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A Systematic Study on The Production of Bioethanol Using Cellulosic Fungi

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Abstract

The major concern for the development of renewable and sustainable energy source is to replace the non-renewable fossil fuels which had a very bad impact on environment and health causing severe issues. The use of cellulase enzymes finds an application to the solution for such a major problem. Cellulase is a hydrolytic enzyme which breaks down cellulose to glucose units. Cellulase enzymes are mainly used in many industries such as food, beverages, textile, pulp, paper and biofuel. The major components of cellulase enzyme system are endoglucanse, exoglucanase and β-glycosidase which convert lignocellulosic biomass to fermentable sugar. So, both saccharification and fermentation steps occur simultaneously by sub-merged fermentation (smf) with the use of mesophilic fungal strain. The isolation of fungal strain producing ethanol are carried out from paddy straw, water hyacinth, ipomea and spathodeaum. Initial selection is made on the basis of strain which has the ability to produce those enzymes. So, by this potential isolate from the strains may be applied for bioethanol production. The mentioned process for bioethanol production may be cheap, however, it may be also affected by different environmental factors such as temperature, pH, incubation time, carbon and nitrogen source.

Keywords
cellulose; bioconversion; lignocellulosic-biomass; water-hyacinth; biofuel.

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INTRODUCTION:

One of the great challenges in this century is production of biofuel from lignocellulosic biomass because biofuels play a particularly important role in decarbonizing transport by providing a low-carbon solution for existing technologies. Biofuels causes lesser damage to the climate in contrast with non-renewable fossil fuels. The most abundant biological compound on terrestrial and aquatic ecosystem is cellulose, which is the main constitute of plant biomass (Shankar et al. 2011). Total annual biomass production from cellulose is about 1.5×10^{12} tons by photosynthesis especially in tropics where cellulose is considered as a dominant raw material for production of different products like in paper and pulp industry, textile industry, bioethanol production, wine and brewery industry, food and animal feed industry, pharmaceutical industry etc. (Klemm et al. 2002). The structure of cellulose is a crystalline polysaccharide which is an unusual feature among other biopolymers. The inter and intra chain of hydrogen bonds harden the crystals of cellulose and weak van der Waals forces held together to adjacent sheets which overlie one another. In the matrix of hemicellulose and lignin cellulose fiber are embedded. An important characteristic of crystalline nature of cellulose is huge impermeability to large and small molecules like enzymes and water respectively. The heterogeneity nature of cellulose makes the fibers

capable of swelling when partially hydrated, with the result that micro-pores and cavities become sufficiently large enough to allow penetration of large molecules including enzymes (Rajeev et al. 2005).

Lignocellulosic biomass mainly composed of three types of polymers like; hemicellulose, cellulose and lignin. All three are strongly connected and chemically bonded (Zhang et al. 2006). Stalks, stems and husks in the form of cellulose are found presiding waste materials from agricultural industry. It is an interesting source as an energy and feed (Sharda et al. 2013).

Cellulose is a long chain of glucose sub-units linked by β -1,4 glucosidic linkages (Azhar et al. 2020). Cellulase is the most important industrial enzyme and covers huge area in the global market. It is considered as the third largest industrial enzyme (Yoon et al. 2014). Cellulase is an enzyme of industrial significance and around the world it covers total 20% of global market (Mrudula et al. 2011). In industry, cellulase enzyme found many novel applications; like production of organic acid, detergents and other chemicals, fermentable sugars and ethanol. This enzyme provides tremendous benefits for utilization of biomass (Jerin et al. 2015). There are two methods for conversion of cellulose to glucose, methods are chemical hydrolysis and enzymatic hydrolysis; here cellulase

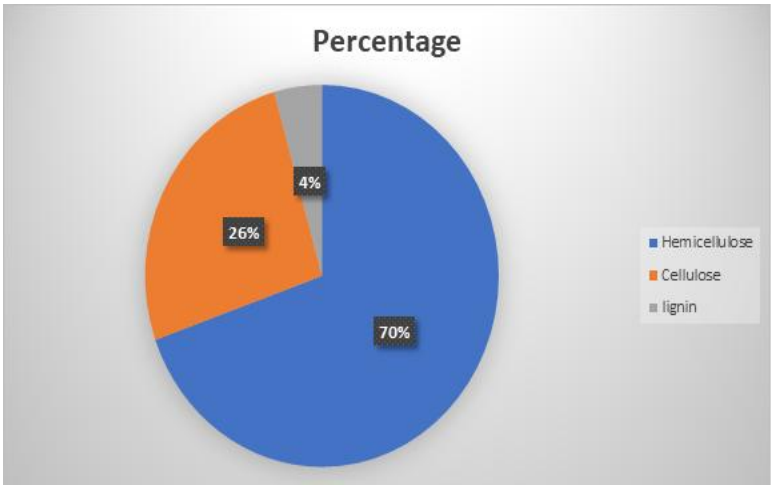

Figure 1: (Amount of lignocellulosic biomass)

were applied as an enzymatic method for the production of bioethanol (Rajeev et al. 2005). In enzymatic hydrolysis renewable lignocellulosic material are convert into biofuels. This process is environmental friendly and remarkably alternative to fossil-derived fuel (Srivastva et al. 2015). During enzymatic hydrolysis cellulase play a vital role in degradation of cellulosic polymer to release monomeric fermentable sugars and produce biofuels (Falkoski et al. 2013). β−1,4-d-glucan linkage in cellulose structure is hydrolyse by this enzyme and release glucose, cellobiose and cello-oligosaccharides. Enzymatic complex includes endo-glucanases (EG), (CBH) cellobiohydrolases and β-glucosidases (BGL) is most comprehensively studied (Rekha rawat et al. 2014). Cellulose degrading microorganisms which can produce cellulase enzyme, this process consists of three major enzyme system namely; exo-β glucanases, endo-β glucanases and β glucosidase (Mrudula et al. 2011).

Endo-glucanases release nicks in the polymeric structure of cellulose, showing reducing and non-reducing ends while exo-glucanases produce cello-oligosaccharides as well as cellobiose units by acting on reducing and non-reducing ends. Merely, β-glucosidases release monomeric sugar molecules during hydrolysis of cellobiose. So, a whole cellulase system is required for effective production of biofuel (Srivastava et al. 2014; Bhatt et al. 1997).

Fungi and bacteria are main source for degradation of cellulose and synthesize cellulolytic enzyme. Filamentous fungi are main inters for commercial enzyme production because fungi produce higher level of enzyme as compare to bacteria. Fungi which are capable to produce enzyme are *Aspergillus, Fusarium, Penicillium, Trichoderma, Phanerochaete chrysosporium* (Rekha rawat et al. 2014). Cellulase has a very high potential for bioconversion to produce important product like ethanol. Identification of new cellulosic strain help to produce cheap ethanol (Lee et al. 2014).

At present, biofuel attracting attention towards substitutes of petroleum-based transportation fuels because of low cost, energy security (Lee et al. 2011).

New research must require for production of novel strain with activity on pretreated biomass substrates. Novel classes of microorganisms will find by screening and engineering cellulases with improved industrial qualities by using genetic engineering (Wilson et al. 2009). The use of lignocellulosic plant biomass is inexpensive, renewable as well as abundantly available. Primary source of lignocellulosic biomass is woody crops, paddy straw, rice straw, water hyacinth and forest residues because theses residues has not any further use in industry. When there is no any particular information about using waste biomass; burning of biomass occur and pollutes the air (Feng et al. 2009).

There are several factors which influence the growth of organisms; factors like pH, temperature, aeration, incubation time, inoculum size, carbon and nitrogen source. Now, a day's increasing air pollution affect the growth of organism and production of ethanol. Air pollution affect the raw materials in farm so, main conclusion is air must be healthy for converting plant wastes to economical products (Azhar et al. 2017).

Lignocellulose biomass includes five major operations: pretreatment, size reduction, hydrolysis, fermentation and product recovery for biofuel production (Lynd et al. 2008). It has been well-established that major developments are required in the enzymatic hydrolysis process to compete economically with the petroleum-derived fuels. The major challenge associated with efficient enzymatic hydrolysis is low rate of reaction, and therefore, high enzyme loading is needed to obtain the desired product concentration. These problems could be solved by using cellulases that can withstand higher temperature for a longer time (Rekha et al. 2014).

Biofuel becomes very useful in avoiding "greenhouse effect" because of burning of fossil fuels release the carbon dioxide and carbon monoxide to the atmosphere (Minal et al. 2010). Ethanol is an alternative to fossil fuel, widely used as liquid biofuel. To reduce ethanol production cost a single microorganism is used which has the ability to hydrolyze cellulose and ferment glucose to ethanol is an attractive (Lynd et al. 2005). Due to high cost of cellulases use of filamentous fungi for production of cellulase is become subject of interest (Zhuang et al. 2007).

For ethanol production, studies of filamentous fungi have largely been driven by attributes that make them appealing for use in industrial fuel ethanol production. The ethanol production is surprisingly high by filamentous fungi comparing with traditional method. Many of these fungi have the enzyme complex of xylanase, cellulase and amylase. This process occurs simultaneously; saccharification and fermentation of biomass with single organism (Christopher et al. 1997).

(1) Current situation and scenario on biofuel production

Nonrenewable fossil fuel makes polluted environment so, by using renewable sources we can decrease depletion in the reserve of fossil fuel and global warming therefore it makes sustainable and clean production of biofuels and other bioproducts (Taha et al. 2016; Sorensen et al. 2013).

This first-generation bioethanol produces from sugar and starch which gain from corn (*zea mays*) and sugarcane (*Saccharum officinarum*) (Taha et al. 2016; Haghighi et al. 2013). For, second generation bioethanol production must use lignocellulosic materials; like forestry and agricultural residues to produce low-cost, abundant and renewable bioethanol (Viikari et al. 2012; Menon et al. 2012). At present, day by day increase the prices of fossil fuels because of demand of crude oil (Taha et al. 2016).

This conversion of lignocellulosic biomass to bioethanol requires many chemicals which create challenge (Menon et al. 2012). According to international energy agency (IEA) total 10% and 25% of forestry and agricultural residues are available for biofuel production and remaining residues uses for other production (Eisentraut et al. 2010). IEA reports said the future perspective of use of lignocellulosic biomass. In year 2030, approximately lignocellulose biomass residues will be enhanced by 50% of roundwood and 28% for crop sources. It is assumed that in year 2030, 25% of total lignocellulose converts to either bioethanol, biodiesel or syngas may supply 385-554 billion liter of gasoline equivalent totally (13.0-23.3 EJ) and about 155 billion liter of gasoline equivalent (5.2 EJ) bioethanol may produce from 10% of total lignocellulose or 4.1% transport fuel (Sunardi et al. 2020).

In bioethanol production biggest challenge on industrial application is conversion of cellulose into fermentable sugars though, this method is environmentally friendly and sustainable (Lozano *et al*., 2014). There are two methods for bioconversion of cellulose to sugar is; chemical method enzymatic method. The common method is chemical method but it is very expensive as compared to enzymatic hydrolysis (Taha et al. 2016). Enzymatic hydrolysis method widely useful because of good specificity, low consumption of energy and chemical is cheap. In this method enzyme cleave from β-1,4 glycosidic bond of cellulose and remove undesired byproduct which adversely affect the fermentation step in ethanol production (Lozano et al. 2014). So, for enzymatic hydrolysis must require the complex of enzyme contain; exoglucanases, endoglucanase and

β-glucosidase (Mrudula et al. 2011). Still several challenges are there for utilization of lignocellulosic biomass for bioethanol production. Because of high price of enzyme, many problems arise so, by using novel strain of fungi can solve this all problem for bioethanol production.

(2) Market scenario of cellulase

Many industrial applications of cellulase increases its demand day by day. From all biofuel, according to literature ranges of bioethanol is $ 0.10/gal (Aden et al. 2009), $ 0.30/gal (Lynd et al. 2008), $ 0.32/gal (Dutta et al. 2010), $ 0.35/gal (Klein et al. 2010) and $ 0.40/gal (Kazi et al. 2010). There are many difficulties arise for techno-economic analysis and commercial production process of biofuels because of the confutation in the cost of enzymes. So, for industrial production of cellulase many factors affect like high substrate loading, low enzyme loading and a short hydrolysis period play important role. When using this factor, it decreases production cost of enzymes (Klein et al. 2010).

There are many companies involve in production of cellulase enzymes but in global stage only two companies are there; Genencor and Novozymes. These companies continuously trying to reduce cost of cellulase enzymes. Accelerase^{R} 1500 name of cellulase complex given by company Genencor, for lignocellulosic biomass processing industries which is considered to be more cost effective than previous one; Accelerase^{R} 1000 (Singhania et al. 2010). New Accelerase^{R} 1500 have higher level of β-glucosidase enzyme activity so, it is very efficient for most of conversion of cellobiose into glucose as compare to other commercial cellulases (Penttila et al. 1998; Singhania et al. 2010). Novozymes, produce different ranges of cellulase depending on their applications. Except Novozymes and Genencor other company also participated in production of enzyme; Amano enzyme Inc., Japan and MAP'S India. Among them so, many participated into the cellulase production but only few companies convert proper cellulase from biomass (Neha et al. 2015).

(3) Cellulase classification

3.1 Cellulase

O-glycoside oxidase (EC 3,2.1) is a brand group of enzyme systems which are present in cellulases. Glycosidic bond is present between non-carbohydrate and carbohydrate molecules or two or more carbohydrates molecules which is hydrolyze by cellulase enzyme. On the basis of carbohydrate-active enzyme database (CAZY), endoglucanses belong to the GH families while exoglucanases or cellobiohydrolases are related to GH families and β-glucosidases are found in the GH families (Juturu et al. 2014). Endoglucanases are regarded as the primary cellulase, because it consists of carbohydrate binding molecules (CBM). These enzymes break the crystalline structure of the cellulosic substrate. There are many fungal strains available for cellulase production. Among them *Trichoderma* and *Aspergillus* spp. are known as model fungal strain. Less production and high cost of traditional cellulase enzyme, researcher focusing on the isolation and screening of the novel fungi with improved cellulosic system (Neha et al. 2017).

3.2 Exoglucanases

Cellobiohydrolases can be classified as exo-acting cellulases because they have ability to cleave β-1,4 glycosidic bonds from site of chain ends and having active site with structure of tunnel-shaped. This tunnel-shaped structure functioning to avoid the re-attachment of once separated molecules from the crystalline cellulosic structure (Divne et al. 1998). Exoglucanases or cellobiohydrolases found to the GH-7 families. Detail study of these enzymes can be understood by *Phanerochaete chrysosporium* cellobiohydrolase (cel 7A) (PDBID: 1GPI). Exoglucanase play role in the solubilization of solid structure of cellulose. Exoglucanase have 431 amino acids chain having 3-dimensional β-jellyroll structure which fit into a functional enzyme. These are simple concept for understanding structural differences, more studies can be done by molecular analysis.

3.3 Endoglucanases

Endoglucanse is also known as CMcase because it participates in the production of cellobiose. Endoglucanse related to GH-5 family. Name endoglucanse came from the fact that enzyme break the inner β-1,4 glycosidic bonds. Endoglucanase degrade the polymerization by increasing glycosidic chain end concentration and these enzymes are more active on the extraneous soluble amorphous region of cellulosic substrate (Tomas et al., 2009). Endoglucanse play role in the transformation of solid structure into sugars. Endoglucanse have 335 amino acid chain having eight-fold (β/α)8-barrel structure which fit into a functional enzyme. Detail study of this enzyme was understood by *Thermoascus aurantiacus* have cleft-shaped active site (Neha et al. 2015).

3.4 β-glucosidases (BGL)

Though β-glucosidases has many functions, but main function is conversion of cello-oligosaccharides into glucose. Structure of BGL properly can be understand by *Bacillus polymyxa* (Bg1A), this species has crystalline structure of BGL-A family. BGL belongs to the GH-1 and clan GH-A family (Juturu et al. 2014). β-glucosidase have wide application in biofuel production from biomass, along with combination of other enzymes for production of reducing sugars (Lin

et al. 2006). *Aspergillus niger* has high potential for production of BGL and use as commercial enzyme in production. However, high cost and fungal strain specificity for production of cellulase, BGL is now becoming subject of research. Though many advantages of BGL, it cannot constantly stable at high temperature because of its mesophilic nature. So, use of thermostable BGL enzyme which stay constant at high temperature (Sun et al. 2008; Rastogi et al., 2010).

(4) Mechanism of cellulose hydrolysis by cellulases

The enzyme complex of endoglucanase (1,4-β-D-glucan-4-glucanohydrolase), exoglucanase (1,4-β-D-glucan cellobiohydrolase) and β-glucosidase (β-glucoside glucohydrolase; cellobiase) is require for enzymatic hydrolysis of cellulosic biomass to sugars (Payne et al. 2015; Srivastava et al. 2014). In addition, endo-glucanases release nicks in structure of cellulose, showing reducing and non-reducing ends as well cellobiohydrolase by acting on reducing and non-reducing ends produce cello-oligosaccharides and cellobiose. Merely, β-glucosidases release monomeric sugar molecules during hydrolysis of cellobiose. So, a whole cellulase system is required for effective production of biofuel (Srivastava et al. 2014; Bhatt et al. 1997). Amorphous region which is more in the cellulose structure is hydrolyse by endoglucanases while cleavage of β-1,4 glycosidic bonds from the chain ends is done by cellobiohydrolases (Divne et al. 1998).

(5) Application of fungal cellulases in saccharification process

In simultaneous cellulase hydrolysis and production it is necessary to maintain optimum environmental condition so, optimization of bioconversion process occurs (Yang et al. 2016). In experimental study of Yang et al, they use rice straw to isolate *Aspergillus niger* and these fungi produce both cellulase and xylanase enzymes. These enzymes inoculate to inoculum medium, after 6 days of solid-state fermentation collect the product and suspend it into buffer, saccharification occur so sugar recovered. It becomes very difficult to simultaneous process of fungal cellulase production and use it to direct bioethanol production. During production of fungi, it reduces sugars and use it for further growth (Yang et al. 2016; Ramussen et al. 2010). If our aim is simultaneous processing it can be possible, by using high number of desired molecules produce through consumption of sugar. This process is acceptable because compare to fungi, yeast and bacteria not produce cellulase.

A consolidated bioprocess (CBP), term defined as use of only one microorganism for both cellulase and bioethanol production (Amore et al. 2010). *Candida tropicalis*, yeast able to demonstrate cellulolytic and hemicellulolytic enzymes; found that it obtains at same time biofuel and cellulase production. When substrate is wheat straw it contains xylose 49 g/L converted into 15.8 g/L xylitol, and 25.4 g/L glucose which converted to 7.3 g/L bioethanol. Here activity of endoglucanase was 98 U/g. (Mattam et al. 2016) Second approach is use of filamentous fungi for production of enzyme complex; xylanase and cellulase. This complex was applied on lignocellulose; produce glucose. Lignocellulosic biomasses like sugarcane, bagasse, rice straw, Japanese cedar are hydrolysed by commercial cellulase enzyme. In addition, cellulose also useful as a substrate in hydrolysis process. Though, cellulase enzyme can be found from lignocellulosic biomass but if there is present of pure form of cellulose then it is very easy for microorganism to produce cellulase (Maeda et al. 2011; Yu et al. 2015; Treebupachatsakul et al. 2015; Saini et al. 2015).

Advantage of on-site production is high than off-site production because enzyme produced in on-site production is more powerful instead of off-site production (Zhao et al. 2019). Many authors find out that fungal strain which use different carbon sources, produces different amount of cellulase (Nazir et al. 2010; Li et al. 2013; Singhania et al. 2011; Zhao et al. 2018). Techno-economic analysis for bioethanol production is reported and published by national renewable energy laboratories (NREL); US department of energy in year 2010. They talk about substrate corn stover biomass which convert to sugar with the use of on-site and off-site cellulase enzyme so, by this process they concluded that value of product from on-site is high because production of huge amount bioethanol but still the value of off-site production is uncertain reason behind it is undecided prices of chemicals and enzymes. This on-site production requires optimize conditions (Kazi et al. 2016).

According to techno-economic studies about on-site production, it is proved that production is more energy efficient as compare to purchase cellulase for off-site production. The one advantages of on-site production is protecting environment by polluted gas. While performing on-site production emits less green-house gases compare to off-site produce (Olofsson *et al*. 2015).

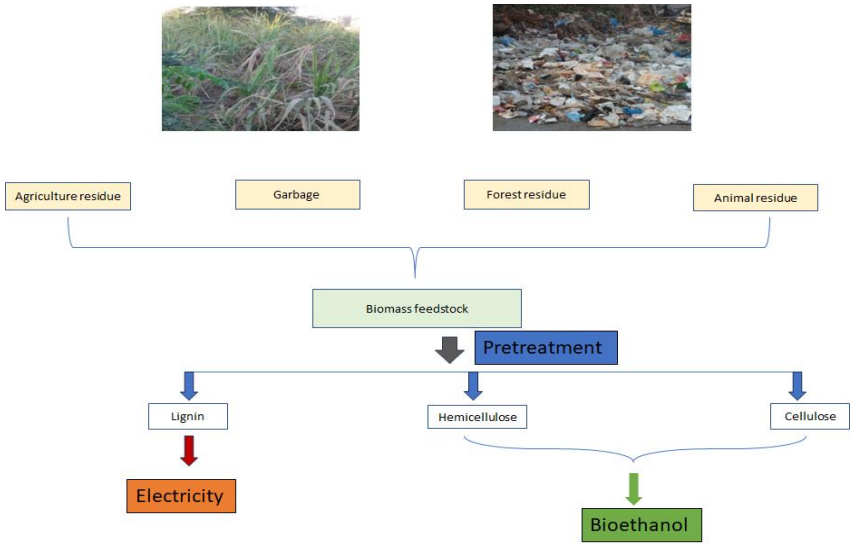

Figure no: II (Process of biofuel production from biomass)

The other advantage of on-site production is it reduce costs during whole production when we are applying on-site production; process occur in presence of low-cost substrate and decreases downstream processing as well as steer clear of enzyme storage and transportation (Fang et al. 2016; Rana et al. 2014). In on-site production of lignocellulosic biomass after using, this substrate burnt and release to environmental; this substrate not harm the environment so it is environmental friendly.

In experimental study here, comparison of single strain produce combination of three different enzyme together so, according to study enzyme extracts produced by; *Trichoderma reesei* RUT-30, *Aspergillus tubingensis* and *P. janthinellum* EMS-Uv-8. The extract, combine of three enzymes used for hydrolysis of avicel-wheat bran by Smf process under same environmental conditions. Fpase activity of mixture is 16.9 U/g, in CMase activity value is 162 U/g and in β-glucosidase activity is 33 U/g. Though β-glucosidase activity is high when using complex mixture, FPase activity is two time higher in complex of three enzyme than using extract of single (Adsul et al. 2014).

In experimental data of (Prevot et al. 2013) they gave data regarding comparison of final production of cellulolytic enzyme by use of two process SSF and Smf. In this process atmospheric condition is similar in both processes, substrate; wheat bran was used. fungal strain *T. reesei* RUT-c30 was used. So, all condition was same in both processes, still in SSF technique high production occur as compare to smf. So, after this process checking of FPase activity (0.5 U/g substrate), glucose concentration is higher in SSF process it is 3.39 g/L, while in smf it is 1.41 g/L (Prevot et al. 2013).

Here our aim is to decrease high energy cost and huge chemical pre-treatment; use of single hydrolysate which reusable. Our interest is use it in second generation bioethanol production. Because during this process the remaining residues is reusable while otherwise it is burnt and cause environmental damage (Ravindran et al. 2015; Larran et al. 2015). If using chemical for pre-treatment it sometimes causes inhibition of by-products (Jonsson et al. 2016).

Use of sugarcane juice for bioethanol production; it reduces cost and easily ferment sucrose. Use of this avoid the chemical pre-treatment but continuous use of this substrate its stoppage occurs because of economically unaviability. Use of corn stover as cellulosic and hemicellulosic substrate with bacterial strain *Lactobacillus pentosus* produces L-lactic acid. This strain has capability to ferment both sugar glucose and xylose (Hu et al. 2016). Use of cellulose and starch as substrate and strain *Propionibacterium freudenreichii subsp,* by hydrolysis process produce propionic acid (Wang et al. 2013). From pre-treated rice straw isolation of fungal strain *A. niger* and *T. reesei* produce cellulase enzyme; collect hydrolysate for production of single cell oil with use of *Mortirella isabelline* fermentation (Yang et al. 2017).

Concluding remark

From reviewing the articles, different lignocellulosic biomass can use for isolation of fungal strain. lignocellulosic biomass like wheat bran, rice straw, corn stover, wheat straw, paper and pulp processing waste and water hyacinth. Cellulase enzyme produce from fungal strain. Now, this enzyme is use for fermentation or hydrolysis and produce sugar; this

sugar helps to produce ethanol. There are many difficulties to produce large amount of bioethanol; because day by day increasing demand of bioethanol so, it is challenge to develop cheap technology for production of bioethanol. Another aspect is process must be environment friendly. According to review to fulfil all the challenge, can utilize consortium of complex of enzyme; which contain 2-3 enzymes together and produce huge amount of sugar. Here one suggestion is use of fungal strain which isolate from water hyacinth and also utilization of water hyacinth as a lignocellulosic biomass because that fungal have high degrading power for same source. Though, bacteria and fungi both have capacity to produce ethanol, fungi have high power for bioethanol production. This process becomes cheap if substrate and enzyme which utilize in production is very cheap. Another factor use organism which have capacity to survive at very high temperature. If here utilization of genetically modified strain which produce cheap enzyme and huge quantity of bioethanol is best.

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CONFLICT OF INTEREST:
The author declares no conflict of interest.

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