8.0. GENERAL DISCUSSION

The natural energy resources such as fossil fuel petroleum and coal are being rapidly utilized worldwide. The utilization of these resources leads to increase the green house gases in the environment. The alternative energy is needed to replace the currently used fossil fuels. Several energy sources such as, ethanol, methane and hydrogen are being produced worldwide. Among them, the bioethanol production by fermentation has received special attention to solve world energy crisis (Ward and Singh, 2006). Although in its industrial infancy, bioethanol is one of the largest volume biofuel produced worldwide through a biochemical conversion from the renewable resources such as, starch, sugar crops, cornstarch, sugarcane, wheat straw and sorghum through microbial fermentation. But, rising prices for the above mentioned resources have had a major impact on the cost. Moreover, the ethanol production from the agricultural crop is unsustainable in the long term because of it clash with the food production for human use and it doesn’t significantly diminish the green house gases (Farrell et al., 2006). Generally, the yeast and bacteria are potential organisms which produce bioethanol as the main fermentation product. According to Saucedo-Costaneda et al. (1992 a & b), the yeast can be used for the ethanol and food production and its fermentation has been well progressed in recent years. However, the bacteria are mainly involved in
composting, ensiling and some food processors. The ocean covers more than 70% of the earth surface. The marine environment is frequently recognized as the largest potential sources of biodiversity such as seaweeds, seagrass, invertebrates and microbes which proved their potential in several fields. Among the biological sources, seagrasses are one of the rich biological resources which furnish the protection to the several marine organisms and used in many ways to human being. They also control the habitat complexity, species diversity, abundance of associated invertebrates and thereby shaping the structure of marine communities. Moreover, they contribute significantly to the coastal productivity of both temperate and tropical waters. Several activities are responsible for the seagrass detachment from the substratum. The accumulation of beach cast is a result of the interaction between dense near shore seagrass meadows and physical factors such as; wind, tide and currents. The accumulations of beach cast create the unpleasant odour along the coastal areas and also give the anoxic condition to the marine organisms. Generally, the marine yeasts produced potential enzymes than the other microbes and there may be a chance to breakdown the disaccharides and polysaccharides also. It has been reported that, *Saccharomyces degradans* was able to degrade many complex polysaccharides including cellulose, pectin, xylan and chitin (Weiner *et al.*, 2008). The marine yeast strain *Cryptococcus aureus* produce inulinase
enzyme and it was used to hydrolyze the inulin for the biofuel production (Sheng et al., 2009). In this connection, the present study has been made an attempt to utilize the deposited seagrasses for possible bioethanol production by fermentation through marine yeast. Moreover, this seagrass renewable source has frequently available along the coastal area of Palk Strait which is also economically feasible.

In this regard, the present study isolate 11 morphologically different marine yeasts from semi-decayed seagrass leaves in the Palk Strait region. Similarly, Chen et al. (2009) also isolated 109 cultures from seawater in China. A novel yeast species; Kazachstania jiainicus was isolated from forest soil in Jiain, Hualein Eastern coast of Taiwan (Lee et al., 2008). Ping et al. (2006) reported that, 327 yeast strains were isolated from seawater and sediments in China. The number of isolates varies from different regions which might be due to geographical, oceanological or biological factors (Chen et al., 2009).

The present study also made an attempt to assess the morphological and biochemical characteristics of isolated yeasts to differentiate among the isolates. Morphological and biochemical characteristics may be used to characterize and differentiate the isolated yeasts (Khan et al., 2000). All the isolates seems to be similar in forms and margin with some differences
between the elevation, colour and size of the colony. The ethanol stress is probably one of the most interesting conditions to be analyzed due to high amount of this substance during the fermentation process. The ethanol tolerance test suggested that, all the isolates showed active growth in various concentrations of tested alcohol levels \textit{viz.}, 100 ml.l\(^{-1}\), 130 ml.l\(^{-1}\) and showed moderate growth at 150 ml.l\(^{-1}\). The high ethanol tolerance might be due to the plasma membrane phospholipids which increases in membrane unsaturated fatty acids and thus helps to survive in high ethanol concentrations. Moreover, the physiological factors such as mode of substrate feeding, intracellular ethanol accumulation, temperature and osmotic pressure all contribute to the ethanol tolerance of yeast. In addition to that, the number of different genes is also involved in the ethanol tolerance mechanism (D'Amore and Graham, 1987). Guimaraes et al. (2006) isolated 61 yeasts from grapes, among them 15 isolates showed the active growth at low concentration of 100 g.l\(^{-1}\) and showed moderate growth at the higher concentration levels.

Flocculation is an important characteristic that allows an easy separation of the cells from the fermentation broth. In the present study, none of the isolates showed active settlement. However, nine isolates showed the moderate settlement and this might be due to the cell aggregation. The flocculation is a non-sexual cell aggregation in which cell
adheres to each other to form flocs and the flo genes are responsible for the yeast flocculation (Stratford, 1989). Guimaraes et al. (2006) reported that, only one isolate has active settlement among the 15 isolates tested. Similarly, Khan et al. (2000) also reported that, only one isolate has active settlement among 30 isolates screened.

The stress induced test suggested that, all the isolates except YPD-11 grew 24 h and 48 h after exposure to -4°C and 55°C respectively. Hence, the isolated marine yeasts have the better ability to survive the excessive heat as well as cold stress treatments and this could be attributed due to heat shock and cold shock genes are responsible for the stress tolerance of the isolated yeasts (Lindquist and Kim, 1996; Rodriguez-Vargas et al., 2002). Khan et al. (2000) reported that, none of the isolate grew at 24 h but they grew at 48 h after 55 °C exposure and all the isolates grew at 24 h after -20°C exposure.

The temperature is one of the prime factors that mainly affect the yeast metabolism during the fermentation. Hence, the present study has made an attempt to find out the temperature tolerance capability of the isolate marine yeasts. All the isolates showed active growth in all the tested ranges except 45°C. This result agrees with the previous reports of Guimaraes et al. (2006).
The present study also made an attempt to find out the total biomass of the seagrasses along the Palk Strait region throughout the year from September 2011-August 2012. The maximum biomass was recorded during the month of December-2011 at Thondi and Manamelkudi which might be due to the sedimentation pattern and environmental parameters of the study sites. Clara et al. (2001) reported that, the higher biomass in Puerto vargas could be attributed to the sediment structure and environmental condition. Estaction and Fortes (1988) reported that, higher biomass are due to the presence of liquid mud. However, the total biomass was not recorded in Kodiyakarai and this might be due to the coarse sand. This is agreeing with the previous results of Estaction and Fortes (1988).

The present study also made an attempt to assess the deposition of seagrasses Cymodocea serrulata and Syringodium isoetifolium throughout the year from September 2011-August 2012. The maximum deposition of seagrass was identified during the month of May-2012. The beach cast deposition might be due to the Southern wind direction with the speed of 12/06 KmPH/Knots and low level of rainfall. It is therefore clear that, hydrodynamics play a major role in the process of detachment and deposition (Ochieng and Erftemeijer, 1999). Mc-Clanahan, (1988) reported that, the high amount of beach cast accumulation due to the wind speed. But, the minimum deposition was identified during the month of
November-2011 and this might be the low wind speed. In addition, none of the coastal area showed the seagrass deposition during the month of December and this could be due to the calm wind. However, the Kodiyanakrai coastal area, neither seagrass standing crop nor deposition was recorded throughout the year and this might be due to the coarse sandy particles.

The present study also made an attempt to utilize the seagrass for bioethanol production through fermentation process. Initially, the seagrasses were subjected for the pretreatment with dilute nitric acid (HNO$_3$). The reason behind is that, this pretreatment showed the maximum release of reducing sugar than the other pretreatment. Del campo et al. (2006) reported that, during the agri food waste pretreatment, most of the fermentable sugars released during the low acid concentration. Rodriguez-Chong et al. (2004) used 6% HNO$_3$ operated at 122°C for the sugarcane bagasse pretreatment process. Xiao and Clarkson (1997) reported that, the acidic acid and nitric acid used for the removal of lignin from waste newspapers. Lignocellulosic materials do not contain readily available monosaccharides for bioconversion. Instead of this, they contain polysaccharides such as cellulose and hemicelluloses. These polysaccharides can be converted in to simple sugars by means of acid, alkali and enzymatic treatments. Generally, the plants contain cellulose
associated with hemicelluloses and lignin. Hence, the pretreatment is required to partially remove the hemicelluloses and lignin to increase the porosity and internal surface area of the materials which enhance the fermentation process (Keller et al., 2003). Several pretreatment methods viz., dilute acid, con.acid, alkali, steam explosion, solvent extraction, thermal pretreatment and liquid hot water have been investigated for the partial removal of hemicelluloses and lignin. Among them, the dilute acid pretreatment is widely used because; the acid attacks the polysaccharides, especially hemicelluloses that are easily hydrolyzed than the cellulose. Moreover, the utilization of lower acid concentrations during pretreatment has reduced the amount of toxic, corrosive and hazardous chemicals. This reduces reactor corrosion and enforces environmental sustainability (Harun and Danquah, 2011).

The present study also made an attempt to find out the crystallinity of the untreated and pretreated samples through XRD analysis. The low CrI values indicate that, most parts of the samples were associated with an amorphous region. The increased CrI values indicate that, some of the non-crystallinity parts including hemicelluloses and lignin were removed during the pretreatment which increased the cellulose portion of the pretreated sample. It has been reported that, the crystallinity index has been increased in the corn stover with aqueous ammonia pretreatment (Kim and
Lee, 2005) and FeCl₃ pretreatment (Liu et al., 2009). Moreover, the rice straw with sodium hydroxide pretreatment also increased the crystallinity index values (He et al., 2008).

To understand the changes in the functional group of untreated and pretreated components, the present study also carried out the FTIR spectroscopic analysis. Eventhough the entire FTIR spectrum seems to be similar, compared with the spectrum band intensity of the untreated sample, the lower intensity was observed in cellulose rich fractions obtained after pretreatment. The lower intensities of the pretreated sample due to the partial removal of hemicelluloses and lignin. The newer peak formation and the removal of peaks suggested that, the HNO₃ penetrated into the interior of the cellulose rich fraction for the formation of new bonds or removal of any bond. Wang et al. (2009) reported that, the low intensity of the pretreated Lespedeza crytobotrya samples occurred due to the partial removal of hemicellulose and lignin.

To study the thermal behavior of the untreated and pretreated samples, the thermal gravity and differential thermal analysis were also carried out by the present study. From the TGA curve, the rapid decrease of weight was mainly from the thermal decomposition of unsteady hemicelluloses and partly from lignin of the samples. The main reason for
this degradation at higher temperature of pretreated sample is due to the lower content of hemicelluloses. Hemicelluloses are easy to be degraded to volatiles at relatively low temperatures due to their amorphous structure and their side chains reduce the aggregation of their molecules and inhibit the formation of hydrogen bonds (Bobleter, 1994; Yang et al., 2007).

The results of DTA curve reveal that, the peak >450°C might be due to the decomposition of lignin for its more resistance to thermal degradation. The reason for the high resistance of decomposition was probably due to the aromatic structures of lignin (Xie et al., 2009). Overall the thermal stability of pretreated samples was higher than that of the untreated sample. This suggested that, the pretreatment slightly increase the thermal stability of the sample, which was related to the partial removal of amorphous components.

In order to understand the structural changes on the surface of the seagrasses during the pretreatment, the pretreated and untreated samples were examined through scanning electron microscope (SEM). The surface of the sample was damaged severely and it has numerous shrinkages and the outer parts of the cell wall. Moreover, the compact structure was lost due to the partial removal of hemicelluloses and lignin. Hence, the partial defibrillation might be taken place in the pretreated seagrasses. The
significant changes after treatments were ascribed to the breaking forces of acid. Ming-Fei et al. (2010) reported that, the morphological changes were observed through SEM in the bamboo during the ultrasound treatment when compared with the untreated sample.

During the lignocellulose pretreatment, in addition to the sugars, aliphatic acids (acetic acid, formic acid and levulinic acid), furan derivatives (furfural and hydroxyl methyl furfural) and phenolic compounds are formed due to the lignin and carbohydrate decomposition (Klinke et al., 2004). Moreover, total phenolic compounds and other organic compounds are known to affect ethanol fermentation performances. The toxic materials could be either removed or transformed into inactive compounds by various physico-chemical treatments such as extraction, neutralization, overliming, evaporation and steaming stripping, adsorbent, adsorption and ion exchange resins (Vanzyl et al., 1988). Among them, overliming the hydrolyzate has been effective detoxification process due to the partial removal of toxic inhibitors such as furfural and 5-hydroxyl furfural and it is also economically feasible. In view of this, the present study also made an attempt to use the overliming for the removal of toxic compounds.

In the present study, the average total phenol detoxification percentage of the hydrolyzate reveals that, the maximum average
detoxification was recorded in the *Halophila ovalis* (40.7%) and *Halodule pinifolia* (40.7%), *Cymodocea serrulata* (39.3%) and *Syringodium isoetifolium* (38.8%) respectively. The low detoxification percentage might be due to the increased level of total phenolic compounds during the fermentation. The increased level of the total phenolic compounds mainly due to the plant polyphenols such as; tannins binding with sugar, protein, cellulose and starch forming glucosidic bonds. These glucosidic bonds are degraded by the acids produced during the natural fermentation and they yield the phenolic compounds (Landbo and Meyer, 2004). The metabolism of some microorganisms might also contribute to the production of phenolic compounds (Xia et al., 2011). Similarly, Martinez et al. (2000) reported that, 41% of the total phenolic compounds have been removed from bagasse hydrolyzate during the overliming detoxification method. However, the detoxification with Ca (OH)\(_2\) remove the phenolic compounds with 35.8% in HCl pretreated sugarcane bagasse hydrolyzate (Chandel et al., 2007). The phenolic compounds and other organic compounds are not removed efficiently by overliming (Cardona et al., 2010).

The present study also made an attempt for the production of bioethanol from the four different pretreated seagrass hydrolyzates by using the marine yeasts as well as the MTCC culture *viz.*, *Zymomonas mobilis* and *Saccharomyces cerevisiae*. Of these, the marine yeasts showed the
maximum ethanol production than the MTCC cultures. The maximum production of ethanol could be due the rapid utilization of multiple reducing sugars by the marine yeast. Bothast et al. (1999) reported that, the conventional ethanol fermenting yeast (Saccharomyces cerevisiae) or bacterium (Zymomonas mobilis) cannot ferment multiple sugar substrates to ethanol. Based on the fermentation duration, it is known that ethanol levels continued to increase by increasing the time of incubation.

The MTCC culture Zymomonas mobilis showed low level of ethanol production. The reason behind that, the low levels of ethanol production are due to aerobic conditions. However, the reduction of sugar tends to be used for energy and cell mass formation. In aerobic conditions, pyruvic acid is converted into acetaldehyde and then ethanol through pyruvate decarboxylase and alcohol dehydrogenase enzymes (Gunasekaran and Chandra, 2007), but it is converted into acetyl-CoA and enter into the Krebs cycle. Therefore, the levels of ethanol produced by Z. mobilis in aerobic conditions tend to be lower (Sulfahri et al., 2011). Z. mobilis ferments hexoses at very high rate under anaerobic conditions (Patle and Lal, 2008). In addition to that, the seagrass acid hydrolyzate contains different reducing sugars including pentose sugar xylose. Xylose is the major pentose sugar in the hemicellulosic fraction of biomass (Kumar et al., 2011). Generally, the yeast Saccharomyces cerevisiae also difficult to use xylose for
the fermentation because, it does not have the genes encoded with xylose reductase (XR) and Xylitol dehydrogenase (XDH) (Jeffries and Jin, 2004; Karhuma et al., 2007). Jeffries (1981) reported that, the yeast Candida tropicalis produce the ethanol from xylose under aerobic condition. Moreover, the yeast Candida sp. has the ability to convert the xylose in to ethanol (Abbi et al., 1996). Ukrit et al. (2009) reported that, the marine yeast Candida tropicalis have been used in the ethanol fermentation and bioremediation. Meng et al. (2009) reported that, the marine yeast Candida tropicalis has also been used in the biodiesel production. In addition, the decrease in ethanol production of Z. mobilis and S. cerevisiae from seagrass acid hydrolyzate medium might be due to high concentration of salts present in the medium that may have raised the osmotic pressure above the acceptable levels which suppressed the ethanol production (Doelle and Doelle, 1990). Hence, the production of ethanol was found higher in marine yeasts when compared with the MTCC microbes.

Among the marine yeast, the YPD-5 (Candida sp.) showed the maximum fermentation in Cymodocea serrulata hydrolyzate at 120 h (2.7 g.l\(^{-1}\)) and found minimum in Syringodium isoetifolium hydrolyzate at 72 h (1.5 g.l\(^{-1}\)). Patel et al. (2007b) reported that, the Saccharomyces cerevisiae showed the maximum (2.5 g.l\(^{-1}\)) ethanol production in wheat straw filtrate as a substrate and 2.2 g.l\(^{-1}\) with rice straw filtrate. Sulfahri et al. (2011) reported
that, the maximum level (2.18% v/v) of ethanol was produced by *Z. mobilis* with the *spirogyra* extract during the aerobic fermentation. *C. tropicalis* is known to produce ethanol from starch at a low rate as it produces glucoamylase (Nakamura, 1970). Patel *et al.* (2007a) also reported that, the *Saccharomyces cerevisiae* showed the maximum (8.5 g.l⁻¹ and 9.8 g.l⁻¹) ethanol production in the rice husk filtrate and bagasse filtrate at the fermentation time of 168 h. When compared with the above results, the ethanol values are found minimum and this might be due to the inhibitory effect of phenolic compounds that cannot be efficiently removed by overliming and that negatively affect the fermentation process. Similarly, Okuda *et al.* (2008) reported that, the low ethanol production from wood hydrolyzate might be due to the phenolic compounds. Moreover, Carvalho *et al.* (2005) also reported that, the conversion of sugars into un-fermentable compounds due to hydroxide catalyzed degradation reaction during the overliming.

The present study also made an attempt to observe the cell density during the fermentation. The cell growth was gradually increased and this might be due to the low level of weak acid and other toxic compounds (Huang *et al.*, 2011). The growth curve gives an overview of the environmental factors that influence the growth of a microorganism such as substrate, ambient temperature and pH (Hogg, 2005). According to Huang
et al. (2011), the main inhibitors of yeast particularly (S.cerevisiae) are weak acids but not furfural, hydroxy methyl furfural (HMF) and phenols. However, the bacteria are not sensitive to weak acids but it may be inhibited by some other toxic compounds. It has been reported that, the level of weak acids in hydrolyzate is an important factor for the production of ethanol from lignocelluloses (Cho et al., 2010). The cell growth decreased when the level of acetic acid is 0.7 g.l\(^{-1}\), whereas the ethanol production was not affected. On the other hand, both the cell growth and the ethanol production decreased severely in the presence of 7.1 g.l\(^{-1}\) of acetic acid (Phowchinda et al., 1995).

The present study also made an attempt to authenticate the most promising ethanol producing strains up to species level through 5.8S-ITS rRNA gene sequencing. The 5.8S-ITS rRNA gene sequencing analysis has been widely applied for the identification of yeasts (Kurtzman and Robnett, 1998). The evolutionary relationship of the marine yeasts was phylogenetically analyzed by neighbor joining method. It reveals that, all the potential strains viz., YPD-1, YPD-2 and YPD-4 showed the maximum similarity with Candida tropicalis and the strain YPD-5 showed maximum similarity with Candida sp. All the identified yeast strains were deposited in the GenBank under the accession number JF922862, JF922863, JF922864 and JF922865. Chen et al. (2009) reported that, Candida tropicalis (9 nos.) and their
evolutionary relationships were phylogenetically analyzed by neighbor joining method. The *Candida tropicalis* is the most frequently isolated yeast found in beach sand (Vogel *et al.*, 2007) and in the bivalves (Buck *et al.*, 1977). The *C. tropicalis* and *Rhodotorula rubra* have been found widely in marine environment (Chen *et al.*, 2009b). *C. tropicalis* has been found in the Indian oceans waters and in the intestines of marine animals (Kutty and Phillip, 2008; Wang *et al.*, 2008). The present study also made an attempt to differentiate among the *Candida tropicalis* through RNA secondary structure prediction method. Eventhough all the species seems to be similar, there is some inherent variation in the genetic system. Ravikumar *et al.* (2012) identified variations among the 5 species of *Streptomyces* sps. by using RNA secondary structure.

The present study also made an attempt to utilize the spent, fresh seagrasses (*Cymodocea serrulata* and *Syringodium isoetifolium* which was predominantly deposited) and paddy straw as a substrate for the milk mushroom *Calocybe indica* cultivation. All the morphometric and proximate values of mushrooms are found maximum in paddy straw however, the total lipid content was found minimum. Generally, the mushrooms contain low level of fat, saturated fatty acids and cholesterol but, it has the high amount of unsaturated fatty acids (Borchers *et al.*, 1999). The percentage of bio-efficiency also found maximum in paddy straw than the other
substrates. This might be due to the presence of various micro and macro elements present in the paddy straw. Mohan and Vinay, (2011) reported that, the maximum bio-efficiency was observed in the substrates *viz.*, brassica straw, radish leaves, pea pod shell and cauliflower leaves due to the presence of major and minor elements. The present study also made an attempt to find out the elements present in the harvested mushroom *Calocybe indica*. The level of nitrogen (N) was found maximum in paddy straw and the level of phosphorous (P), potassium (K) and zinc (Zn) were found maximum in sterilized *Syringodium isoetifolium*. The manganese (Mn) was found maximum in sterilized *Cymodocea serrulata*. The level of ferrous (Fe) and copper (Cu) were found maximum in the pretreated *Syringodium isoetifolium* and the level of carbon (C) was found maximum in pretreated *Cymodocea serrulata*. The variation in elements might occurs due to the type of substratum and the variation among the elements present in the substrates. Urben (2007) reported that, the mineral content of the *A. sylvaticus* mushroom varied due to the type of crops, climate, region and genetic mutations of the mushroom. Moreover, the proximate composition and elemental level mainly depends on the substratum, atmospheric conditions, age and part of the fructification (Alam *et al.*, 2008).