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Abstract	Lignocellulosic biorefineries are expected to combat the problems of depletion of fossil resources by large-scale production of chemicals and biofuels in future. Research studies in the past two decades have resulted in the systems for the production of bio-based novel products with improved ecological footprints. Among other challenges, the large scale operations would result in the huge consumption of fresh water (1.9–5.9 m^3 water per m^3 of biofuel) which will, in turn, question the sustainability of biorefineries. Fresh water shortage which already exists in many parts of our overpopulated planet has led to more uncertainties to biorefinery industries. Hence, some enzymatic and chemocatalytic applications using sea water as a reaction medium for large-scale biorefineries have been reported by several research groups, which emphasize the use of nonpotable water resources of coastal regions for locally available biomass. Many of those reports concluded that the use of such nonpotable water system is highly promising and hence the opportunities at the interface between biology and chemistry are predictable for holistic innovation and further research. The recent National Water Policy of India strongly advocated the implementation of new technology to minimize the fresh water consumption in industries. This critical situation warrants the design and development of economically, environmentally and socially sustainable practices for accomplishing sustainability in the global fine and speciality chemicals industry. The chapter has been planned to review the basics and research studies conducted to use the sea water as a reaction medium in bioethanol industries to reduce the usage of fresh water.

Keywords (separated by " - ")	Bioethanol - Sea water - Saccharification - Halotolerant - Biorefinery

Chapter 9
Sea Water as a Reaction Medium for Bioethanol Production

Dash Indira, Baskar Das, P. Balasubramanian, and R. Jayabalan

9.1 Introduction

Due to the depletion of fossil resources, it is expected that biorefineries will be preferred for the production of biofuels. Efficient biocatalytic systems are being made to produce biofuels with reduced ecological footprints. Among the challenges in biofuel industries, fresh water consumption is an important challenge which has become the global concern. Fresh water shortage which is already existing in our overpopulated world has also brought more pressure and uncertainities in this regard. Several research groups ~~aroudn~~ around the world have reported some enzymatic, fermentative, and chemocatalytic applications using sea water as a reaction medium for large scale biorefineries. The use of nonpotable water resources as reaction medium is promising. This chapter contextualises in detail about seawater-based applications for biorefineries.

9.2 Ethanol Production

Two types of ethanol production process are generally in practice: wet mill and dry grind. Around 80% of corn ethanol is produced through the dry grinding process. Corn is milled, and then the slurry is prepared with water. Either enzymes or chemical saccharification is performed to hydrolyze the starch to glucose. Yeast is then

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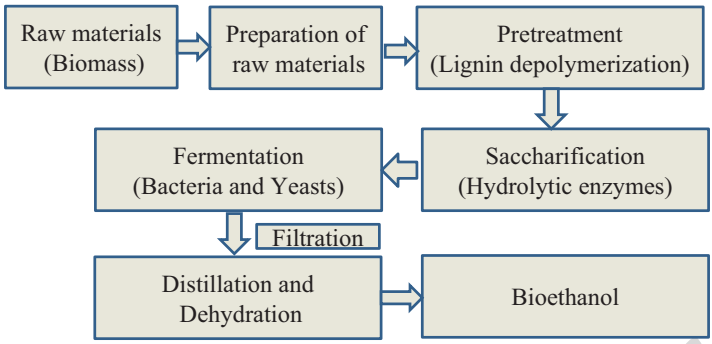

Fig. 9.1 Production processes of the bioethanol

added to the hydrolyzed biomass to ferment sugars into ethanol, which is then purified by distillation and molecular sieve dehydration to create the fuel. The processes involved in production of ethanol from biomass are given in Fig. 9.1. These ethanol plants have either a very little or almost no waste water discharge. They recycle a significant portion of industrial waste water through centrifuges, evaporation, and anaerobic digestion. The majority of water demand is primarily associated with cooling units or the boilers. As calculated by IATP (2006) an estimated water usage per gallon of ethanol production ranges from 3 to 4 gal. Few other reports calculated it to be in 3:1 ratio. Thus, 50 million gal of ethanol facility per year is expected to consume 15–200 million gal of water per year, i.e. 438 acre-feet.

9.3 Cellulosic Ethanol

Sugar and starch rich biomass gained popularity for fuel production which increased the competition with their use as food (Pimentel et al. 2008). The supply of sugar crops for ethanol industry was insufficient. Lignocellulosic biomasses are the most abundant renewable source, which can be used as an attractive alternative biomass for biofuel production. Lignocellulosic biomasses can be obtained from agricultural, municipal, forestry, and industrial source. The security of supply of biomass is ascertained by the widespread geographical distribution of lignocelluloses sources, which is not the case with fossil fuel reserves. Lignocellulose sources also put an end to the food vs. fuel conflict. Lignocellulosic biomasses are less expensive, can be produced with the low input of fertilizers, energy, and pesticides. The major cost involved in biomass production is mainly the purchase and processing of feedstock. Lignocellulosic feedstocks are the least expensive when compared to the recent prices of sugarcane and corn (Lynd et al. 2009). The conversion cost of feedstocks using current technology is, however, opposite to that of the purchase cost of feedstocks:

Cellulosic biomass > Corn > Sugarcane

Both, the feedstock purchase price and the conversion technology accounts for the fuel cost.

Sugarcane < Corn ethanol < Cellulosic ethanol

The growing technological advancement has the potential to reduce the selling price of cellulosic ethanol which is cheaper than the purchase cost of feedstocks (Lynd et al. 2009). Cellulosic ethanol is beneficial to the environment, can be produced in large-scale, and has a potential at a low fuel-production price, and thus cellulosic biomass is considered as the best candidate for energy production in the long run. However, they have high processing cost and not produced at a competition level (Lynd et al. 2008).

9.4 Cellulosic Ethanol Plants

Cellulosic ethanol production at different development stage is a part of many public and private international projects in few countries in the bio-renewable energy sector. Iogen's demonstration plant, located in Ottawa, Canada is operational since 2004 and is designed to process 20–30 t of feedstock per day and to produce approximately 5000–6000 L of cellulosic ethanol per day. It achieved a production capacity of 1,464,978 L/year in 2009 from wheat straw. Sekab's plant in Ornskolsvik, Sweden started producing cellulosic ethanol from sawdust in 2005. Currently, Sekab sells ethanol to 1400 E85 petrol pumps in Sweden, and the number of flex-fuel cars is about 147,000.In 2003, the U.S Department of Energy and Abengoa Bioenergy New Technologies, signed a 4-year contract, $35.5 million to develop technology for "Advanced Biorefining of Distillers Grain and Corn Stover Blend: Pre-Commercialization of Biomass-derived Process Technologies". In agreement to the contract, Abengoa Bioenergy is developing pilot scale processes integrating both lignocellulosic and cereal ethanol production to achieve best economic results. The major objective of the project is the conversion of residual starch, cellulose, and hemicelluloses, mainly corn stover to ethanol. The first phase of the project is completed and is in the testing phase with successful conversion of residual starch to bioethanol and improved co-product production. Inbicon has installed biorefineries for cellulosic bioethanol production at different sites. At Kalundborg, Denmark, 1.4 million gal of cellulosic ethanol per year is produced from bagasse, miscanthus grass, and fruit bushes. In Malaysia, projects with production estimated between 5 and 10 million gal of cellulosic ethanol per year are in the plan. In the United States, the first commercialisation produced 20 million gal/year. POET has 27 plants in seven states in the United States, which produces more than 1.6 billion gal of ethanol annually. For commercial cellulosic ethanol plant construction at Emmetsburg, Iowa with a capacity of 25 million gal/year using corn cobs, over $40 million had been invested.

NREL is working hard to develop and standardize technology for production of ethanol from agriculture residue, woody feedstocks, and switchgrass. NREL (2002)

report demonstrates a detailed process design and economy of conversion of corn stover to ethanol. This design can be used to produce 69.3 million gal of ethanol per year at a consumption rate of 6 gal of water per gallon of ethanol produced. This process being non-optimized has the further scope of improvement. Another report by NREL (2007) documents a detailed process design and economy for conversion of wood chips to ethanol by the thermochemical approach. This report suggests a minimal usage of 1.9 gal of water per gallon of ethanol produced. In this process in place of cooling water, forced air cooling was used to minimize water usage.

9.5 Water Consumption in Ethanol Production and Issues

Growing demands and growth of biofuel industry has triggered concern over many issues other than food security. One of the major concerns is water availability and its utilization for biofuel production. In the Midwest, the growing conflict over water use in agriculture facilities, livestock management units, and urban areas raised many eyebrows. Institute for Agriculture and Trade Policy (IATP) has warned in 2006 that the utilization of fresh water in biofuel industries is a serious concern. Life Cycle Inventory and Assessment (LCA) method is being used to analyse and quantify the consumption of water. It involves the quantification of material and energy flow during crop production, harvesting, transportation, ethanol production, and its final utilization in engines. Major areas of focus are crop production and ethanol production.

9.6 Freshwater Usage in Energy Industries

Unlike fossil resources, the sources for the production of biofuels are widely available. Amount of water consumed in biofuel industries and in irrigating the feedstocks vary significantly based on the processing technology and growing regions, respectively. For each liter of ethanol produced from switchgrass, 1.9–9.8 l of water is consumed (Wu et al. 2009). On average, corn ethanol production tends to consume more water than cellulosic ethanol on a life-cycle basis. Net water use for cellulosic ethanol production is comparable to that of gasoline from conventional crude or oil sands. Water use is declining because of rapidly evolving technologies for second-generation biofuel (cellulosic ethanol) and steady improvement of existing first-generation corn ethanol production. This is also true for crude oil recovery.

Crop sector leads in the consumption of freshwater accounting for 91.85% (1237 $km^3 year^{-1}$) of the 1314 $km^3 year^{-1}$ of global annual freshwater consumption. Agricultural production is considered as the principal driver of pressures on freshwater resources globally. Industrial and domestic demand accounts for 5.88% (77 $km^3 year^{-1}$) of the remaining fresh water consumption. In 5.88% of industrial

and domestic utilization, 23.78% utilized directly by energy sectors. Although this figure is comparatively small, the importance of considering freshwater consumption associated with energy sectors arises for two reasons (Holland et al. 2015).

9.7 Water Footprint of Bioethanol

Water footprints (WFs) refer to the volume of water directly and indirectly used by producers or consumers. Blue, green, and grey are the three key water elements suggested. The blue WF refers to the amount of fresh water (surface and ground) consumed in producing goods and services. The green WF is the consumption index of green water resources, which refers to rain water that falls and remains on the ground without flowing away or becoming part of ground water. The grey WF is a measure of pollution and is expressed as the volume of water required to assimilate the pollutant to load to meet ambient water quality standards (Hoekstra 2002). According to WF assessment manual published by the Water Footprint Network (WFN) (Hoekstra et al. 2011), a WF is an indicator used to measure water use based on the perspectives of freshwater resource use and pollution. Table 9.1 lists out the WF of bioethanol produced from various biomass.

Table 9.1 Water footprint of bioethanol from various raw materials (Chiu et al. 2016)

Raw materials	Country	Volume of fresh water (L) per L ethanol
Sugarcane	Brazil	2450
	US	2775
	India	2995
	Thailand	1396–2196
	Global average	2855
Sugar beet	France	790
	Germany	845
	US	1290
	Russian Federation	2075
	Ukraine	2780
	Global average	1355
Maize	US	1220
	Global average	1335
Cassava	Thailand	2374–2841
Sugarcane molasses	Thailand	1976–3105

9.8 Importance of Fresh Water

Water is the driving force of all nature with a little quantum of 3% as fresh water that has been intensively consumed in agriculture, industrial sectors and domestic purposes. Today's freshwater scarcity at alarming rate sensitized the global researchers and environmentalists that could trigger the next great global crisis given third world war. World Water Development Report for the year 2014 by United Nations has estimated that global industrial sectors consume 19–23% of the fresh water available in the world. The recent climb in oil prices and consumer demand for environmentally friendly products has now opened new windows of opportunity for bio-based chemicals. In the establishment of biomass-based industries, consideration should be given to possible unintended consequences such as the competition for food and biomass resource, the impact on water use and quality, changes in land-use, soil carbon stocks and long-term fertility, the net balance of greenhouse gasses and impacts on biodiversity. With the increase in the production through biomass-based industries, there is a need to discuss the impact of these industries on the usage of water. Water is the main component of all the biomass-based industries irrespective of the products produced. With the continued threat of the depletion of fresh water sources, the rapid growth of biomass-based industries adds more threats on the usage of fresh water shortly.

Also, very few or no research studies are being done to address this issue. The recent National Water Policy (NWP) of India strongly advocated the implementation of new technology to minimize the fresh water consumption in industries. This critical situation warrants the design and development of economically, environmentally, and socially sustainable practices for accomplishing sustainability in the global biofuel industries. It is necessary to find processes that are not only efficient but also sustainable. The biorefinery of lignocelluloses has drawn the central attention of researchers as it could meet the global energy and fine biochemical demands sustainably due to much better economics and ecological footprint. The conversion of lignocellulosic biomass to value-added chemicals such as bioethanol, furfural, and vanillin are gaining popular globally. Recent advancements in life cycle assessment and water footprint analysis revealed that around 6–10 l of fresh water are being consumed to produce 1 l of bioethanol. Utilization of sea water in industries to produce the biofuels could reduce the dependence of fresh water significantly as the sea water contains 97% fresh water and rest as minerals. Though desalination technology to generate fresh water has been explored in the recent decades as an excellent option, it has been reported as expensive and unsustainable. Although scientific literature on hydrolytic enzymes derived from marine microbes is available in plenty, only very few studies targeted the utilization of sea water as a nutrient medium.

9.9 Government Policies

Several Governments around the world have started to reduce the usage of fossil fuels through several policies. National Alcohol Program was launched by Brazilian Government in the 1970s after the oil crisis to utilize sugarcane as the primary energy crop for bioethanol research, development, and production. Brazil has produced approximately 32.5 billion litres of bioethanol in 2011 due to the encouragement of research on improvement in cultivation technologies of sugarcane species by the Brazilian Government (Azadi et al. 2012). The United States announced the implementation of the Energy Independence and Security Act (EISA 2007) to augment the standards of fuel efficiency and to decrease the crude oil dependency. EISA 2007 also involved a motto to increase the usage of bioethanol produced from maize by more than six times with an annual use of 36 billion gal in 2022 (Wallner and Mike 2011). The European Union has mandatory volumes of using renewable energy and biofuels in road transportation to 10% in 2020 (Gerbens-Leenes and Hoekstra 2012). Thailand Government has proposed a strategy to inspire the use of bioethanol produced from sugarcane molasses and cassava as transport fuels (Silalertruksa and Gheewala 2009). The Vietnam Government has declared to use rice straw as raw material for bioethanol production. In 2011, the global ethanol production volume was 22,742 million gal, 87.4% of which was produced only by US and Brazil. From 2007 to 2012, the global bioethanol production volume was tremendously increased from 13,089 to 22,715 million gal, indicating a 74% increase in 5 years. This increase suggested the importance of bioethanol as renewable energy in transportation sector which indirectly increased the competition over freshwater resources (Gerbens-Leenes and Hoekstra 2012). In 2030, the global annual WF of biofuels will be ten times that in 2005 (IEA). There is a huge threat imposed on fresh water resources due to a global increase in water consumption for the production of bioethanol. Along with energy problems, the water-resource related problems are also gradually increasing which is significantly critical and worthy of exploration (Earth Policy Institute 2012).

9.10 Opportunities for Saving Water

From various reports it is clear that energy demands and water consumption are interrelated, reduced water demands can be achieved through reduced energy consumption. Various options are being considered for reducing water utility in ethanol production. Production of broth with higher ethanol concentration reduces the energy required for distillation. One of the methods to potentially decrease water consumption involves pervaporation instead of distillation. Replacing the use of water in heat transfer process by forced air fans for cooling will cut short water loss due to evaporation and blowdown losses. Owen (2007) demonstrated a patented water conservation technology to be used in cooling towers to reduce 20% of water

consumption and also proposed a new high-efficiency dryer design. These technologies are modelled in NREL laboratory and are under review. DOE is examining the water use associated with the cultivation of fuel crops as most of them use ground water for the same. Optimization of cultivation condition is necessary to cut down the water consumption and to meet the renewable fuel goals. Apart from this, replacing fresh water with sea water in all bioprocessing steps in ethanol industries will save the fresh water to a larger extent.

9.11 Sea Water as Production Medium

The assessment of nonpotable water resources as a reaction medium for lignocellulosic biorefineries has been recently started. Despite its great potential, very few investigations of the use of seawater in fermentations have been reported to date with its utilization been strictly limited to seafood culture, the production of salts, and vegetable pickling (Al-Hotti and Kamel 1981; Sabu et al. 2000; Komives et al. 2005). The major concern in its use is its high salinity. In general, the salinity of seawater is around 25–35 practical salinity units (PSU), depending on the oceanographic conditions. Chemical composition of sea water is given in Table 9.2. These values are three times higher than those normally employed in conventional bacterial fermentation media (e.g. ten PSU for 10 g L^{-1} NaCl in the Luria-Bertani (LB) broth). Figure 9.2 explains the production of bioethanol using sea water based approach. Some proof-of-concept application has been developed by using sea water in succinic acid fermentation, chemoenzymatic, and chemocatalytic processes (Dominguez de Maria 2013; Lehmann et al. 2012; Grande et al. 2012; Lin et al. 2011). No significant inhibition of cell growth of *Actinobacillus succinogens* and succinic acid production was observed, even if fresh water was replaced by 100% synthetic sea water (Lin et al. 2011). Vom Stein et al. (2010) studied the depolymerisation of cellulose using (bio-based and biodegradable) organic dicarboxylic acids in concentrated sea water. The use of seawater-derived media as fermentative broth has received very little attention, with only some examples to produce

Table 9.2 Composition of sea water (Indira et al. 2016)

Elemental composition	Concentration ($\times 10^3$ ppm)
Na	5.5
Mg	3.8
Ca	3.7
Mn	1.2
Cu	1.2
K	<0.2
Co	<0.1
Fe	<0.1

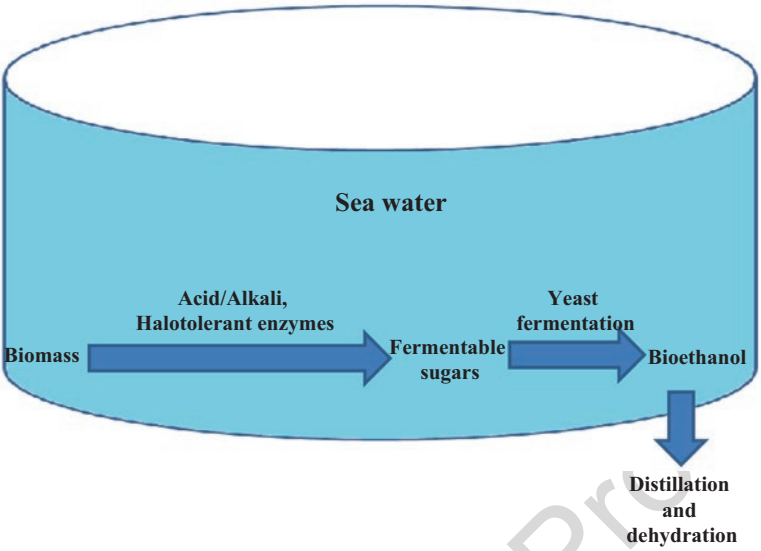

Fig. 9.2 Sea water approach for the production of bioethanol

carboxylic acids like lactic acid (Al-Hotti and Kamel 1981) and succinic acid (Lin et al. 2011), for the biosynthesis of enzymes (L-Glutaminase) (Sabu et al. 2000), or for growing *Bacillus methanolicus* with methanol as the sole source of carbon (Komives et al. 2005). Lignocellulosic (LC) biomass has to be pretreated to overcome its recalcitrant nature and to increase the yield of hydrolysis. Various methods using different physical treatments by mechanical utilities, chemical (acids, alkali, ionic liquids, oxidizing agents, organic solvents, steam explosion, ammonia, supercritical CO_2 explosion, etc.) and biological (enzymes like laccase and peroxidase) methods have been proposed for depolymerization of lignin. However, these technologies suffer from relatively low sugar yields, the formation of compounds inhibiting subsequent fermentation, severe reaction conditions, and high processing costs (Kumar et al. 2009). Nevertheless, to our knowledge, there is very less number of a report on the utilization of seawater for pretreatment of lignocellulosic biomass in the literature, where seawater based hydrothermal pretreatment (Fang et al. 2015) and ionic liquid pretreatment (Ren et al. 2016) of biomass were conducted. Apart from this, few saccharification studies have been conducted using seawater as the reaction medium. In the process of depolymerization of several amorphous and crystalline cellulose by commercially available enzyme cocktails (Accellerase 1500), only slightly lower production rates (~90%) were observed in seawater media about those in pure citrate buffer (Grande and de Maria 2012). The C5 and C6 sugars derived from the lignin depolymerization can be converted to various platform chemicals like 1,4-diacids (succinic acid, fumaric acid, malic acid), 2,5-furan dicarboxylic acid (2,5-FDCA), 3-hydroxy propionic acid (3-HPA), aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxy

butyrolactone (3-HBL), glycerol, sorbitol, and xylitol/arabinitol (Werpy et al. 2004). Though there are well-established procedures available for the conversion of sugars to value-added chemicals, none of these reactions was performed using seawater.

9.12 Development of Seawater Based System for Saccharification

The increase in the proportion of water for human use due to changing lifestyle and increasing population coupled with spatial and temporal variation in water availability denotes that the water for agriculture, industrial usage, and other human usage is becoming scarce. By decreasing fresh water usage in industries, fresh water usage can be cut short manifolds. Around 70% of world fresh water is used in agriculture, by using marine algae as a substrate for biofuel production total water intake for energy crop production can be reduced. Use of saline water in the cultivation of marine algal biomass as a substrate for biofuel production and use of the saline system in pre-treatment (saccharification) and fermentation process will result in water management. However, the substrate, i.e. marine algal biomass and the saline system for saccharification demands halotolerant enzymes.

Cellulases and other hydrolytic enzymes are inhibited at higher salt concentration. Reports suggest the isolation of cellulases from halotolerant organisms with optimal activity in between 6% and 22% of NaCl. Xylanolytic and ligninolytic activity have been reported in several marine fungal isolates. Bonugli-Santos et al. (2010) reported the production of laccase, manganese peroxidase, and lignin peroxidase from Brazilian marine-derived fungi. Wejse et al. (2003) reported purification and characterization of two extremely halotolerant xylanases from a novel halophilic bacterium strain, CL8 with optimal activity at 1 M NaCl concentration. Khandeparker et al. (2011) reported a novel halotolerant xylanase from marine isolate *Bacillus subtilis* cho40. Salt tolerant cellulases can tolerate high salt levels and ionic liquids better than normal cellulases. Use of salt tolerant cellulase in biofuel industry for pre-treatment of biomass shall lead to advancement in the usage of seawater/brackish water. Grant et al. (2004) reported the presence of cellulase-producing microorganisms in Wadi al Natrum Soda Lake that are capable of depolymerisation of polysaccharides in ionic solvents or a saline solvent (4 M NaCl) (Liang et al. 2011; Pottkämper et al. 2009). The commercially available Accellerase-1500 is capable of depolymerising amorphous and microcrystalline cellulose with 90% of the original rate in a seawater-based media (Grande and De Maria 2012). The other reports involves utilization of seawater for bioprocessing of carboxylic acids like lactic acid (Al-Hotti and Kamel 1981) or succinic acid (Lin et al. 2011), for the biosynthesis of enzymes (L-Glutaminase) (Sabu et al. 2000), or for growing *Bacillus methanolicus* with methanol as the sole carbon source (Komives et al. 2005). Despite such promising and immense prospect, use of seawater for biomass processing is still in infancy. The present chapter is aimed to

minimize the fresh water consumption in biomass-based industries by developing seawater based systems to convert agricultural biomass into fermentable sugars. Enzymatic conversion of biomass to bioethanol in seawater would be done using halotolerant hydrolytic enzymes produced by the potent isolates. The envisaged outcome could open up a door to utilize the seawater based system in industries to make it sustainable and assist the Mother Nature to solve freshwater scarcity problems of the world.

Grande and De Maria (2012) assessed the cellulolytic activity of commercially available Accellerase-1500, an enzymatic cocktail of different glycosidases (cellulase, hemicellulase, and a higher level of β-glucosidase, derived from *Trichoderma reseii*) in different concentrated seawater systems. This was the first report published regarding the enzymatic hydrolysis of cellulose in seawater. Cellulose molecule with varying degrees of crystallinity was assessed for hydrolysis in seawater, and the reports proved that hydrolysis is possible with slight diminishing rates (~90%) as compared to that of the reaction carried out in a controlled buffer system. The enzyme effectively hydrolyzed both amorphous and microcrystalline cellulose in seawater. Hydrolysis of amorphous cellulose Sigmacell-101 using Accellerase-1500 remained same in buffer and in seawater, which may be due to low crystallinity of the samples. For (Sigmacell-20 and Avicel) the hydrolytic activity of enzyme in seawater was 90% to that of the activity achieved with buffer.

9.13 Production of Bioethanol in Sea Water Based Medium

Water plays a major role in the production of energy, both in the process of extraction of sugars as well in the processing of fuels. Growing demands of an alternative source of energy from conventional and non-conventional renewable resources are going to increase the demand for water (Wu et al. 2009). According to the Energy Independence and Security Act (EISA 2007), U.S. is committed to producing 36 billion gal of biofuel by 2022 and for which the production rate is increasing at an unprecedented rate crossing the record of 9.0 billion gal of ethanol in 2008 (RFA 2007). Total water consumption in biofuel production can be divided under two heads, one being the production of energy crops and the other is processing of biofuel. Ethanol has been produced from sugar or starch rich crops since ancient times, and the water for the growth of energy crops was supplied through either precipitation or irrigation. Replacement of sugar-rich crops with lignocellulosic feedstock has subtracted the water consumption in this regard. Studies conducted by the U.S. Department of Energy (DOE) and USDA reported the availability of more than a billion tonnes of biomass for fuel production (Perlack et al. 2005). The biochemical process for pretreatment of biomass requires additional water that sums up the total water consumption for cellulosic ethanol-equivalent to the water use for corn ethanol production. The current technology of biochemical pre-treatment process of biomass for ethanol production consumes 9.8 l of water for producing one litre of ethanol, which is estimated to be reduced to 5.9 l with an increase in ethanol yield

(Aden et al. 2002). Increasing demand for water and global climate change is going to pose a serious threat to the availability of freshwater (WWAP 2012). It is estimated that requirement of water for biofuel production may arise by 5.5% by 2030 exerting an extra load on scanty freshwater resources (Gerbens-Leenes et al. 2012).

Development of a seawater-based biorefinery strategy could make a strong impact in these areas with a holistic utilisation of seawater, aiming at more efficient, low cost, and small carbon footprint processes. There are reports for the use of seawater in enzymatic hydrolysis of lignocellulosic biomass (Grande and de María 2012; Klement et al. 2012; Vom Stein et al. 2010), fermentation process using halotolerant yeasts (Senthilraja et al. 2011) also, few marine yeasts were isolated and tested for their fermentation capacity in seawater (Urano et al. 2001).Utilization of seawater for biofuel production reduces stress on freshwater resources while enabling cultivation of biomass, saccharification, and processing of biofuel over a common platform (Goncalves et al. 2015). Fermentation of ethanol in seawater using *S. cerevisiae* has reported production of 0.5 g ethanol per gram of glucose (Goncalves et al. 2015). To utilize sea water as a reaction medium for saccharification, the enzymes must be salt tolerant and can saccharify in the presence of salt. Hence, halotolerant cellulase enzymes and microorganisms producing halotolerant cellulase are needed to develop sea water based system to produce ethanol.

9.14 Halotolerant Cellulase Producers

Strong acids and alkali solutions are used at a higher temperature during the pretreatment process, the neutralisation of these acids and bases results in the formation of salts. Removal of salt is done using tonnes of water and energy to facilitate further downstream processing. Also, ionic liquids (ILs), i.e. a liquid form of salts at room temperature have found recognition as an alternative to chemical solutions as a green solvent for lignocellulosic pretreatment (Mäki-Arvela et al. 2010; Gunny and Arbain 2013). One of the major shortcomings of ILs is their ability to inhibit cellulase enzyme in subsequent saccharification process, rendering the whole process ineffective. The high salinity of ILs may be the reason behind enzyme inhibition (Turner et al. 2003; Zhao et al. 2009; Salvador et al. 2010). To overcome the risk of enzyme inhibition, the residual ILs are washed after the pretreatment process, which again adds up to the energy consumption and processing cost (Engel et al. 2010; Zhang et al. 2011). This issue was addressed by proposing ideas like utilisation of one-pot process for pretreatment and saccharification of biomass by utilising salt tolerant cellulases (Kamiya et al. 2008). Given the above discussion, identification and utilisation of halotolerant cellulase, which are stable at saline conditions of neutralised acids, bases, and compatible with salts of ILs, is important. Thus, halotolerant enzymes with stability and good catalytic activity are in vigorous search, which will be suitable for industrial production and consumer affordability (Xing et al. 2012; Kuhad et al. 2011; Garg et al. 2016).

Hydrolysis of cellulose is a common ability found in many bacterial and fungal species within the Eucarya domain, although from the domain Archaea cellulolytic organism is yet to be identified (Holt et al. 1994). Many aerobic and anaerobic cellulolytic bacteria have been isolated from diverse environments (Sukumaran et al. 2005). There are a good number of reports over alkaliphilic cellulase-producing microorganism but limited reports on haloalkaliphilic cellulase producers (Aygan et al. 2011; Sukumaran et al. 2005; Zhang et al. 2012). *Bacillus sp. BG-GS10* isolated from Zabuye Salt Lake (Tibet) was reported to have high cellulolytic activity in 0–18% NaCl concentrations (Zhang et al. 2012). *Aspergillusterreus* UniMAP AA-6 showed halotolerant cellulolytic ability accompanied with thermotolerance (Gunny and Arbain 2013). The cellulase stability and relative activity was more with less viscous solvents and decreased with increase in viscosity, which affects the enzyme-substrate interaction (Romero et al. 2008; Samayam and Schall 2010).

The genus *Streptomyces* is an attractive industrial organism due to the growth rate, its extracellular secretion and biosafety reasons. *Streptomyces roseosporus* and *S. griseus* showed CMCase activity at pH 8 and in the presence of 5% NaCl and 2% NaCl, respectively, at 37 °C. Highest enzyme activity was detected after 90 min incubation under shaking at 37 °C (Hakobyan et al. 2013). Dasilva et al. (1993) reported *Streptomyces sp*. S36-2 to produce alkaliphilic cellulase at optimum pH and temperature of 6.0–7.0 and 55 °C, respectively.

Halophilic microorganisms can produce halotolerant enzymes that are active in a high saline environment (Oren 2010). There are reports suggesting stability of halotolerant enzymes at a wide range of temperature, i.e. from 40 to 80 °C, though a gradual decrease in enzyme activity was noticed. The enzyme displayed 55% residual activity at 70 °C, which is a thermophilic characteristic (Oren 2006; Mesbah and Wiegel 2005). The stability of halophilic cellulase at a higher temperature may be due to increasing in surface charge of the microbial cell membrane, ionic interaction and change in the cytoplasmic membrane to adapt and acclimatise to high-temperature conditions (Karan et al. 2012). Few halotolerant cellulases have been described from metagenomic studies; *Thermotoga maritima,* Archaea and *Pseudoalteromonas* sp. (Datta et al. 2010; Ilmberger et al. 2012; Trivedi et al. 2013; Raddadi et al. 2013).

Paenibacillus tarimensis was characterised for CMCase activity over a wide range of pH (3.0–10.5) and salt concentration (9 mM–5 M NaCl). At high salt concentrations, i.e. 20% of ILs at 80 °C, >76% of relative activity was retained, and at 40% concentration of ILs, >40% relative activity was detected (Raddadi et al. 2013). Stability at such high concentration of salts makes these enzymes a promising candidate for application in detergents, textiles, paper/pulp industry; and ILs treatment-saccharification of lignocellulose. *Paenibacillus tarimensis* L88 showed highest CMCase activity at 72 h after incubation with a decrease in viscosity of the growth medium suggesting that cellulase(s) to be β-endoglucanase(s) (Percival et al. 2006). Earlier reports of CMCase from other *Paenibacillus* strains include *Paenibacillus curdlanolyticus* B-6 and *Paenibacillus campinasensis* BL11 with enzyme activity of 0.05 U/ml and 0.1 U/ml, respectively (Ko et al. 2007; Pason et al. 2006). Highest endoglucanase activity was obtained by the recombinant enzyme purified from

Paenibacillus barcinonensis expressed in the yeast *Saccharomyces cerevisiae* with 1.2 U/ml (Mormeneo et al. 2012) or the cellulase from *Paenibacillus cookie* expressed in *Escherichia coli* and that exhibited 39.1 U/ml CMCase activity (Shinoda et al. 2012). Multiple cellulases have been reported from *Paenibacillus* genus. *P. campinasensis* BL11 (Ko et al. 2007) was reported with three different cellulases, zymogram analysis of cellulolytic system from *P. curdlanolyticus* strain B-6 showed the presence of 12 xylanases, and 9 CMCases (Pason et al. 2006). Liang et al. (2011) reported that Cel5A to be stable at pH range 4.5–10; Hirasawa et al. (2006) showed a cellulase from *Bacillus agaradhaerens* was active in a pH range 5–11.5 and Trivedi et al. (2010) reported cellulase activity from *Bacillus flexus* having optimal activity at pH 10 with stability in the pH range 8–12.

Haloarcula G10 strain isolated from the saline soil of Yuncheng Salt Lake, China showed endoglucanase activity at an optimal temperature of 60 °C, and pH of 9.0 at 17.5% NaCl. Endoglucanase activity and stability was reported over broad ranges of temperature (40–80 °C) with 60% residual enzyme activity at 80 °C, pH (7.0–10.0) and NaCl concentration (12.5–27.5%), showing its thermostable, alkali-stable and halostable properties (Li and Yu 2013). A similar range of optimal temperatures has been reported for halophilic endoglucanases from *Thalassobacillus* sp.LY18 and *Bacillus* sp. L1 (Li and Yu 2012). EDTA, phenylmethylsulfonyl fluoride (PMSF) and diethyl pyrocarbonate (DEPC) lead to some significant inhibition of enzyme activity revealing it as a metalloenzyme with serine and histidine residues essential for enzyme catalysis. The surfactants tested had little effects on the enzyme activity. The *Haloarcula* G10 endoglucanase was active and stable in the nonpolar hydrophobic organic solvents with log $P_{ow} \geq 0.88$. All the characteristics mentioned above make this endoglucanase an ideal choice for applications in the industrial process under harsh conditions.

Bacillus vallismortis RG-07 was reported for maximum cellulase production from sugarcane bagasse (4105 U ml^{-1}) with optimum temperature and pH of 65 °C and 7.0, respectively. The enzyme retained its residual activity of 95% and 75% of activity even at a temperature of 95 °C, and pH 9.0, respectively. The presence of organic solvents like (30%) n-dodecane, iso-octane, n-decane, xylene, toluene, n-hexane, n-butanol, and cyclohexane enhanced enzyme activity on prolonged incubation (7 days). Ca^{2+}, mercaptoethanol, Tween-60, and sodium hypochlorite promoted whereas Hg^{2+} strongly inhibited the enzyme activity. Kinetic analysis of purified enzyme showed the *Km* and *Vmax* to be 1.923 mg ml^{-1} and 769.230 μg ml^{-1} min-, respectively (Singh and Kumar 1998).

Bacillus, Clostridium, Cellulomonas, Rummminococcus, Alteromonas, Acetivibrio, Bacteroides, etc. are among the most commonly reported bacterial genera for endo-cellulase activity. Among *Bacillus* sp., *B. brevis* (Singh and Kumar 1998), *B. pumilus* (Gachomo 2003), *B. amyoliquefaciens* DL-3 (Lee et al. 2008), and *B. subtilis* YJ1 (Yin et al. 2010) are well studied for cellulase production under submerged conditions (Jo et al. 2008; Mayende et al. 2006).

The Cel5R (belong to GH5 family) identified by soil metagenomic approach shows thermostability up to 58 °C and pH stability from 5 to 9 with halotolerance and extreme halostability in 4M NaCl, 3M LiCl and 2M KCl (Garg et al. 2016)

which is higher than other known halostable cellulases (Zhou et al. 2016; Zarafeta et al. 2016). The halostability of Cel5R may be due to the presence of acidic residues (Asp and Glu) on the surface of the protein, which is supported by the crystal structure analysis of Cel5R with acidic residues (16.7% with 52 residues) present on the surface of the protein. The mutation of surface residues in malate dehydrogenase from *H. marismortui* (Madern et al. 1995) and glucose dehydrogenase from *H. mediterranei* (Esclapez et al. 2007) altered only the halophilic properties of mutant without affecting the kinetic parameters and enzyme activity of the protein.

Two strategies generally opted to obtain better biocatalysts are either protein engineering through rational design or directed evolution (Dalby 2011; Bornscheuer et al. 2012; Bornscheuer and Pohl 2001) or mining nature's genetic reservoir for genes that encode enzymes with novel properties from previously uncharacterized organisms either bioinformatically or by functional screening (Lorenz et al. 2002). Extremophiles are a rich source of such enzymes, as they have evolved to thrive in extreme environments. Culturing or culture-independent approaches are applied to retrieve genomic or metagenomic material from extreme habitats followed by functional or bioinformatics screening to reveal novel enzymes with the desired properties (Kim and Peeples 2006; Demirjian et al. 2001).

The cellulolytic enzyme, CelDZ1 which is predicted to be located on the outside of the cell membrane, requires halotolerant features in order to maintain the enzyme activity, which could be achieved by lowering the affinity of the active site of the enzyme to the chloride and potassium/sodium ions, to avoid competitive inhibition with the substrate. The surface potential of CelDZ1clearly shows an overall negative charge in the active site channel which does not favour binding of chloride ions. Monovalent cation binding sites are usually formed by a carboxylic side chain and at least one carbonyl from main protein chain. CelDZ1 ligand groove revealed no carbonyls exposed to solvent near carboxyl side chains which would form an alkaline ion-binding site (Zarafeta et al. 2016).

High extracellular CMCase activity was reported in *Marinimicrobium* sp. LS-A18 is grown on mineral salt medium with CMC as the sole carbon source (Zhao et al. 2012). Maximum CMCase activity was recorded at 55 °C and pH 7.0 in the absence of NaCl. Optimised fermentation conditions yielded CMCase up to 2.5 U/ml, which was 3.1-fold higher than non-optimized process. Eighty-four percent of enzyme activity was retained after incubation at 60 °C for 1 h and more than 88% of enzyme activity was retained after incubation for 72 h at a pH range between (5–11) and NaCl concentrations (0–25%, w/v), indicating it was halotolerant, thermostable and alkali-stable.

9.15 Indian Scenario

India has a vast variety of agricultural and forest bioresources, which can be efficiently used for the sustainable biorefinery industries. An estimated 50 MMT (million metric tons) of liquid fuels are consumed annually in India, but with the actual

biomass potential and its full utilization, India is capable of generating almost double that amount per annum. Several studies have been carried out using various treatment methods for the depolymerization of lignin available in different biomass and subsequent saccharification and fermentation of the hydrolyzed products to value added products (biodiesel, biogas, bioethanol, acetic acid, etc.) for many decades. Singh et al. (2014a, b) studied the enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by *Saccharomyces cerevisiae, Scheffersomyces stipitis,* and their co-culture. Separate hydrolysis and fermentation methods are adopted to produce bioethanol from dilute acid pretreated Indian bamboo variety (*Dendrocalamus* sp.) (Sindhu et al. 2014). Other researchers also actively contributed to bioethanol production from lignocellulosic materials (Behera et al. 2014; Singh et al. 2014a, b, 2015a, b). Besides such studies, Biswas et al. (2014) developed the methods for efficient enzymatic hydrolysis by wet explosion pretreatment of sugarcane bagasse. Sharma et al. (2015) carried out a pilot-scale study on the steam explosion and mass balance for higher sugar recovery from rice straw. Microwave and ultrasonic waves assisted depolymerization of biomass are being studied with or without alkali treatment by several India researchers on green coconut fiber (Jeyanthi and Subramanian 2011), rice straw and hulls (Singh et al. 2011), rice straw (Singh et al. 2014a, b), paddy straw (Kaur and Phutela 2016), and waste newspaper (Subhedar et al. 2015). Many researchers in India have performed chemical transformation of sugars to various value added chemicals, but none of these works was attempted in sea water-based reaction media. Recently in India, €110 million joint venture cellulosic ethanol project of Chempolis Ltd. and Numaligarh Refinery Limited (NRL) is moving towards construction after getting approval from NRL's board. By 'Hydrocarbon Vision 2030 for Northeast", the big project will be started to produce bioethanol from bamboo with the co-production of furfural and acetic acid. Although many of researchers are potentially contributed, the methods and strategies to adopt different methods for biorefineries of biomass favourably, there is no one till now subject the water issue during the production process. It will be a significant problem in India also if fresh water usage in such biomass-based industries continues. Therefore, it is the time of using some alternative source of water like sea water for the use in such industries.

9.16 Conclusion

Water is precious, and depletion of water may lead to the unfavourable situation between countries. Several initiatives have been taken by international agencies to save the fresh water. It has been already recommended to reduce the usage of freshwater in industries for bioprocessing. Sea water would be an excellent alternative to replace the fresh water in industries as it has 97% fresh water and 3% salts. Halotolerant microorganisms and their enzymes can tolerate the salts in sea water and saccharify the biomass in sea water. Halotolerant yeasts can ferment the sugars in sea water to produce ethanol. However, there must be a suitable technology to

measure the ethanol present in sea water and to concentrate the ethanol from sea water. Sea water based systems will tremendously reduce the requirement of fresh water in industries not only in saccharification processes but also in several other bioprocesses.

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Author Queries

Chapter No.: 9 454192_1_En_9_Chapter

Queries	Details Required	Author's Response
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