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Engineering for Cellulases from Escherichia coli and Thermostable Organisms for increased Production of Biofuels

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Abstract

In order to mitigate climate change there is a possibility of producing biofuels from renewable sources. The design of biocatalysts can efficiently convert cheap lignocellulosic raw material into liquids fuels. Genetic and metabolic knowledge associated with Escherichia coli, makes this bacterium is the most appropriate starting point for engineering catalysts for biofuel production. Hemicellulose and lignin, together "lignocellulose" prevents access of cellulolytic enzymes to the cellulose. Cellulases are subjected to tight induction and regulation systems and additionally suffer inhibition from numerous end products. Bacterial cellulases are considered to be more stable. Work has been done on the cellulases to improve its thermostability which could increase the hydrolysis performance but still there is no thermostable cellulase available for commercial applications. Approaches like protein engineering, reconstitution of protein mixture are gaining importance to improve thermostability. Further the details about the importance of biofuel application and the distinctive challenge that protein engineering faces in the method of changing lignocelluloses to biofuels and their advances in this field has been highlighted.

Keywords: *Biofuels, Cellulase, Escherichia coli, Lignin, Lignocellulose*

1. Introduction

Use of fossil fuels has increased rapidly in the twentieth century and the need for its use is continuously increasing [1]. This enhanced using of petroleum reserves has increased concerns like depletion of reserves for future need, unequal distribution of reserves, and global climate change due to increased greenhouse gas emissions. In order to reduce the province on fossil fuel reserves, important attention has been paid in the recent past few years to develop alternate renewable energy sources such as biofuels through cellular transformation of biomass into fuels. Remarkable success

has been achieved in the production of bio ethanol in industrial scale as major biofuel alternatives to the traditional transportation fuels [2].
By now, the exceeding concept of carbon neutrality has gained sufficient political and social impetus so as to enable the recent entry of at least two types of biofuels into the marketplace-ethanol from sugarcane or corn, and biodiesel from soybean, rapeseed, or palm oil. Given that neither product can be made up to completely replace petroleum-derived transportation fuels, it is widely expected that biofuels of the future will likely be derived from agricultural waste or dedicated lignocellulosic crops. Polymeric cellulose, hemicelluloses and lignin must be broken down into simpler compounds such as sugars or organic acids. Second, these monomers (or oligomers) must be deoxygenated into liquid fuel. For such fuels to economically compete with fossil fuels, both processes must operate with maximum possible atom economy and volumetric productivity.
Genetic engineering of a biocatalyst for fuel production is the choice of a microbial host. In this review, we highlight on *Escherichia coli* [3, 4] because of its excess of sophisticated genetic tools as well as its recent track record in the biotechnology industry. The advances enabled us to improve natural pathways, to construct new biosynthetic pathways de novo for the optimal production of the desired biofuel products. In addition, the development of new sequencing technologies enabled the identification of the genetic variations, understanding the diverseness, and characterization of the genetic makeup of organisms, which could play a role in generating new classes of biofuels [5]. All the biofuels derived from *E. coli* so far are derived from the moderation of central carbon catabolism and the process includes the conversion of hexose/pentose sugar molecule into C_2 molecules, and the further modification of C_2 molecules. Given the recent advances in technologies for the microbial production of
biofuels we climax the metabolic engineering and systems biology approaches utilized in *E. coli* for making biofuels.
Biofuels can also be derived from plant biomass through microbial fermentation; here thermophiles exhibit higher potential for ethanologenic fermentation due to thermodynamic advantages with decreased processes costs by degrading input energy. Here we will be highlighting about the using of thermophilic bacteria for bio ethanol production [6].
Increased energy use duo with depleting petroleum reserves and increased greenhouse gas emissions have regenerated our interest in making fuels from renewable energy sources by microbial fermentation [7]. To this the problem is the choice of microorganism that catalyses the production of fuels at high volumetric productivity and yield from cheap and copiously available renewable energy sources. Microorganisms that are metabolically engineered to deflect renewable carbon sources into desired fuel products are observed as best choices to obtain high volumetric productivity and yield. Contemplating the availability of vast knowledge in genomic and metabolic fronts, *Escherichia coli* are regarded as a primary choice for the production of biofuels [8].
Biofuels represent a sustainable, renewable, and also the just predictable energy supply to fossil fuels. During the green production of biofuels, several processes take place in the conversion of biomass to sugars by engineered enzymes, and the succeeding conversion of sugars to chemicals by designed proteins in microbial production hosts [9]. Enzymes are necessary within the effort to provide fuels in an ecologically friendly manner. They have the potential to catalyse reactions with high specificity and potency while not using dangerous chemicals. Nature provides an in-depth variety of enzymes, however usually these should be altered to perform desired functions in needed conditions. Presently available enzymes like cellulose are subject to tight induction and regulation systems and additionally suffer inhibition from numerous end products. Therefore, more vulnerable and economical catalyst preparations ought to be developed for the enzymatic method to be more economical. Approaches like protein engineering, reconstitution of protein mixtures are gaining importance [10]. Advances in enzyme engineering allow the planning and/or directed evolution of enzymes specifically tailored for such industrial applications. Recent years have seen the production of improved enzymes to help with the conversion of biomass into fuels [11].
Climatic changes have laid further stress on already decreasing fossil fuels. In response to the emerging energy needs, biofuels can be considered as the safest and sustainable energy resources. At present, ethanol fermentations have been successful in fuelling motor vehicles in some countries [12,

13]. However, with the existing population, obtaining ethanol from food competing commodities might not be a desirable option. Therefore, non-food competing second-generation biofuels are the right choice to help the increasing energy demand. In this review potential of lignocellulosic biomass, the largest renewable natural resource for biofuels' generation has been discussed in reference to economical consideration. The strategies will likely involve thermophilic microbes possessing cellulolytic as well as ethanologenic potential [14]. The process economics might be supported to some extent by procuring by products of some value from the bio energy fermentations. Nutritional rating of the fermentation residues for animal feed may improve the biofuel economics**.**

The development of a potential single culture that can produce ethanol which is beneficial for industrial application. Strain improvement by molecular approach was proposed on ethanol producing bacterium, *Escherichia coli* SS1. *E. coli* SS1 is subjected to fermentation using 10 g/L of glycerol at initial pH 7.5. Nevertheless, wild-type SS1 reported hydrogen yield of 0.57 mol/mol glycerol and ethanol yield.

Glucose fermentation was also conducted for comparison study. The performance of wild-type SS1 is studied [15]. Lignocellulosic biofuels represent a sustainable, renewable, and the only computable alternative energy source to transportation fossil fuels. However, the uncooperative nature of lignocellulose poses technical hurdles to an economically viable bio refinery. Low enzymatic hydrolysis efficiency and low productivity, yield, and titer of biofuels are among the top cost contributors [16]. Protein engineering has been used to improve the performances of lignocellulose-degrading enzymes, as well as proteins involved in biofuel synthesis pathways. Unlike its great success seen in other industrial applications, protein engineering has achieved only moderate results in improving the lignocellulose-to-biofuels efficiency [17]**.**

Biofuels

Biofuels which are mainly obtained from biomass produced by the very slow geological processes that might play a significant role for renewable energy provider for transportation. It plays an important role in reducing carbon dioxide emissions. Biofuels are one of the largest sources of renewable energy in use today [18].

Types of Biofuels

Biofuels are classified into 3 totally different generations, based on biomass feedstock. The first-generation biofuels can even be referred to as standard biofuels, and they are basically obtained from food crops and edible oil seeds; their technologies are mature and comparatively cheap. However, first generation biofuels draw wide disapproval owing to their competition with food and fibre production, in addition of large consumption of chemical and H_2O. Excess production of first-generation biofuels can considerably increase food costs [19].

Second generation biofuels are principally made from lignocellulosic biomass, non-edible oil seeds and wastes. They gain the benefits of getting less food crop competition. Conversion of waste lignocellulosic remnants to biofuels by eco-friendly technologies would be useful for the making. The energy price in the yield of substance in second generation processes is also low compared with food crops (e.g., prices in ploughing, composting and harvesting). Second generation biofuels are also less costly than first generation biofuels, if the capital prices and additional advanced pre-treatment processes will be seen by a budget substrate resource.

Third generation biofuels are principally obtained from algae. Algae, which may be grouped as microalgae and macro algae (seaweeds), are identified for chemical action efficiencies and productivities, thereby rising to lower space needs compared with land-based plants, like maize, corn and panic grass. Algae can be grown in non-freshwater sources, like salt water and H2O on non-arable land, and don't contend with regular food resources.

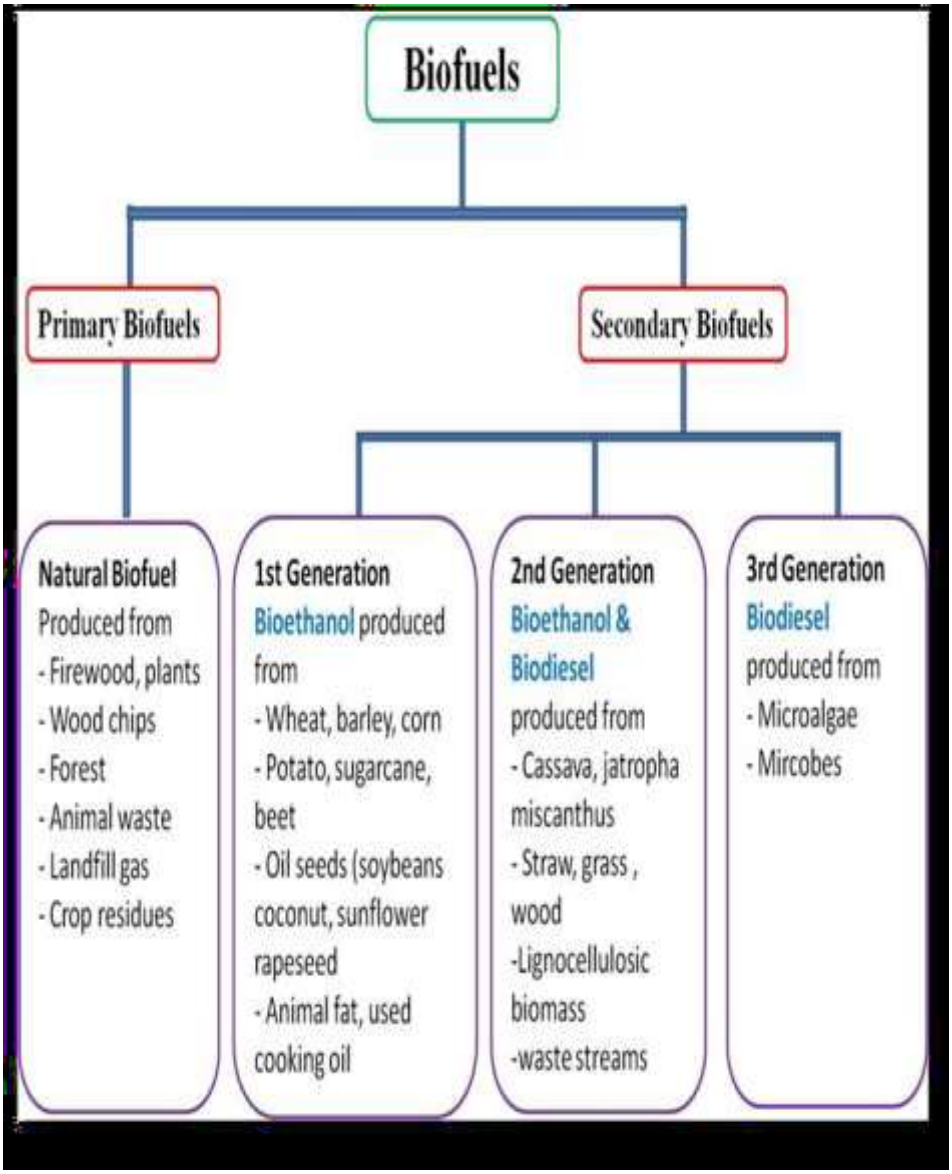

Figure (1): Biofuel production sources [14]

Process of Biofuel Production

Bioethanol

Lignocellulosic biomass is an important possible resource for the production of biofuels because of its being ample, cheap and production of such resources is environmentally achievable. Agricultural remnant like stems and stalks from sources such as corn fibre, corn fodder, biogases, rice hulls, woody crops are a great source of lignocellulosic biomass which are renewable, chiefly unutilized, and cheap. Also, there are waste from industrial and agricultural processes like citrus peel waste, sawdust, paper pulp, municipal solid waste, paper mill sludge and energy crops including switch grass and other fodder feedstock like *Miscanthus*, Bermuda grass, Elephant grass, etc. add up to the multiple sources of lignocellulosic biomass [20]. Lignocellulosic biomass comprises mainly of cellulose, hemicelluloses and lignin. The prime component cellulose is a homopolysaccharide consisting of glucose units, linked by β-(1→4) glycosidic bonds. Cellobiose is the smallest transpire unit of cellulose and could be converted into glucose. Hemi cellulose is a heterogeneous polymer, made up of mainly pentose (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. Hemi cellulose has mainly xylans in hardwood, while glucomannans are present in softwood. Hydrolysis of cellulose and hemicelluloses needs various types of enzymes. Briefly, cellobiose degradation needs endoglucanase while xylan degradation needs endo-1-4,-β-xylanase, β-xylosidase,

as well as acetylxylan esterases. In glucomannan degradation β-mannanase and β-mannosidase are needed to cleave the polymer backbone.

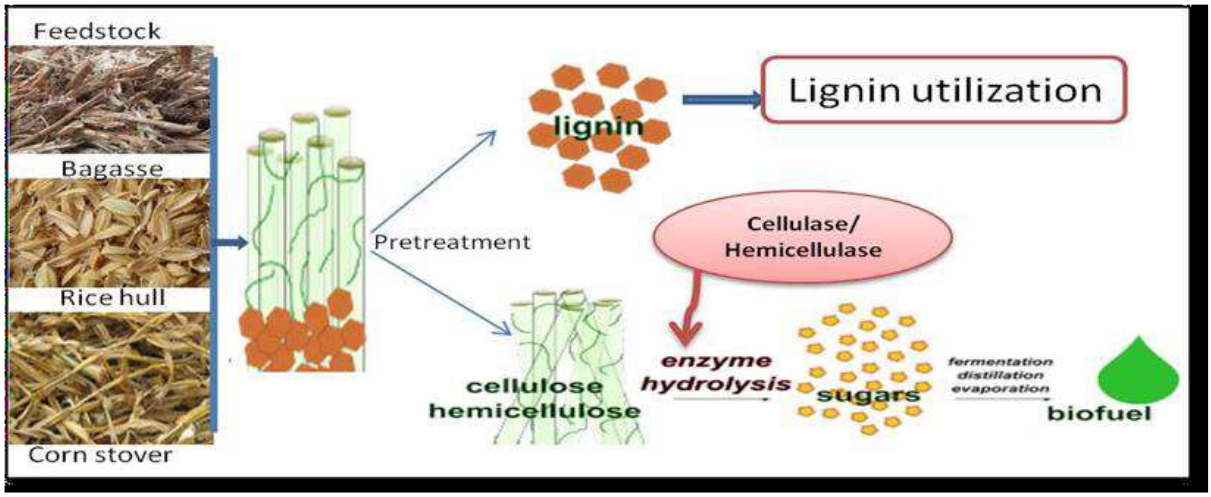

Figure (2): Production of biofuels from lignocelluloses biomass [20]

Biodiesel

Biodiesel is a form of biofuel obtained from renewable sources that has free fatty acids (FFAs) and triglycerides (TGs). Biodiesel is obtained by esterification of FFAs or transesterification of TGs and may provide limited solution as an alternative of fossil fuels. T Now, the main feedstock for biodiesel production is virgin oil such as soybean and rapeseed oil which increases the production cost and food competition therefore, non-edible oils (e.g., castor bean, jatropha, pongamia, etc.), low value lipids (e.g., animal fat, waste cooking oils, etc.) and microalgae have recently attracted notable interest. Biodiesel is a CO_2 neutral fuel and is now broadly supported as a sustainable renewal to diesel fuel for transportation applications. It can be obtained by two processes firstly, chemical-catalysed conversion processes, using alkali catalysts like NaOH or KOH and secondly by, the enzyme (lipase)-catalysed process. The enzyme (lipase)-catalysed process is more achievable because it avoids soap formation, do not produce large amount of waste water as compared to the chemical-catalysed method.

Despite their remarkable promise, there are essential challenges in adapting the lipase-catalysed method or cellulase process to industrial scale, including performance, stability, catalytic activity, and production of these enzymes. Mainly, the rate of enzymatic reactions is generally low. Improving the performance and durability of these enzymes and lowering their manufacturing cost will thus sustain the key to large-scale commercialization of enzyme-catalysed biofuel production.

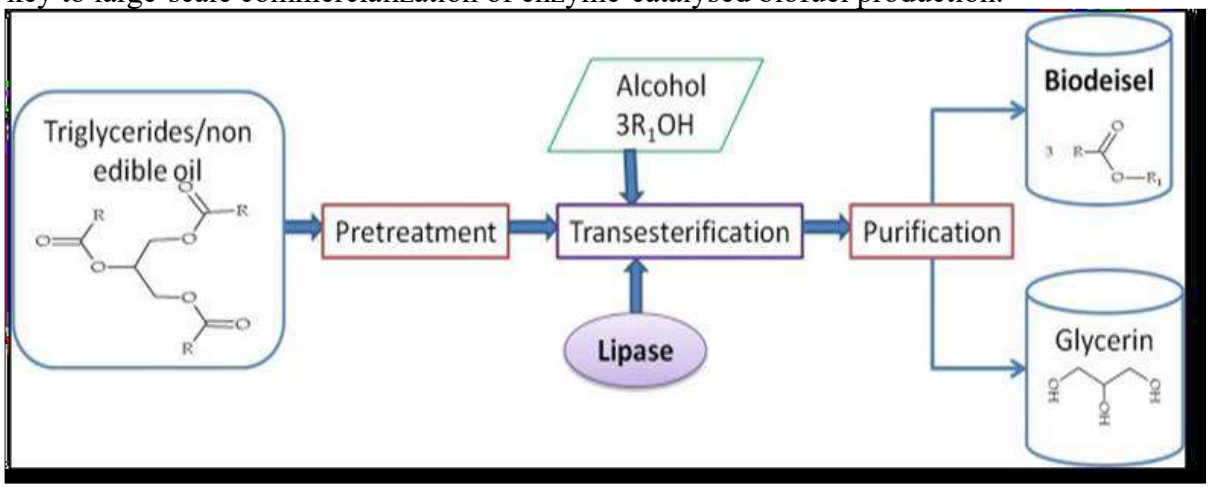

Figure (3): Production of biodiesel from lipase catalyzed transesterification reaction [20]

Engineering *E. coli* to Produce Bioethanol

Presently, ethanol is influencing the biofuel industry and is commercially being produced as a possible renewable fuel irrespective of its limitations such as corrosiveness and low energy. The major source of ethanol production is lignocellulosic feed stock material (composed of lignin, hemicelluloses, and cellulose) and is considered a less costly available renewable energy source for ethanol production. The hemicelluloses component of lignocellulosic biomass converts into hexose sugars (mannose, glucose, and galactose) and pentose sugars (xylose and arabinose), which are basically converted into ethanol through the fermentation process. Organisms such as *S. cerevisiae* and *Zymomonas mobilis* are mainly used as front runners to produce ethanol through fermentation. However, these organisms cannot use pentose sugars and thus limit our ability to harness maximum productivity. In search of other alternatives, organisms such as *E. coli* and *Clostridia* sp are considered because of their ability to use both pentose and hexose sugars. Here, we focus on the plan that is being used to produce ethanol from *E. coli.*

The domestic *E. coli* is capable of producing ethanol through an endogenous process in which under anaerobic conditions one mole of glucose is metabolized into two moles of formate, two moles of acetate, and one mole of ethanol. The last step in the endogenous ethanol production process involves the reduction of acetyl-coA into ethanol by AdhE [21, 22]. The reduction reaction absorbs two NADH molecules, while the initial glycolysis in order to convert glucose to puruvate produces only one NADH (1NADH for each glyceraldehyde 3 phosphate to 1,3-Bisphosphoglycerate) leading to redox variance. To control the redox imbalance, the domestic *E. coli* balances the production of ethanol by oxidation of acetyl-coA into acetate, which needs no NADH. This domestic fermentation process leads to the minimal level of production of ethanol, which is estimated to be 0.26 g ethanol/g of glucose, whereas the maximum possible conceptual yield is 0.51 g ethanol/g of glucose [23].

To reduce the problems existing in the endogenous ethanol production process, Ingram et al3 have made successful attempts of genetic engineering in *E. coli* to produce high quantities of ethanol by placing genes such as *pdc* and *adhB* from *Z. mobilis*. The *pdc* and *adhB* genes were expressed in operon from a plasmid under an essentially expressed artificial *pet* (production of ethanol) promoter to produce pyruvate decarboxylase and alcohol dehydrogenase II, respectively. This heterologous fermentation pathway produces 95% of the final products as ethanol without creating any redox variance (consumes only one NADH). In order to further support the *E. coli* to continuously generate ethanol production, Ohta et 2005 constructed an *E. coli* ATCC 11303 strain KO3 through chromosomal integration of *pdc*, *adhB* genes along with a selective chloramphenicol resistance gene.

To further increase ethanol production, an *frd* gene (encoding fumarate reductase) was deleted from a KO4 strain (isolate of KO3) leading to 95% reduction in succinic acid in the resulting KO11 strain. Relative to the KO4 strain, this KO11 strain observed higher ethanol productivity (41.6 g/L ethanol over 72 h as opposed to 36 g/L of KO4) and theoretical yield (104% as opposed to 94% in KO4) in 8% xylose and equal productivity (52.8 g/L) and yield (~110%) in 10% glucose. KO11 strain successfully produced ethanol from various lignocellulosic hydrolysates at 10,000 L capacity.4,40 Directed evolution of KO11 was carried out to enhance its ethanol-tolerance capabilities through alternate cycles of selection in liquid media (to increase ethanol tolerance) and solid media (to increase ethanol production) leading to the LYO1 strain.41 A lactate-producing isolate of KO11, the SZ110 strain, was reengineered to delete all fermentative routes for NADH and insert complete ethanol-producing pathway genes *pdc*, *adhA*, and *adhB* into chromosomes. The generated LY160 strain has produced high ethanol (46 g/L) in minimal medium and with lower-grade carbon source xylose; leading an economical way to produce ethanol [24].

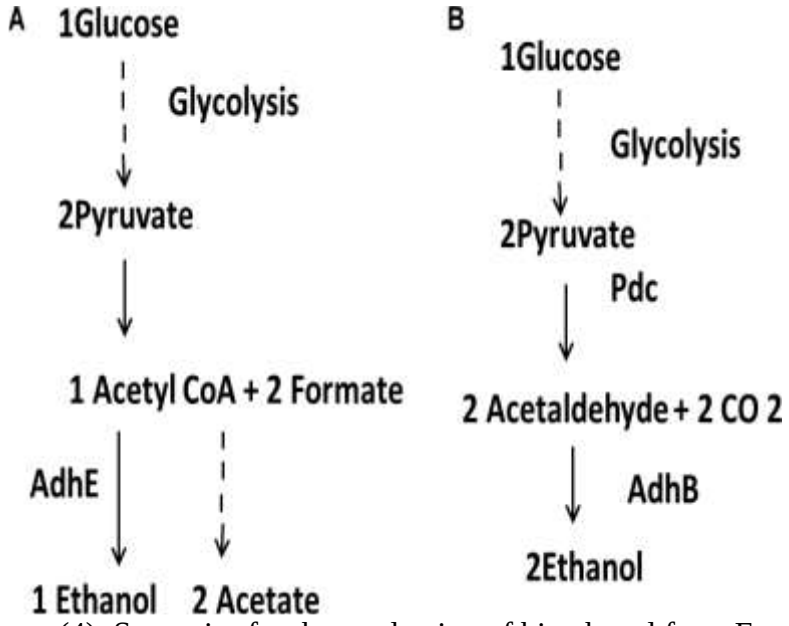

Figure (4): Strategies for the production of bioethanol from E. coli.

(A) Endogenous ethanol production pathway/Heterofermentative pathway for ethanol production in *E. coli*. (B) Metabolic engineering pathway for ethanol production in which endogenous *E. coli* ethanol production pathway was engineered by expressing *pdc* and *adhB* genes of *Zymomonas mobilis*. Broken arrows represent the pathways that involve multiple enzymes and steps (Abbreviations: Pfl, pyruvate formate lyase; AdhE, alcohol dehydrogenase; Pdc, pyruvate decarboxylase; AdhB, alcohol dehydrogenase II [21]).

Engineering *E. coli* to Produce Biodiesel

Biodiesel along with bioethanol component almost contain 90% of the industrial production of biofuels. The main source of the current industrial biodiesel production is from triacylglyceride-rich vegetable oils such as rape seed oil. The production process involves a catalytic transesterification of vegetable oil with petro chemical–derived methanol. Given the stand up public concerns of utilizing vast land area to produce vegetable oils for diesel rather than for food, another ways such as using microalgae and bacteria are being seen. Although the use of *E. coli* to produce biodiesel is still in its early stage, we made an effort to put together the pertinent available knowledge in this field [25].

Kalscheuer in 2007 introduced the idea of transesterification of fatty acids with bioethanol (instead of currently petro chemically derived methanol) to produce fatty acid ethyl ester (FAEE) biodiesel (micro diesel). First, *E. coli* was engineered to produce bioethanol by introducing *Z. mobilis* genes *pdc* (encodes puruvate decarboxylase) and *adhB* (encodes alcohol dehydrogenase) as outlined in Figure 1B. To esterify ethanol with fatty acid–derived Acyl CoA, *E. coli* was engineered with gene *atfA* (encodes unspecific acyl transferase) from *Acinetobacter baylyi*. The process needed external addition of fatty acids, as the acyl transferase did not use the fatty acids produced in *E. coli* when grown on glucose. In 2011, Steen developed an *E. coli* strain, A2A, which is capable of using hemicellulose or glucose to produce fatty acids that can be used for biodiesel production. Using this strain, they observed the production of FAEE biodiesel with a yield of 9.4% of the theoretical maximum. In 2012, developed a dynamic sensor-regulator system (DSRS) in A2A *E. coli* to improv the stability of the strain [26]. They engineered *E. coli* with transcription factors that regulate the expression of genes involved in biodiesel production leading to increased titer to 1.5 g/L and increase in yield by threefold to 28% of the abstract maximum. Attempts to make fatty acid methyl esters (FAME) diesel using *E. coli* by transesterification of fatty acids with methanol is also progressing rapidly. Although these are good starting points in using *E. coli* for biodiesel production, further advance in yield and productivity are needed to practically replace petroleum-derived diesel.

Ethanol Production from *E. colli* and its Recombinant Strains used During Fermentation

Ethanol was produced equally by wild-type SS1 approaching a maximum concentration of 3.20 g/L after 48 h during glycerol fermentation at initial pH 7.5. The recombinant hybC produced ethanol achieving approximately 2.44 g/L within 12 h of fermentation.

As for glucose fermentation at initial pH 7.5, the ethanol production of both wild-type SS1 and recombinant hybC occurred exponentially within 12 h yielding 1.30 g/L and upland after 12 h. The ethanol yield and productivity of wild-type SS1 and recombinant hybC

at initial pH 7.5 are seen. The ethanol yield of recombinant hybC obtained in glycerol fermentation was lower than the wild-type SS1, whereas no important change of ethanol yield was observed between both strains during glucose fermentation. This showed that the bioconversion of glycerol into ethanol was affected by the additional copy of hybC gene. The ethanol yield of recombinant hybC was found to decrease further under acidic condition and achieved approximately 0.31 mol/mol glycerol at initial pH 5.8[27] The lower ethanol yield (0.50 mol/mol glycerol) was also seen in the recombinant hycE at initial pH 5.8 Albeit lower ethanol was surrendered by recombinant hybC during glycerol fermentation, the ethanol productivity was 3-fold higher in comparison to wild-type SS1 at initial pH 7.5, probably due to higher usage rate of glycerol and higher cell growth [28] [29]. It was noted that the ethanol yield of wild-type SS1 obtained in glucose fermentation was lower than glycerol fermentation. In theory, both glycerol and glucose could yield similar molar of ethanol which is one mole of ethanol per mole substrate. However, wild-type SS1 tends to produce higher ethanol yield from glycerol rather than glucose. Yet, the ethanol productivity of glucose fermentation was higher than glycerol probably due to higher cell growth [30].

Extremophiles sued in the Production of Biofuels

There are a number of advantages of using extremophiles in industrial applications, mainly in the production of biofuels. Extremophiles are vigorous organisms producing stable enzymes, and are often able to tolerate changes in environmental conditions, such as pH and temperature [31]. In evaluating the information available on the use of extremophiles in biofuel production, it became evident that the majority are of thermophilic source. This is not shocking since thermophiles have a extraordinary ability to tolerate variation in pH, temperature and environmental change, an allocate which offers a clear advantage in the development of a commercially feasible process. Thermophiles readily ferment pentose and/or hexose sugars from biomass and, in some cases, even structurally complex carbohydrates, a quality which is mainly important for production of second-generation biofuels. Moreover, thermophilic industrial fermentations are less susceptible to microbial contamination and require lower energy inputs as a result of the reduced cooling steps needed between the fermentation steps. Also, the removal of any explosive products, which in turn reduces the problem of product inhibition, is facilitated. Despite the dominance of thermophiles in biofuels, other extremophiles groups have also been used in this field, including methanogens (typically thermophilic, anaerobic archaea) and psychrophiles. Methanogens play a crucial role in the production of biogas, whereas psychrophiles are being utilized for their cold-adapted lipases for use in biodiesel [32]. The application of these extremophilic organisms and their enzymes in the production of biofuels, particularly for bioethanol and to a lesser extent in the production of other biofuels [33].

Bioethanol

Perfect microbiological strains for bioethanol production should produce high yields of ethanol, with few side products, and have low inhibitor sensitivity and high ethanol tolerance. Industrial production of bioethanol, using improved strains of *Saccharomyces cerevisiae* from sugar cane molasses or enzymatically hydrolysed starch, yields as much as 20% (v/v) of ethanol. The bacterium *Zymomonas mobilis* has also been used for bioethanol production as it contains an ethanol fermentation pathway, resulting in a higher ethanol yield than *S. cerevisiae*, and it can tolerate up to 120 g/L of the product. However, for bioethanol to become economically applicable, the use of lignocellulosic material as a source of bioethanol production is a need. This process requires the hydrolysis of cellulose, which is catabolised into hexose sugars and hemicelluloses, containing mostly of pentose sugars. Not like *S. Cerevisiae* and *Z.mobilis*, which can use only hexose sugars, a large number of thermophiles are able

to brew both hexose and pentose sugars derived from biomass and hydrolysates. This allows for high growth and metabolic rates of organisms growing on both cellulose and hemicelluloses [35, 36].

Biodiesel

Production of biodiesel is a grown-up technology for use in compression-ignition (diesel) engines. The cost of the plant raw materials averages 70% of the total production cost which involves processing of the vegetable oils by transesterification into mono alkyl esters of the plant fatty acids. Regrettably, these fulsome plants produce fatty acids that account for around only 5% of their total biomass, providing small quantities of biodiesel to be used for mixing with petroleum diesel [37]. If biodiesel is to become an economically applicable resource, more efficient novel sources of oil, such as microalgae as well as from extremophilic organisms, need to be researched [38, 39].

Thermophilic Ethanologenesis and its Significance

Deriving from LCB, sugars need for ethanol fermentation can be homogenous or heterogeneous. Conventional baker's yeast, *Saccharomyces cerevisiae* can degrade hexose and disaccharides but unable to use pentose and complex biopolymers present in LCB feedstock [40]. That is why S. Cerevisiae has been widely used for ethanol production. This yeast has multiple importances compared to other known ethanologenic microbes which include higher ethanol yields i.e. (1.9 mol of ethanol/mole of hexose, higher ethanol tolerance i.e. 12 %), higher robustness and higher confrontation to the toxic inhibitors. Another highly efficient ethanologenic microbe is *Zymomonas mobilis* which is a mesophilic bacterium and tolerates roughly up to 12 % ethanol and has the potential of 2.5 times faster growth than yeasts. Compact preview of lignocellulosic bioethanol production is outlined. The processes of saccharification and the capacity of micro-organisms to use both categories of monomeric sugars (C6/C5) and their potential of withstand lignocellulose derived chemical inhibitors, ethanol tolerance and feedback mechanisms at various steps that potentially affect the overall ethanol yield [41]. Thus detailed information and novel solutions of congestion of the process are obligatory to develop efficient lignocellulosic bioethanol production plants. Thermo stability is another important characteristic affecting the fermentation efficiency positively. Thermophiles might have the same ways of oxidation and fermentation process familiar to the mesophiles. However; their thermozymes are more systematic as the biochemical reactions occur at raised temperatures [42, 43].

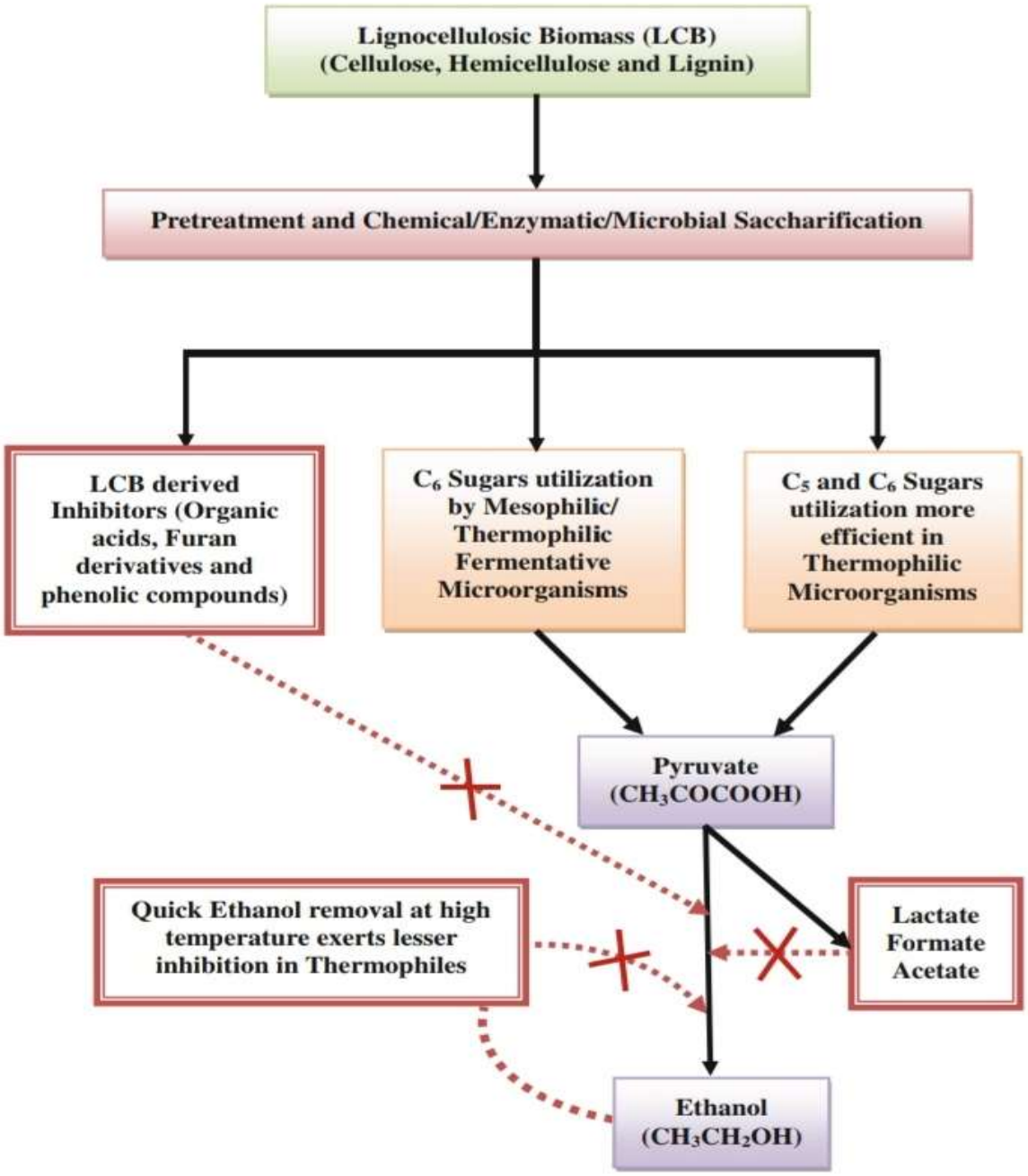

Figure (5): Simplified mechanism of ethanologenesis from lignocellulosic biomass.

Thermophilic bacteria advantageously utilize both C5 and C6 sugars of complex biopolymers (Cellulose and Hemicelluloses) for ethanologenesis and here ethanol intolerance is of lesser significance than mesophiles. Inhibitory effects on the LCB ethanologenic process are indicated as red dotted lines [50].

Engineering Cellulases

Cellulose is a linear homopolymer of glucose linked by β-1,4-glycosidic bonds. As the most ample, yet the most intractable constituent of plant cell wall, cellulose hydrolysis is a censorious and challenging step, involving the action of three major types of cellulases: endoglucanases, exoglucanases (including cellodextrinases and cellobiohydrolase), and β-glucosidases. Microorganisms have developed two strategies of using their cellulases: distinct noncomplexed cellulases that are mainly secreted by aerobic bacteria and fungi, and complexed cellulases (cellulosome) that are basically expressed on the surface of anaerobic bacteria and fungi. While

cellulosome engineering has mainly focused on enhancing the cellulosomal components, protein engineering has been applied to improve the performance of individual non-complexed cellulases. In spite of continuing efforts to enhance non-complexed cellulase performances, the improvements obtained so far using protein engineering approaches have been additive, mainly due to the difficulty of the insoluble substrates and the lack of high throughput screening/selection methods [51, 52] .Limited knowledge of the biochemical mechanisms involved in cellulose hydrolysis has limited the success obtained by rational and semi-rational design strategies in cellulase engineering, and no notabe activity enhancement has been reported to date. Although cellulase activity on insoluble substrates is hard to forecast, the stability of the cellulase itself could be very well modeled by the SCHEMA energy function. Using a SCHEMA structure-guided recombination method, 15 highly diverse thermostable cellobiohydrolase hybrids (up to 7 °C higher than the most thermostable parent) were obtained by screening only a total of 73 variants. Considering the fact that protein stability enhances both mutational vigorous and evolvability, this group of wide cellobiohydrolases provides a better platform for improving their catalytic efficiency [53].

In an effort to adapt directed evolution to cellulase engineering, a high throughput selection method was recently developed based on chemical addition to improve endoglucanase activity. In this study, the researchers gracefully designed an oligosaccharide [54] surrogate by imbedding a cellotetraose between a methotrexate and a dexamethasone, which acted as a transcription inducer linking the hydrolysis activity of endoglucanases to the survival of a URA3-FOA counter-selection yeast strain. This method was of very high output and yielded two variants with upgrade catalytic efficiency (3.7- and 5.7-fold) from a family DNA shuffling library with a size of 108. However, since the selection was based on cleavage of a soluble substrate (methotrexate-cellotetraose-dexamethasone) by intracellular enzymes, it could not be used to engineer cellulase activity toward insoluble substrates. The fact that there is no clear connection between enzyme activity on soluble substrates and that on insoluble substrates. By using ultracentrifugal milling and a robotic multi-pipetting workstation, the problem of irreproducible solid substrate delivery was solved. Although no application of this system was reported, the integration of high throughput pretreatment, fermentation and microplate format described here has the potential to allow high throughput engineering of the entire lignocellulose-to-biofuels process in a miniature biorefinery.

Protein Engineering for Improvement of Enzymes

In spite of the broad spectrum of cellulases, hemicellulses and lipases being isolated, no single catalyst is completely suitable as it is, for the hydrolysis of cellulose or the chemical process of the transesterification reaction in the bio refining industry. However, these enzymes provide a sufficient place to begin for the development of these enzymes in steps towards increasing the economics of biofuel production [55]. Basically, the utilization of protein engineering technology has been pointed towards the study of catalytic activity of those enzymes. Recently, modifications to enzymes isolated from microorganisms through the utilization of protein engineering is taking a stage within the production of economical hydrolytic enzymes utilize in a vast scope of industries and comprises targeting structural amino acids, on the far side amino acids within the catalytic site.

Future Perspectives

Biofuels are of quickly growing interest thanks to energy security, sustainability, and climate change. The first-generation biomass has been used to produce ethanol from corn and sugarcane on a large scale in some of the developed countries. Although, this will lead to food competition and thus, the second-generation biofuel technology based on lignocelluloses, non-edible oil is under great study. Several factors will affect the economic viability of second-generation bio refinery. With the development of high-throughput screening/selection methods, protein engineering plays an important role in producing new, more active enzymes for the hydrolysis of biomass to sugars, succeeding microbial conversion of sugars to biofuel molecules and catalysis of transesterification reactions although the progresses reported to date have been advancing.

2. Conclusion

One possible reason for the limited success of protein engineering might be that the enzymes used as engineering templates so far were obtained from a very limited sequence space – namely culturable microorganisms, which, on average, represent <1% of the genetic diversity found in nature. To overcome this limitation imposed by traditional microbiological techniques, new strategies such as metagenomics and single-cell genomics were developed. A recent metagenomic study of a wood-degrading termite revealed hundreds of hitherto unknown glycoside hydrolase genes. These narrative cellulolytic proteins might expand the current plant-cell-wall-degrading enzyme model and allow more high-yield protein engineering studies. With the continuing development of new tools and scientific knowledge, appropriate progress will be there towards the production of next generation biofuels. Collaborative research programs combining protein engineering, metabolic engineering, chemical catalysis, and chemical process engineering for high enzyme activity will lead to an economically viable bio refinery in the near future.

3. References

1. Kerr RA. Climate change. Global warming is changing the world. Science. 2007; 316(5822):188–190.
2. Stephanopoulos G. Challenges in engineering microbes for biofuels production. Science. 2007; 315(5813):801–804.
3. Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol production in Escherichia coli. Appl Environ Microbial. 1987; 53(10):2420–2425.
4. Liu T, Khosla C. Genetic engineering of Escherichia coli for biofuel production. Annu Rev Genet. 2010; 44:53–69.
5. Antoni D, Zverlov VV, Schwarz WH. Biofuels from microbes. Appl Microbiol Biotechnol. 2007; 77(1):23–35.
6. Sheridan C. Making green. Nat Biotechnol. 2009; 27(12):1074–1076.
7. Wen M, Bond-Watts BB, Chang MC. Production of advanced biofuels in engineered E. coli. Curr Opin Chem Biol. 2013; 17(3):472–479.
8. Becker J, Wittmann C. Advanced biotechnology: metabolically engineered cells for the bio-based production of chemicals and fuels, materials, and health-care products. Angew Chem Int Ed Engl. 2015; 54(11):3328–3350.
9. Nigam, P. and Singh, A. Production of liquid biofuels from renewable resources. Prog Ener Comb Sci. 37 (2011) 52-68.
10. IEA, 2011. Technology roadmap: biofuels for transport.
11. Kiran, B., Kumar, R. and Deshmukh, D. Perspectives of microalgal biofuels as a renewable source of energy. Ener Convers Manag. 88 (2014)1228-1244.
12. Demirbas, D. Use of algae as biofuel sources. Ener Conver Manag. 51 (2010) 2738-2749.
13. Jones, C. and Mayfield, S. Algae biofuels: versatility for the future of bioenergy. Curr Opin Biotechnol. 23 (2012) 346-351.
14. Kim, S. and Dale, D. A distributed cellulosic biorefinery system in the US Midwest based on corn stover. Biofuels Bioprod Bioref. 7 (2013) 1-4.
15. Maki, M, Leung, KT. and Qin W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci. 29 (2009) 5(5):500-16.
16. Andrade, J., Pacrez, A., Sebastian, P. and Eapen, D. A review of bio-diesel production processes. Bioma Bioen. 35 (2011) 1008-1020.
17. Cui, J.D., Liu, R.L. and Li, L.B. A facile technique to prepare cross-linked enzyme aggregates of bovine pancreatic lipase using bovine serum albumin as an additive. Korean J Chem Eng. (2016) 33: 610–615.
18. Meher, L., Vidyasagar, D. and Naik, S. Technical aspects of biodiesel production by transesterification - A review. Renew Sustain Ener Rev. 10 (2006) 248-268.
19. Suehara, K., Kawamoto, Y., Fujii, E., Kohda, J., Nakano, Y. and Yano, T. Biological treatment of wastewater discharged from biodiesel fuel production plant with alkali-catalyzed transesterification. J Biosci Bioengg. 100 (2005) 437-442

20. Gray, KA, Zhao, L, Emptage, M. Bioethanol. Curr Opin Chem Biol. 2006; 10(2):141–146.
21. Kessler D, Leibrecht I, Knappe J. Pyruvate-formate-lyase-deactivase and acetyl- CoA reductase activities of Escherichia coli reside on a polymeric protein particle encoded by adhE. FEBS Lett. 1991; 281(1–2):59–63.
22. Schmitt B. Aldehyde dehydrogenase activity of a complex particle from E. coli. Biochimie. 1975; 57(9):1001–1004.
23. Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. Genetic improvement of Escherichia coli for ethanol production: chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol. 1991; 57(4):893–900.
24. Bi C, Zhang X, Ingram LO, Preston JF. Genetic engineering of Enterobacter asburiae strain JDR-1 for efficient production of ethanol from hemicellulose hydrolysates. Appl Environ Microbiol. 2009; 75(18):5743–5749.
25. Yomano LP, York SW, Ingram LO. Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. J Ind Microbiol Biotechnol. 1998; 20(2):132–138.
26. Yomano LP, York SW, Zhou S, Shanmugam KT, Ingram LO. Re-engineering Escherichia coli for ethanol production. Biotechnol Lett. 2008;30(12):2097–2103
27. Kalscheuer R, Stolting T, Steinbuchel A. Microdiesel: Escherichia coli engineered for fuel production. Microbiology. 2006; 152(pt 9):2529–2536.
28. Steen EJ, Kang Y, Bokinsky G, et al. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature. 2010; 463(7280):559–562.
29. Zhang F, Carothers JM, Keasling JD. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol. 2012;30(4):354–359.
30. Soo CS, Yap WS, Hon WM, et al. Improvement of hydrogen yield of ethanol-producing Escherichia coli recombinants in acidic conditions. Electron J Biotechnol. 2017;26: 27–32.
31. C. Gerday and N. Glansdorff, Physiology and Biochemistry of Extremophiles, ASM Press, Washington, DC, 2007.
32. B.S. Dien, M.A. Cotta, and T.W. Jeffries, Bacteria engineered for fuel ethanol production: Current status, Appt Microbiol Biotechnol. 63 (2003), pp. 258–266.
33. Zaldivar, J., Nielsen, J. and Olsson, L. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol. 56 (2001), pp. 17–34.
34. D. Antoni, V. Zverlov, and W. Schwarz, Biofuels from microbes, Appl. Microbiol. Biotechnol. 77
35. P. Sommer, T. Georgieva, and B.K. Ahring, Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. Biochem. Soc. Trans. 32 (2004), pp. 283–289.
36. P.L.K. Rogers, J. Lee, M.L. Skotnicki, and D.E. Tribe, Ethanol production by Zymomonas mobilis, Adv. Biochem. Eng. 23 (1982), pp. 37–842007), pp. 23–35
37. R. Kumar, S. Singh, and O.V. Singh, Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives, J. Ind. Microbiol. Biotechnol. 35 (2008), pp. 377–391.
38. J. Zaldivar, J. Nielsen, and L. Olsson, Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration, Appl. Microbiol. Biotechnol. 56 (2001), pp. 17–34.
39. S. Behzad and M.M. Farid, Review: Examining the use of different feedstock for the production of biodiesel, Asia-Pac. J. Chem. Eng. 2 (2007), pp. 480–486.
40. Taylor, M.P., Eley, K.L., Martin, S., Tuffin, M.I., Burton, S.G., Cowan, D.A.: Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends Biotechnol.27(7), 398–405 (2009)
41. van Maris, A.J.A., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A.H., Wisselink, H.W., Scheffers, W.A., van Dijken, J.P., Pronk, J.T.: Alcoholic fermentation of

carbon sources in biomass hydrolysates by Saccharomyces cerevisiae:current status. Antonie Van Leeuwenhoek 90, 391–418 (2006)
42. Rogers, P.: Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 31, 1–60 (1986)
43. Turner, P., Mamo, G., Karlsson, E.N.: Potential and utilization of thermophiles and thermostable enzymes in biorefining. Microb. Cell Fact. 6, 9 (2007).
44. Qazi, J.I.: Biotechnological potential and conservatory of extremophiles from climatically wide ranged developing countries: lesson from Pakistan. Crit. Rev. Microbiol. 39(1), 1–8 (2013).
45. Klinke, H.B., Thomsen, A.B., Ahring, B.K.: Inhibition of ethanol- producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26 (2004)
46. Nardon, L., Aten, K.: Beyond a better mousatrap: a cultural analysis of the adoption of ethanol in. Brazil. J. World Bus. 43, 261–273 (2008)
47. Singh, A., Pant, D., Korres, N.E., Nizami, A.-S., Prasad, S., Murphy, J.D.: Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: challenges and perspectives. Bioresour. Technol. 200);101, 5003–5012.
48. Ibraheem, O., Ndimba, B.K.: Molecular adaptation mechanisms employed by ethanologenic bacteria in response to lignocellulose- derived inhibitory compounds. Int. J. Biol. Sci. 2013;9(6), 598–612.
49. ynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506–577.
50. Weng JK, Li X, Bonawitz ND, Chapple C. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. Curr Opin Biotechnol. 2008;19:166–172.
51. Wen, F.; McLachlan, M.; Zhao, H. Directed evolution: novel and improved enzymes. In: Begley, TP., editor. Wiley Encyclopedia of Chemical Biology. John Wiley & Sons, Inc.; 2008.
52. Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006;24:452–481.
53. Heinzelman P, Snow CD, Wu I, Nguyen C, Villalobos A, Govindarajan S, Minshull J, Arnold FH. A family of thermostable fungal cellulases created by structure-guided recombination. Proc Natl Acad Sci USA 2009; 106: 5610–5615.