present. Though unprecedented success has been made in the agriculture sector, there is a need for developing alternative food and energy sources for future sustainability. Seaweeds have a long history of being utilized in diverse applications for the welfare of mankind. Seaweeds have long been recognized as valuable food source because of their biochemical constituents with proven nutritional value. There has been a greater awareness worldwide especially in the past one decade about the positive effects of seaweed foods in human health. Another emerging application gaining considerable importance worldwide is use of seaweeds as sustainable feedstock for bioenergy with co-production of commercially important bio-products such as proteins, carbohydrates, minerals, lipids, pigments etc. The increased utilization of seaweeds has led to farming even in developing countries like India. The attractiveness of farming will be further aided possibly with selection/development of seaweed strains with desired agronomic traits such as growth, nutrition and hydrocolloid content. Though the importance of selective breeding in seaweeds was clearly stated by van der Meer in 1990, the fruits of these efforts are yet awaited. Somatic hybridization by protoplast fusion is an alternative approach which merits the potential of mixing of two genomes irrespective of their genetic distance and sexual incompatibility barrier. Though 1980s witnessed a spurt in research on protoplast isolation, regeneration and fusion, there is no evidence of somatic hybrid development till date and this area of research remained abandoned for the past one decade. The present study is an attempt to renew the interest of researchers towards this classical technique which has made unprecedented success in agriculture by developing various varieties of major agriculture crops with improved traits. The major technical constrains before making attempts for protoplast fusion in seaweeds are:

1. Optimization of protoplasts isolation from different species.
2. Differentiation of protoplasts to whole plant.
3. Genotyping of parent intend for protoplast fusion.
4. Determination of heterotic traits.
5. Basis for evolution of heterotic traits resulted from genetic recombination.
Protoplasts isolation and regeneration was successfully accomplished from various seaweed species belonging to the genus *Ulva* with viability and regeneration rate >90%. Protoplast isolation was also optimized for different agarophytic species such as *Gracilaria dura* and *G. verrucossa* using various combinations of commercial enzymes. But the isolated protoplasts were failed to regenerate. Therefore an attempt was made to isolate protoplasts using a crude enzyme preparation of agarase isolated from a bacterium endophyte to *G. dura*. Previous studies have shown isolation of viable protoplasts from agarophytic species using crude enzyme prepared in the laboratory. An extracellular exo-β-agarase was characterized from an endophytic bacterial strain *Pseudomonas* sp. isolated from the red alga *Gracilaria dura*. Using this enzyme along with other enzymes like cellulase and pectinase protoplasts were isolated but yields were inferior to that of the same obtained from commercial enzyme combination. Though protoplasts yield was inferior, some protoplasts showed cell wall regeneration and division up to 4 cell stage only. There is no full plant regeneration from protoplasts despite the best efforts. It is presumed that agarophytes being anatomically complex lacks regeneration potential when disassociated into single cells like protoplasts.

Unlike higher plants, seaweed protoplasts regenerate and differentiate into a full thallus without any amendments of phytohormones to culture medium. Nevertheless, the protoplasts from green seaweeds followed different types of regeneration patterns and gave rise to a several phenotypically variable morphotypes such as sporangia, microthalli, saccate (or spherical), tubular (or spindle), irregular, and frondose. This developmental polymorphism may be misleading while identification of hybrids at morphological level. In this study protoplast-derived polymorphic development was observed for species *U. reticulata* which showed two developmental patterns (1) normal reticulate thalli (2) hollow disc like structure. The regeneration of protoplasts into different morphotypes as a result of epigenetic variations was discussed for the first time in this study. The present study reveals the distinct expression of cytosine methylation and is thus correlated to differential morphogenesis of plants regenerated from cultured cells. The hypermethylation condition was apparent in the disc type thalli with methylation ratio of 72.97% compared to that of normal filamentous thalli with 67.56%. Therefore frequency and distribution of DNA methylation is shown to
be an important factor that perhaps regulates the morphogenesis of protoplast-derived regenerants.

Another hindrance towards the success of genetic improvement program in seaweed is high genetic diversity which leads to miss-identification of cultivars. The most recent example is that the most cultivars of *Porphyra* in Asia after extensive genetic analysis were shown to be species of the genus *Pyropia*. Therefore, there is a need to confirm the genetic identity of the species. Considering this fact, genetic identity of *Ulva* species employed for protoplast fusion and development of hybrids was confirmed by different molecular markers. For this four new molecular markers (*accD, ndhJ, rpoC* and *ycf5*) were used along with the three most utilized markers (*nrDNA ITS, rbcL* and *tufA*). Yet another advantage of these markers is to characterize the inheritance of extra-nuclear genome so as to confirm the genetic recombination if any at organelle level.

The major advantage of hybridization is to realize the heterosis in developed genotype. Heterosis can be determined as growth, stress tolerance and improved nutritional and biomass characteristics. Though a few studies on seaweed protoplast fusion have characterized improvement in growth and biochemical composition, the basis for heterosis is not been studied. Therefore, in this study, endogenetic phytohormone analysis and metabolic composition were selected to evaluate the biochemical basis for heterosis. Along with it epigenetic regulation as change in DNA methylation was considered as molecular basis for heterosis. To accomplish these biochemical and molecular basis for heterosis, protocols were developed for quantitative analysis of endogenetic phytohormone using HPLC and metabolome analysis by NMR spectroscopy. The plant growth regulators determined includes gibberellic acid (*GA_3*), indole-3-acetic acid (*IAA*), abscisic acid (*ABA*), indole-3-butyric acid (*IBA*), salicylic acid and kinetin riboside (*KR*). The parameters optimized for distinct separation of PGRs were mobile phase (60:40 methanol and 0.6% acetic acid in water), column temperature (35°C) and flow rate (1 ml/min). In comparison to earlier methods of PGR analysis, sample preparation and analysis time were substantially reduced while allowing determination of more classes of PGRs simultaneously.
A facile and tractable method suitable for metabolite profiling in marine macroalgal species was also developed and validated against the one commonly employed for terrestrial plants. The method developed distances the need of deuterated solvents as the spectra were acquired directly on water extract. Spectral information from both aqueous and solvent extract was found similar. Interestingly, the spectral profile of the aqueous extract remained unchanged after an incubation of 12 h at 20 °C thereby overcame the stability issue. The untargeted metabolite information generated for the first time for marine macrophytic algae provided newer insights into the key aspects about their functionality and biochemical regulations. The identification of lignin precursors in green alga *Ulva lactuca* supports the hypothesis of conserved evolution of lignification trait in terrestrial plant from aquatic algal ancestors. Also, biochemical regulatory mechanisms including non-neuronal cholinergic system and sulfinic acid switch for redox signalling were determined.

After establishment of different protocols, protoplast fusion was successfully accomplished at both inter-/intra generic levels. Electric field mediated protoplast fusion was carried out and the fusion was confirmed based on the increase in size and presence of two different chloroplasts in fused cell. Various lines of evidences including morphological, cytological, molecular, and biochemical characterization was carried out to confirm the genetic introgression in the regenerated hybrids. Hybrids were then analyzed for improvement in functional traits like growth, thermal tolerance, proximate biochemical composition. The results revealed either heterosis or mid-parent heterosis for growth, thermal tolerance and other proximate composition analysis. The genotype obtained from protoplast fusion at intragenus level showed DGR (%) in the order of hybrid 3 > *U. reticulata* > hybrid 2 > hybrid 1 > *U. fasciata*. The fatty acid (FA) profile revealed an interesting result with higher amount of essential PUFAs in the regenerated hybrids compared to the parent plants. The proximate composition analysis revealed improvement in the traits compared to the parent having inferior traits and at some instances better than both the parents.

The hybrids regenerated from protoplast fusion at intergenus level showed growth rate either equal or higher than mid-parent value but none of the hybrid showed growth better than both the parents. Further, the proximate composition
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analysis showed that all the hybrids have the carbohydrate content average of both the fusing partners except for hybrid 9 having this inferior to mid-parent value. The protein content of hybrid 1 was found to be higher than the average of both the fusing partners while others showed protein content higher than the parent with inferior trait. Further, hybrid 1, 6 and 9 showed lipid content higher than the average while others showed lipid content almost double to the content of parent with inferior trait. FA profile revealed that there was an increase in unsaturation in all the hybrids as compared to both the parents except hybrid 6. Further, the content of C18 PUFAs was higher than both the parents with the only exception of hybrid 6. Among MUFAs, both C17:1 (n7) and C18:1 (n9) showed the traits of heterosis (superior hybrids than both the parents) while C16:1 (n9) showed heterosis in three hybrids (4, 6 and 9) while three hybrids (1, 2 and 8) had the traits of mid-parent heterosis. Similarly, among PUFAs, C18:4 (n3) (except hybrid 6) and C22:6 (n3) showed higher contents than both the parents.

In conclusion, the present study for the first time successfully established the genetic and biochemical distinctness of intra- and inter-generic somatic hybrids of Ulva following the protoplast fusion. The genetic recombination of species led to develop seaweed varieties with improved growth rate and proximate composition. The hybrids with improved growth rate and biochemical constituents can be exploited for aquatic biomass programme aimed at meeting the both food and energy security. Also, large scale farming of such improved seaweeds with higher growth rate in the sea can mitigate the global warming and climate change effects on the biosphere.