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Protein engineering for biofuel production: Recent development

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Abstract: The unstable and unsure handiness of crude oil sources moreover the rising price of fuels have shifted international efforts to utilize renewable resources for the assembly of greener energy and a replacement which might additionally meet the high energy demand of the globe. Biofuels represent a sustainable, renewable, and also the solely predictable energy supply to fossil fuels. During the green production of Biofuels, several in vivo processes place confidence in the conversion of biomass to sugars by engineered enzymes, and the subsequent conversion of sugars to chemicals via designed proteins in microbial production hosts. Enzymes are indispensable within the effort to provide fuels in an ecologically friendly manner. They have the potential to catalyze reactions with high specificity and potency while not using dangerous chemicals. Nature provides an in depth assortment 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 impregnable and economical catalyst preparations ought to be developed for the enzymatic method to be more economical. Approaches like protein engineering, reconstitution of protein mixtures and bio prospecting for superior enzymes are gaining importance. 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. The assembly of the many of those fuels is feasible due to advances in protein engineering. This review discusses the distinctive challenges that protein engineering faces in the method of changing lignocellulose to biofuels and the way they're addressed by recent advances in this field.

Keywords: Bioefuel, Protein engineering, cellulase, lipase.

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1. Introduction: In recent years, as a result of rising economies and increasing population, the worth of gasoline and diesel remains high. Supported the present consumption rate, the available supply of fossil fuels might last for <50 years **[1]**. Additionally, increasing CO_2 emissions owing to burning fossil fuels place pressure on the ecological cycle and will account for international temperature change. The growing concern towards the continued use of fossil fuels, unsustainable demands, speedy depletion of fuel reserves, international temperature change, rising oil prices and environmental degradation have forced governments, policymakers, scientists and researchers to search out energy sources. In recent years, several microorganisms, as well as algae, fungi, and bacteria, are used for the assembly of fuel bio-chemicals **[2]**. The biofuel production from renewable sources is wide thought-about to be one in all the foremost alternatives to fossil fuels and a viable suggest for environmental and economic property. Design to built microbes for transformation of biomass into renewable fuels and chemicals remains a nascent and growing field **[3]**.

1.1 Biofuels: Biofuels which are predominately obtained from biomass might play a significant role for renewable energy provider in transportation [4]. Biofuels like biogas, biomethanol, bioethanol and biodiesel, are thought to be a substitute to fossil fuels in the upcoming years as a result of which they will scale back transport emissions and increase the security of supply [4]. The International Energy Agency (IEA) urged that biofuels will give twenty seventh of world transport fuel (equal to thirty two EJ) by 2050; meantime, the worldwide bio-energy potential of "low-risk" biomass feedstocks might reach 475 EJ [5].

1.2 Types of Biofuels: Biofuels is classified into 3 totally different generations (figure 1), betting on biomass feedstock. The first generation biofuels can even be referred to as standard biofuels, and they are principally obtained from food crops and edible oil seeds; their technologies are mature and comparatively cheap [4][6]. However, 1st generation biofuels draw wide criticism owing to their competition with food and fibre production, in addition of large consumption of chemical and H_2O. Excess production of 1st generation biofuels can considerably increase food costs [6].

Second generation biofuels are principally made from lignocellulosic biomass, non-edible oil seeds and wastes [4]. They obtain the benefits of getting less food crop competition. Conversion of waste lignocellulosic remnants to biofuels via eco-friendly technologies would be useful for the setting. The energy price in the yield of substrates in second generation processes is also low compared with food crops (e.g., prices in ploughing, composting and harvesting). Second generation biofuels are also cheaper than 1st generation biofuels, if the capital prices and additional advanced pre-treatment processes will be offset by a budget substrate resources.

Third generation biofuels are principally derived from algae. Algae, which may be classified as microalgae and macroalgae (seaweeds), are identified for chemical action efficiencies and productivities, thereby leading to lower space needs compared with land-based plants, like maize, corn and panic grass [7]. Algae can be cultured in non-freshwater sources, like salt water and H_2O on non-arable land, and don't contend with regular food resources [8]. In this review we will focus on the production of 2nd generation biofuels from lignocellulosic biomass, non edible oil catalysed by various enzymes and protein engineering methods used to enhance the property of enzymes catalyzing the biofuel production process.

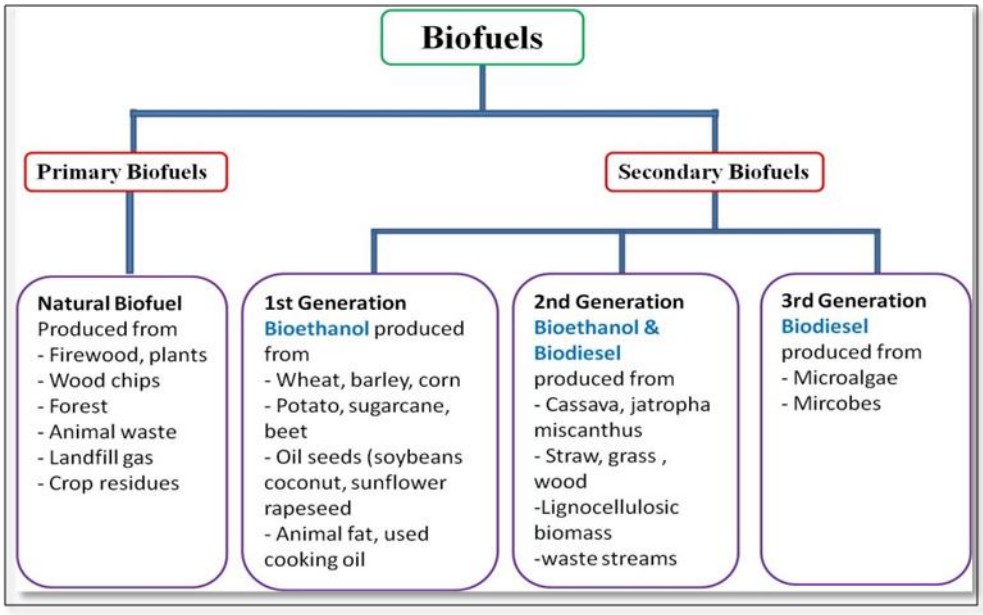

Figure (1): Biofuel production sources [6]

2 Process of biofuel production:

2.1 Bioethanol: Lignocellulosic biomass is an important potential resource for the production of biofuels because of its being plentiful, cheap and production of such resources is environmentally feasible (Figure 2). Agricultural residues like stems and stalks from sources such as corn fibre, corn fodder, bagasse, rice hulls, woody crops are a great source of lignocellulosic biomass which are renewable, chiefly unexploited, and inexpensive. 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 switchgrass and other forage feedstocks like *Miscanthus*, Bermuda grass, Elephant grass, etc. add up to the multiple sources of lignocellulosic biomass **[9]**. Lignocellulosic biomass comprises mainly of cellulose, hemicelluloses and lignin. The prime constituent cellulose is a homopolysaccharide consisting of glucose units, linked by β-(1→4) glycosidic bonds. Cellobiose is the smallest reoccurring unit of cellulose and could be converted into glucose. Hemicellulose is a heterogeneous polymer, made up of mainly pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. Hemicellulose contains mainly xylans in hardwood, while glucomannans are present in softwood. Hydrolysis of cellulose and hemicellulose needs various types of enzymes. Briefly, cellobiose degradation needs endoglucanase while xylan degradation requires 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.

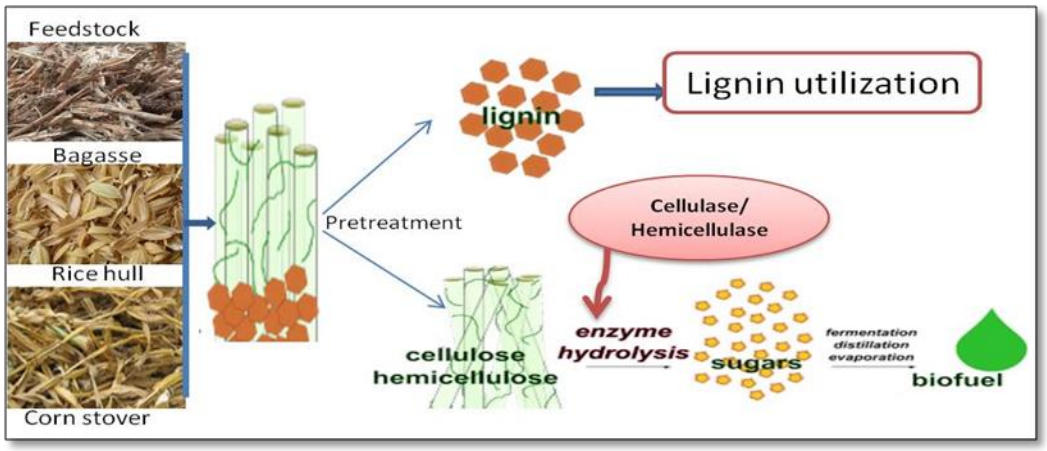

Figure (2): Production of biofuels from lignocelluloses biomass **[10]**

2.2 Biodiesel: Biodiesel is a form of biofuel obtained from renewable sources that contain free fatty acids (FFAs) and triglycerides (TGs). Biodiesel is obtained by esterification of FFAs or transesterification of TGs **[11]** and may provide partial solution as an alternative of fossil fuels. The typical chemical reactions involved in biodiesel production are shown in Figure 3. Currently, the main feedstock for biodiesel production is virgin oil such as soybean and rapeseed oil **[12]** 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 significant interest **[12]**. Biodiesel is a CO_2-neutral fuel and is now widely supported as a sustainable replacement to diesel fuel for transportation applications. It can be obtained by two processes firstly, chemical-catalyzed conversion processes, using alkali catalysts like NaOH or KOH **[13]** and secondly by, the enzyme (lipase)-catalyzed process. The enzyme (lipase)-catalyzed process is more feasible because it avoids soap formation, do not produce large amount of waste water as compared to the chemical-catalyzed method **[14]**.

Despite their significant promise, there are vital challenges in adapting the lipase-catalyzed method or cellulase process to industrial scale, including performance, stability, catalytic activity, and production of these enzymes. Specifically, 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-catalyzed biofuel production.

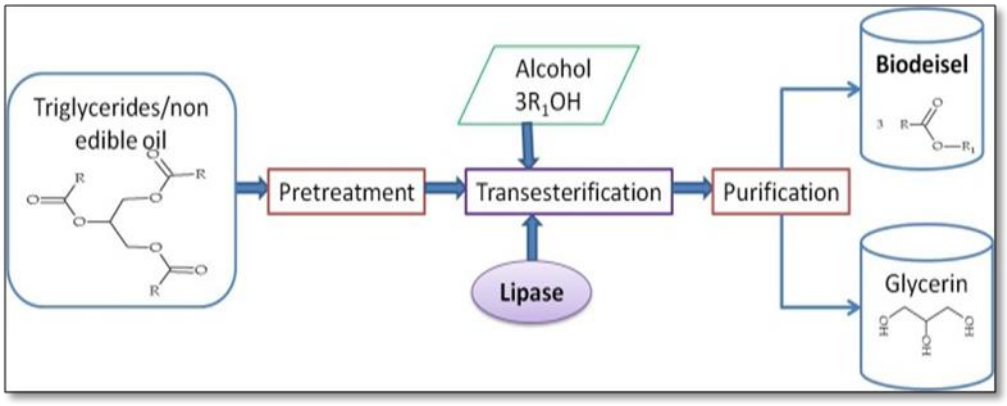

Figure (3): Production of biodiesel from lipase catalyzed transesterification reaction **[12]**

3 Protein engineering for improvement of enzymes: Despite the broad spectrum of cellulases, hemicellulses and lipases being isolated, no single catalyst is completely appropriate 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 decent place to begin for the development of these enzymes in steps towards enhancing the economics of biofuel production. Typically, the utilization of protein engineering technology has been aimed towards the study of catalytic activity of those enzymes. Additionally recently, modifications to enzymes isolated from microorganisms through the utilization of protein engineering is taking a stage within the production of economical hydrolytic enzymes exploited in a vast scope of industries and comprises targeting structural amino acids, on the far side amino acids within the catalytic site. There are two major ways for the improvement of an enzyme component: 1) rational design and 2) directed evolution. A close correlation of the two kinds of protein engineering methods is illustrated in Figure 4.

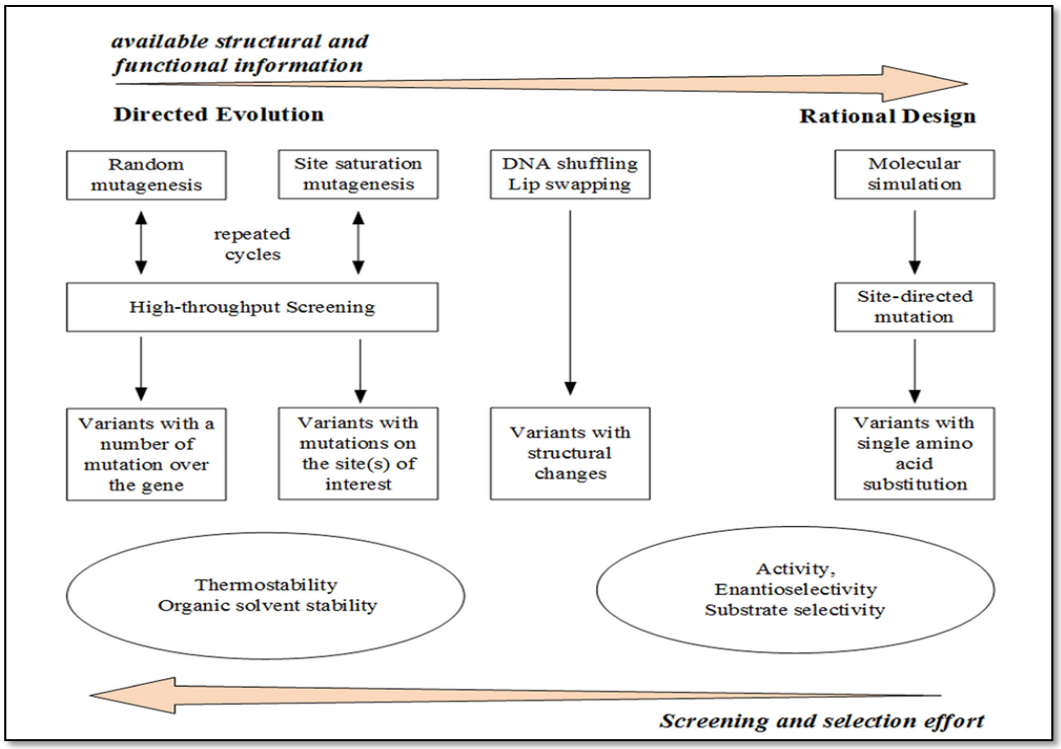

Figure (4): Schematic of strategies to select protein engineering methods **[15].**

3.1 Rational Design: Rational design involves 1) selection of an appropriate catalyst, 2) identification of the amino acid sites to be modified, based mostly typically on a high resolution crystallographic structure, and 3) identification of the mutants **[15]**. The utilization of rational design needs elaborate information of the enzyme structure: what makes the catalytic site active, a theoretical molecular structure-based model of the protein, and most preferably structure-function relationship. With at least part of this information, modification of amino acid sequence may be achieved exploiting site-directed mutagenesis, in some cases components of secondary structure may be altered and even interchange of whole domains and/or generation of fusion proteins **[15]**. If the structural data of a selected variety of protein is missing, the structure of a homologous catalyst may be used to facilitate the modification method **[16]**.

Baker and colleagues (2005) designed and mutated Tyrr 245 of Cel5A of *Acidothermus cellulolyticus* to Gly and this was found to lower the inhibition of the endoglucanase by cellobiose. Solubilised sugars were hydrolyzed 400th greater by the mutant Cel5A (with *T. reesei* cellobiohydrolase-I) compared to the wild-type. Structural and kinetic studies correlated enhanced enzymatic activity to lowered product inhibition **[17]**. Additionally, mutation of a single active-site cleft tyrosyl residue to a glycyl residue considerably modified the mixture of products discharged from phosphoric acid-swollen cellulose (PSC) from the catalytic domain of the endoglucanase-I from *A. cellulolyticus*. The proportion of glucose found within the product stream was close to 400th greater for the Y245G mutant they created compared to the wild-type catalyst **[18]**. Microbial cellulases and hemicellulases improved by rational design are summarized in Table 1.

Brady et al. (1990) first used X-ray crystallographic analysis to know the structure of *Rhizomucor miehei* lipase. The structures of other major lipase varieties are known in recent years, as well as *Bacillus thermocatenulatus, Candida antarctica, Pseudomonas cepacia*, and *Bacillus subtilis* **[19 - 21]**. This data provides a concrete content to rationally pick potential modification sites on lipases. The recent advances in computer-assisted protein design by molecular dynamic simulation tools have enabled forecasting of point mutation(s) on functional properties of **[22]**. Microbial lipases improved by rational design are summarized in Table 2.

3.2 Directed evolution: Contrary to rational design, irrational style or directed evolution is an application to non-informational protein engineering that exploits the ability of natural selection to evolve enzymes and choose for those with desired attributes. Specifically, directed evolution requires the utilization of deoxyribonucleic acid techniques like error-prone PCR (epPCR) and dna shuffling to randomly produce a large library of gene variants. It's an excellent advantage over rational design as a result of it's being independent of enzyme structure and of the interactions among catalyst and substrate. However, a significant problem of this technique is to develop a method to accurately measure the performance of mutants generated by recombinant DNA techniques and therefore the choice of superior mutants. Screening strategies generally include such tests as CMC agar with congo red staining or the utilization of chromogenic or fluorogenic substrates, as previously mentioned. The success of directed evolution depends on a large library of gene variants, the larger, the larger the prospect of mutants with desired properties.

The method of directed evolution has been used to enhance the thermal stability of *Clostridium cellulovorans* cellulosomal endoglucanase (EngB) in vitro by DNA recombination with non-cellulosomal endoglucanase EngD. The screening was done using CMC agar and marking with congo red **[48]**. Further, DNA shuffling was used to produce a library of mutated endoglucanases from *B. subtilis.* Apparently, a bacterial surface display technique was used to selectively screen for variants with increased activity on CMC agar with congo red staining. By joining the genes with the ice nucleation protein (Inp) the resulting fusion enzymes would be present on the bacterial cell surface for simple

screening [49]. Microbial cellulases and hemicellulases improved by directed evolution are summarized in Table 1. In a directed evolution approach, Yu et al. enhanced thermostability of lipase from *Rhizopus chinensis* considerably by two stage of ep-PCR and two stages of DNA shuffling [25].

Table (1): Examples of cellulases and hemicellulases improved by protein engineering techniques.

Enzymes	Property Altered	Method	References
Cellulases		***Rational Design***	
Acidothermus cellulolyticus Endoglucanase	Type of products released	Site-directed mutation	[18]
Acidothermus cellulolyticus Endoglucanase	Product inhibition	Site-directed mutation	[17]
Pectobacterium chrysanthami Endoglucanase	Activity	Nonsense mutation	[27]
Pectobacterium chrysanthami Endoglucanase	Activity	Insertional truncation	[28]
Thermobifida fusca Endoglucanase	Activity	Site-directed mutation	[29]
Thermotoga maritime Endoglucanase	Activity	Site-directed mutation CBD engineering	[30]
		Directed Evolution	
Agrobacterium sp. Mutated α-glucosidase	Activity	epPCR	[31]
Bacillus subtilis Endoglucanase	Activity	DNA shuffling	[24]
Clostridium cellulovorans Endoglucanase	Thermal stability	Family shuffling	[23]
Paenibacillus polymyxa α-D-glucosidase	Thermal stability	epPCR	[32]
Paenibacillus polymxa α-D-glucosidase	Thermal stability	epPCR + family shuffling	[33]
Pyrococcus furiousus α-glycosidase	Activity	Family shuffling	[34]
Thermotoga neapolitana α-D-glucosidase	Activity	epPCR	[35]
Hemicellulases		**Rational design**	
Bacillus circulans Xylanase	Thermostability	Site-directed mutagenesis	[36]
Bacillus amyloliquefaciens Bacillus subtilis xylanase-β-glucosi	Substrate usage	Peptide linker fusion	[37]
Clostridium thermocellum xylanase, arabinofuranosidase/ xylosidase	Substrate Usage	Peptide linker fusion	[38]
Thermobacillus xylanolyticus GH-AA xylanase	Thermostability	Site-directed mutagenesis	[39]
		Directed evolution	
Bacillus subtilis Xylanase XylA	Thermostability	epPCR, DNA shuffling	[40]
Dictyoglomus thermophilum Xylanase XynB	Thermostability Alkalinity	DOGS, epPCR	[41]
Streptomyces halstedii Xylanase Xys1	Activity	epPCR	[42]

They found that, by enhancing the hydrophilicity and polarity of the protein surface and making hydrophobic contacts within the protein, the melting temperature of a variant was 22^0C higher and half-

lives at 60^0 and 65^0C were 46 and 23-time longer, as compared to the parent. They also investigated the link between lid rigidity and lipase activity by introducing a disulfide bond within the hinge region of the lipase lid **[26]**. They showed that, as compared to the wild-type, the cross-linked variant showed 11-fold increase in half-life at 60^0 and 70^0C increase in melting temperature. Microbial lipases improved by directed evolution approach are summarized in Table 2.

Table (2): Examples of lipase improved by protein engineering techniques.

Enzymes	Property Altered	Method	References
		Directed Evolution	
Candida antarctica lipase B	Thermostability	site saturation mutagenesis	**[43]**
Aspergillus niger	Thermostability	iterative saturation mutagenesis	**[44]**
Bacillus sp.	Thermostability	ep-PCR	**[45]**
Bacillus subtilis	Thermostability	site saturation mutagenesis	**[46]**
Bacillus subtilis lipase A	Thermostability	ep-PCR	**[47]**
Bacillus subtilis lipase A	Thermostability	iterative saturation mutagenesis	**[48]**
Candida antarctica lipase B	Thermostability	ep-PCR	**[49]**
Geobacillus sp.	Thermostability	ep-PCR and site saturation mutagenesis	**[50]**
Pseudomonas aeruginosa	Thermostability	iterative saturation mutagenesis	**[51]**
Bacillus subtilis	Solvent tolerance	site saturation mutagenesis	**[52]**
Bacillus subtilis	Solvent tolerance	(iterative saturation mutagenesis	**[53]**
Candida antarctica lipase B	Substrate selectivity	***Rational Design***	**[54]**
Pseudomonas fragi	Substrate selectivity	Rational Design	**[55]**
Rhizopus delemar	Substrate selectivity	Rational Design	**[56]**
Candida antarctica lipase B	Thermostability	Rational Design	**[57]**
Fervidobacterium changbaicum	Thermostability	Rational Design	**[58]**
Geobacillus sp.	Thermostability	Rational Design	**[59]**
Pseudomonas aeruginosa	Solvent tolerance	Rational Design	**[60]**
Burkholderia cepacia	Catalytic activity	Rational Design	**[61]**
Bacillus thermocatenulatus	Catalytic activity	Rational Design	**[62]**
Candida antarctica lipase B	Catalytic activity	Rational Design	**[63]**
Candida rugosa LIP4	Catalytic activity	Rational Design	**[64]**

4 Conclusions and future perspectives: Biofuels are of rapidly 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. However, this will lead to food competition and thus, the second-generation biofuel technology based on lignocelluloses, non edible oil is under intense investigation. Several factors will influence the economic viability of 2nd generation biorefinery. 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, subsequent microbial conversion of sugars to biofuel molecules and catalysis of transesterification reactions although the progresses reported to date have been incremental. With the continuing development of new tools and scientific know how, significant progress will be there towards

the production of next generation biofuels. Concerted research programs combining protein engineering, metabolic engineering, chemical catalysis, and chemical process engineering for high enzyme activity will lead to an economically viable biorefinery in the near future.

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