CHAPTER 1
INTRODUCTION

1.1 BIOFUEL: BIOETHANOL AS TRANSPORTATION FUEL

Increase in human population, simultaneously increased the standard of living. Simultaneously the growth of industrial affluence also increased leading to excess consumption of fossil fuels especially in production of electricity and transportation purpose. A decline in annual global oil production is anticipated due to this ever increasing dependence. Therefore today, biofuel serves as an alternative to fossil fuels. This has a great potential to fill the gaps for energy security faced domestically and globally especially in transportation sector without increasing any new carbon led pollution [1]. The raw materials for biofuel production like bioethanol, biobutanol, biodiesel etc are sourced from the wastes of forests, agricultural lands, marine or even from domestic house-hold kitchen wastes.

Bio-ethanol can be blended with petrol in varying ratio and can be used as petrol for engines. Preference for bio-ethanol over other alcohols is due to its physico-chemical characteristics. To name few it has greater octane number, broader flammability limits; higher heats of vaporization; higher flame speeds etc, which eventually enables a higher compression ratio and shorter burn time for IC engine. When ethanol blended petrol combusts particulate and nitrogen oxides (NOx) emissions will reduce highly [2].

Implementation of bioethanol as renewable fuel by all governments globally has impacted a significant growth in global ethanol market (85% of ethanol produced is consumed as biofuels). Countries like USA, Brazil, China, Canada and Thailand are the top 5 ethanol producers processed from plant materials [3]. In India, the new biofuel policy aims for a 20% blend with petrol (E20) by 2017 which will create a demand more than 4 billion gallons of ethanol per year. Ethanol production in India mainly depends on the availability of sugarcane. In the present situation India can produce 6,000-7,000 litres of ethanol from one hectare of sugarcane (only from molasses) and if bagasse is included total of 12,000-15,000 litres per hectare can be produced. However due to the down cycle in sugarcane production and lack of molasses owing to unpredicted rainfall; India is forced to shift to other alternatives resources. Backbone of Indian economy is the agricultural sector which eventually has the potential to shovel the path in developing second generation ethanol
industry. By 2020, annually in India 125 million and 183 million tonnes of biomass residues will be available just for bioethanol production. And expected theoretical conversion of these biomasses to ethanol will be 34-50 billion litres [4].

1.2 CELLULOSIC TECHNOLOGY: VITAL ROUTE FOR CELLULOSIC BIOETHANOL

The lignocellulosic substrates (LCS) are a significant renewable resource which is being looked upon as an alternative for petroleum [1]. Plant biomass majorly consists of cellulose, hemi-cellulose, lignin, ash and other macro-molecules (composition varies based on the type of wood). Structural sturdiness for plants are extended by cellulose, which upon hydrolysis releases glucose moieties. Chain of pentose sugars make up the hemi-cellulose which generally consist of non-fermentable sugars. Hemi-cellulose binds and holds all the cellulose fibrils together. Another compound found abundantly in plants are the lignin made up of aromatic molecules. These insoluble molecules provides structural strength and acts as water repellent [5]. Dependence on agricultural lignocellulosic biomass is a better option for bioethanol production since it does not compete with food availability like in the case of starchy biomass. Thereby woods, straws, grain husks all can be a better option as bio-resource for second generation biofuels. Efficiency of biomass material conversion to ethanol depends on the type of pre-treatment and hydrolysis technique involved during the process. Some of the process pathways carried out during ethanol production from different raw materials is depicted in Table 1.1. Despite the high complication involved in biological cellulosic ethanol production, the first second generation commercial plants have been put into operation and some are scheduled to open soon (Table 1.2).
**Table 1.1** Process pathways of different raw materials to ethanol [6,7]

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Process involved</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood</td>
<td>Mechanical/Physicochemical</td>
<td>Enzymatic/Acid hydrolysis</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>Chopped</td>
<td>Enzymatic/Acid hydrolysis</td>
</tr>
<tr>
<td>Corn straw</td>
<td>Chopped, Steam explosion, alkali pre-treatment</td>
<td>Enzymatic hydrolysis</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>Ball milling, Acid pre-treatment</td>
<td>Enzymatic hydrolysis</td>
</tr>
<tr>
<td>Sweet sorghum bagasse</td>
<td>Acid pre-treatment</td>
<td>Enzymatic hydrolysis</td>
</tr>
</tbody>
</table>

**Table 1.2** Examples of commercial-scale cellulosic ethanol projects currently in operation or expected to open [8]

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
<th>Feedstock</th>
<th>Pre-treatment</th>
<th>Capacity (10^6 L/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta Renewables</td>
<td>Crescentino, Italy</td>
<td>Wheat straw</td>
<td>Steam explosion</td>
<td>76</td>
</tr>
<tr>
<td>Abengoa Bioenergy</td>
<td>Hugoton, Kansas, USA</td>
<td>Corn stover, wheat straw, grasses</td>
<td>Acid impregnation, steam explosion</td>
<td>95</td>
</tr>
<tr>
<td>Poet DSM</td>
<td>Emmetsburg, Iowa, USA</td>
<td>Corn stover and cobs</td>
<td>Two stage steam explosion</td>
<td>76</td>
</tr>
<tr>
<td>GranBio</td>
<td>Alaogas, Brazil</td>
<td>Sugarcane straw</td>
<td>Steam explosion</td>
<td>83</td>
</tr>
<tr>
<td>DuPont Danisco</td>
<td>Nevada, Iowa, USA</td>
<td>Corn stover</td>
<td>Dilute ammonia</td>
<td>95</td>
</tr>
</tbody>
</table>

**1.3 COMPETENT ETHANOL PRODUCING MICRO-ORGANISMS**

Many traits are considered when developing an ethanologenic organism for a commercial level. These organisms should have a broad substrate utilization range for fermentation rather depending only on mono-sugars. It should be tolerant to increasing ethanol concentration, changes in pH, salt concentration, temperature *etc*. Certain thermostrains were reported to be tolerant up to 12% ethanol and was able to survive even at 50°C.
And these strains must be resistant to inhibitors as the fermentation medium contains hydrolysates of lignocellulose [10]. *Clostridium cellulolyticum* [11], *Ruminicoccus albus* [12], *Thermoanaerobacter ethanolicus* [13] are the general bacterial strains used in industries. And among fungal *Aspergillus niger* [14], *Fusarium oxysporum* [15], *Aspergillus nidulans* [16], *Rhizopus oryzae* [17]. Among fungal strains *Saccharomyces cerevisiae* [17], *Kluyveromyces marxianus* [18], *Pichia stipites* [19], *Candida shehatae* [20], *Pachysolan tannophilus* [21] are used.

### 1.4 CLOSTRIDIUM THERMOCELLUM: ORGANISM OF INTEREST

*Clostridium thermocellum* is a strict anaerobic cellulolytic, thermophilic bacterium which has the rare property of degrading and hydrolysing recalcitrant plant biomass majorly into cellobiose and cellodextrins. Since they do not engulf these materials for hydrolysis, robust hydrolytic enzymes are produced from cellulosomes found on the outer surface of the microbial cell [22]. Its cellulosome comprising of 72 different proteins as well as 25 free enzymes [23] and also lichenase, chitinase, mannanase, and five xylanases that can even release lignin from hemicellulose. This complex consists of a non-catalytic primary scaffold (CipA) supporting up to nine catalytic enzymes through secondary scaffoldins. Ttype I cohesion (nine types present) found on CipA when in contact with type I dockerin; a synergistic action occurs in degrading the substrate. Another mediated action is between type-II dockerin module and the internal type II cohesin domain of S-layer anchor proteins. Cellulose binding module (CBM) further aids in the anchorage of the cellulosome to lignocellulose [24,25]. In 2016 Qi Xu and team have established the use of another type of cellulosomal system which is not attached to the cell wall of the microbe. They named it as “cell-free” cellulosomal system. The peculiar property of this complex is that it diffuses away from the bacteria and can degrade polysaccharide substrates in a distant (Figure 1) [23]. The presence of all these unique catalytic properties justifies *Clostridium thermocellum* as the most efficient biomass degrader among all cellulolytic organisms [26].
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**Figure 1** Cellulase systems found in *Clostridium thermocellum.*
A) Free-enzyme system. B) Cell-free cellulosome systems. C) Cell-bound cellulosome systems [23]

### 1.5 GENETIC MANIPULATION IN CLOSTRIDIUM THERMOCELLUM

*C. thermocellum* utilises the cellobiose further for the production of ethanol, acetic acid, lactic acid, hydrogen, and carbon dioxide. One of the cost-consuming step in fermentation industry is the provision of adequate oxygen transfer. This step will be negated completely since this bacterium is obligate anaerobe and various routes for contaminations can be reduced as the optimal temperature for organism survival is above 60 °C. Its hydrolytic enzymes are considered to be stable due to the toughness of growth conditions [27]. Acetate and lactate are the two by-products produced along with ethanol which acts as weak uncouplers and thereby reducing the growth rate and ethanol yield [28].

Researchers started genetically modifying *Clostridium thermocellum* for commercial purpose considering its robustness nature. Most of the works were increasing the ethanol production or its tolerance towards ethanol. By direct evolution a mutant strain was developed for improved growth rate with a tolerance upto 17.5% v/v of ethanol. The substrate used in the above case was poplar hydrolysate, mildly pre-treated with dilute acid [29]. Many new strains of *C. thermocellum* were isolated with improved ethanol to organic acid ratio and enhanced cellulase and activity [30,31]. For industrial application, a
consortium of the multi-enzyme complexes was designed through targeted cellulosome engineer. An improved result was observed in switch-grass hydrolysis due to the enhanced cellulose activities in aggregate [32]. In some case studies when ethanol tolerance of the organism was improved up to 80 g/L, an inconsistent and slow growth of the organism was reported. However in another study a stable growth was observed when the intolerance was increased to 50 g/L of ethanol. This stability was attained by altering the natural carbon flow by disrupting the end product formations in the fermentation pathway [33–35].

1.6 HINDRANCE IN BIOCONVERSION OF LIGNOCELLULOSE

Various research and developments are happening throughout the globe for converting lignocellulose into bio-ethanol. Many reasons accounts to the hindrance for the conversion of LCS into sugars. Starting from the grass-root level, the type and nature of the biomass vary resulting in resistance towards pre-treatments. Since LCS on hydrolysis release different mono-sugars, the necessity for organisms capable enough of fermenting both hexose and pentose have to be identified. Thirdly is the logistics and storage of these biomass materials. Expenditures incurred during the bioprocess primarily depends on the enzymes used, type of sugar produced and in parameters considered during ethanol production [36]. There are less chemical and structural explanations of various pre-treatment methods which can help the researchers further for an improvised conversion. Presently in industries, the lignocellulose is hydrolysed to reducing sugars either with dilute acid and steam explosion or by hydrothermal/enzymes techniques [37,38]. Though acid can break down cellulose, it generates unwanted inhibitors such as furans, phenolics, weak acids, raw material extractives, heavy metal ions and entails more corrosion to the operating systems. [39,40]. Enzymatic hydrolysis claims a better prospect and advancement over acid hydrolysis [1]. A range of synergistically acting enzymes required in hydrolysing a lignocellulosic biomass to liberate different mono-sugars are always prepared with a consideration of optimal specific activity, stability, binding and end-product inhibition [38]. Again enzyme hydrolysis and its entire related parameters add to the overall cost when scaled up to an industrial level making it a lesser option for large scale ethanol production. This thesis extrapolates the venue of microbial hydrolysis through consolidated bioprocess using *Clostridium thermocellum* DSM1313 with a goal of enhanced ethanol titre.
1.7 AIM

Enhancing ethanol production of lignocellulose substrates using an anaerobic thermophilic organism *Clostridium thermocellum*

1.8 RESEARCH HYPOTHESES

Categorical methodology was designed and conducted for increasing the bioethanol titre produced by *C. thermocellum* as well as for quantifying ethanol using RP-HPLC.

1.8.1 Approach I: Recombinant DNA technology

Genetic modification of the metabolic pathway of organism where deletion of the lactate dehydrogenase enzyme gene (*ldh*) can divert the pyruvate pool towards alcohol dehydrogenase for enhanced ethanol production.

1.8.1.1 Objectives

i. Construction of knockout vector for *ldh* gene in *C. thermocellum*

ii. Transformation of *C. thermocellum* with knockout vectors and selection of recombinants

1.8.2 Approach II: Bioprocess Technology

Acclimation of *C. thermocellum* in lignocellulosic substrate can induce the innate hydrolytic characteristic and thereby the ethanol production. Concomitant fermentation of this lignocellulosic spent media by Zymomonas *mobilis* / Saccharomyces *cerevisiae* will further increase the total bioethanol yield.

1.8.2.1 Objectives

i. Physical and chemical pre-treatment of lignocellulosic substrates

ii. Microbial hydrolysis of lignocellulosic substrates using wild and acclimated *Clostridium thermocellum*

iii. Concomitant cultivation of the lignocellulosic hydrolysate using *Zymomonas mobilis* / *Saccharomyces cerevisiae*
1.8.3 Development of an Analytical Method

A simple, user-friendly, and a technique devoid of any chemical modification at the same time cheap and accurate can be conducted in RP-HPLC by estimating the negative peaks.

1.8.3.1 Objectives

i. Method optimization : Wavelength determination and protocol optimization

ii. Peak Confirmation : Confirmation of water, acetone and ethanol peaks

iii. Peak Occurrence : Confirmation of acetone and ethanol peak with GC-MS

iv. Method Validation with statistics analysis

v. Test Sample Analysis
1.9 OUTLINE OF THE THESIS

The primary aim of this research is to tap the potentials of Clostridium thermocellum DSM1313 through genetic engineering as well as through a conventional microbiological method for enhanced ethanol production. Only agricultural lignocellulosic biomass were sourced as substrates feeds. The main emphasis was to develop a strategic approach which can enhance bioethanol production. This thesis is organized into four chapters.

The first (current) chapter, Introduction, provides the insight of bioethanol and its role as transportation fuel. The relevance of cellulosic ethanol is highlighted in context to world-wide production. Clostridium thermocellum the organism of interest is introduced along with its major characteristics. The drawbacks faced in industries during bioethanol production and possible solution with the application of this organism is hypothesized here and eventually leading to the objectives of the research.

In Chapter 2, a detailed literature review regarding bioethanol and its production using different ethanolgenic organisms; pre-treatment and hydrolysis techniques followed by cellulosic bioethanol production and its quantification; in depth scrutiny of Clostridium thermocellum and its genetic breakthroughs till date is reported.

Chapter 3 deals with detailing of all the methodologies followed throughout the research. It includes all the regents, buffers and media preparation. The procedures carried out in approach I, approach II and the newly developed and standardised methodology for ethanol quantification are all explained here.

Chapter 4 brings out the results & discussion of each approach in two separate sections. A final discussion of the research is included analysing both the approaches in relevance to the present need of the hour. Third section includes the methodology development for ethanol quantification using RP-HPLC.

Chapter 5 contains the summary and conclusions of the thesis as a whole. At the end of the thesis, in the bibliography section, all of the referenced articles, books and other resources are listed.