Metabolic engineering of *Saccharomyces cerevisiae* for consolidate bioprocessing of lignocellulosic biomass for bio-ethanol production

Mr.Sujan Bishwakarma, Mr.Sandesh Maharjan, Mr.Mitesh Shrestha, Dr.Tribikram Bhattarai
Central Department of Biotechnology
Tribhuvan University

Abstract

Lignocellulosic biomass is found abundantly in the biosphere of earth which can be a good source for bioethanol production. The sugar polymers of cell wall like cellulose and hemicelluloses are bound with lignin, resisting their hydrolysis. Pretreatment of biomass is necessary to expose cellulose by degradation of lignin, which is a major problem in pretreatment. *Saccharomyces cerevisae* has been taken as the best sugar fermenter used in industry to ferment glucose based agricultural products to ethanol but *Saccharomyces cerevisiae* is unable to degrade lignin. The basidiomycetes such as white rot fungi produce laccase enzyme that degrade lignin. The three copper centered metalloezyme, laccase of class oxidoreductase (EC 1.10.3.2) catalyze oxidation of lignin of lignocellulose so that lignin free hemicellulose and cellulose can be extracted. The metabolic engineering of *Saccharomyces cerevisiae* expressing laccase gene enables to degrade lignin. For such purposes, laccase gene from *Ganoderma lucidum* is most widely utilized for the heterologous expression in the *Saccharomyces.* The development of such yeast can degrade lignin that will reduce the cost of pretreatment. The further step for release of hexose and pentose, in this case will be the heterologous expression of cellulase and hemicellulase genes in *Saccharomyces*. Finally, introduction of pentose- especially xylose utilization pathway should be extensively explored and if succeeded will be a great step in conversion of lignocellulosic biomass in to high value ethanol fuel.

Key words; Laccase, lignin, *Saccharomyces cerevisae*, Second generation bioethanol.

Introduction

The bioethanol produced through carbon neutral approach has a near zero greenhouse gas emission (GHG) which can simultaneously substitute crude oil partially for energy source and lead to economic and environment benefits (Kuhad *et. al*.2011). Utilization of lignocellulosic biomass for ethanol production is ideal over the traditional procedure that uses glucose rich hydrolysates from sustenance crops like wheat and corn (Leandro *et al*., 2006). The major focus had been drawn on bioethanol as liquid fuel source after the major energy crisis in 1970s. Ethanol is being used as an additive to the fossil fuel as E10 that contains 10% ethanol and 90% gasoline and E15 (15% ethanol and 85% gasoline). The EISA (EISA, Energy independence and security act, 2007) policies mandate to produce 21 BGY (Billions of gallons per year) of second generation bio-ethanol from renewable source other than corn starch to mitigate 50% of GHG emission. Lignocellulosic biomass is the most promising substrate which substitute food and feed substrates used for ethanol production. However, the process is challenging due to lack of efficient pretreatment technology for depolymerization of lignin component (Canilha, 2012). There are tons of agro-industrial wastes that are generated throughout the year after processing of agricultural product such as sugar cane, corn-strover, bagasses etc. that are rich in fermentable sugar polymer such as cellulose and hemicelluloses. Lignin is another most abundant component of biomass covalently cross-linked with hemicelluloses making biomass inherently recalcitrant (Rodgers et. al, 2010). These sugar polymers along with lignin are main structural parts of plant cells. Lignocellulosic biomasses are typically composed of 30 to 50% cellulose, 15 to 35% hemicellulose and 10 to 20% lignin (Limayema,

2011). The most abundant component of biomass is cellulose followed by hemi-cellulose and lignin. The percentages of cellulose, hemicelluloses and lignin vary depending on type of biomass (Tab 1).

Lignocellulosic materials	**Cellulose %**	**Hemicellu-lose %**	**Lignin %**
Hardwood stems	40-55	24-40	18-25
Softwood stem	45-50	25-35	25-35
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Papers	85-99	0	0-15
Wheat straw	30	50	15
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Switch grass	45	31.4	12
Cotton seed hair	80-95	5-20	0
Costal Bermuda grass	25	35.7	6.4

Table 1: Content of cellulose, hemicelluloses and lignin in common agricultural residues and waste (Adapted from Yang and sun, 2002)

The production of bio-ethanol from lignocellulosic materials needs destruction of structural complexity of lignocelluloses and depolymerization of hemicellulose and cellulose to simple sugars followed by fermentation. Traditionally, lignocellulosic biomass can be pretreated in various ways-mechanical, chemical, thermal and thermochemical processes (Limayem, 2012) which need high thermal energy and costly chemical and may also produce fermentative inhibiters.

Saccharomyces cerevisiae is the best ethanol producer and being industrially used in making wine; traditionally even in the household level. The end product of *Saccharomyces cerevisiae* is ethanol and can tolerate low pH, high sugar and high temperature; these are the reason why *Saccharomyces cerevisiae* is superior ethanol producer than other. The *S. cerevisiae* is GRAS (Generally regarded as safe) organism approved by US department of FDA (Food and drug administration) and easily cultivable eukaryotic host with well characterized genome sequence. The *Saccharomyces cerevisiae* possess its own genome databases such as Saccharomyces genome database (SGD) and comprehensive yeast genome database (CYGD) which enables ease of gene manipulation.

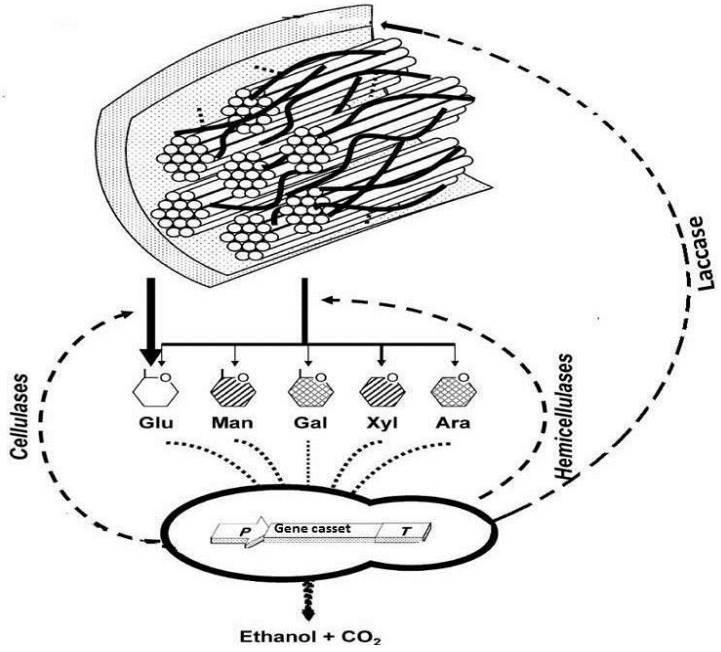

Figure 1: Schematic consolidate bioprocessing of lignocellulosic biomass into Bio-ethanol using model recombinant strain of *Saccharomyces cerevisiae* (Modified from Zyl *et.al*.,2007). Recombinant yeasts produce laccase to depolymerize lignin and cellulases, hemicellulases to hydrolyze the cellulose and hemicellulose respectively that is followed by ethanolic co-fermentation of hexose and pentose in a single bioreactor.

The biological processing of lignocellulosic biomass represents an environment-friendly and economically justified process (Knezevic, 2013). Recently, intense research has been focused on consolidate bioprocessing of lignocellulosic biomass. The consolidate bioprocessing include three steps in single bioreactor- i) Production of enzyme- Laccase, Cellulases and Hemicellulases ii) Hydrolysis of biomass into sugar monomer hexose and pentose iii) Fermentation of hexose and pentose sugars degrade it (Bottcher *et. al*., 2013). The degradation of lignin is foremost and crucial steps to release sugar polymer. Laccase from white rot fungi have been recognized for oxidation of lignin. Now, most research has been immensely motivated towards heterologous expression of laccase gene in easily cultivatable and safe host, *Saccharmoyces cerevisiae, Pichia pasto*ris etc and found to be better producer of recombinant laccases.

alcohol (Menon and Rao, 2012) held together by most stable bonds precludes the ability of any single enzyme to properly recognize and In 2013, You *et. al.,* isolated laccase gene from *Ganoderma lucidum* and expressed in *Pichia pastoris* and it was larger in molecular weight and more stable in low pH as a result of 18% hyperglycosylation in comparison to native laccase.

The three copper centered metalloezyme, laccase of class oxidoreductase (EC 1.10.3.2) catalyze oxidation of lignin in presence of molecular oxygen. The enzyme contains three types of copper center molecule, type I copper atom absorb intensely at 600 nm due to the covalent Cu-Cys bond which imparts the blue color of the enzyme and considered as a primary electron acceptor whereas the type II and two type III copper center is the electron carrier which involves in the reduction of molecular oxygen into water. The type II

copper atom is invisible in the absorption spectrum and the type III copper atom absorbs at 300nm. The type II and two type III copper center forms the triangular structure (Santhanam et.al, 2011; Rodgers et.al, 2010).

The mechanism of the lignin degradation is complex; four copper ions of enzyme mediate the production of reactive molecules which can undergo the number of chemical reaction. The cleavage of alkyl-aryl covalent bond in presence of mediator results in the production of the monomer of substrate and it also mediate the cleavage of ring aromatic compound. The substrate is oxidized by donating one electron to the type I Cu-atom located near by the substrate binding pocket. Now type I copper atom is re-oxidised by donating one electron transferred to the type II - type III tri-nuclear Copper complex. The molecular oxygen binds to the tri-nuclear copper complex and reduced into water by the 4 electrons. For the complete reduction of molecular oxygen required four electrons which is achieved by the four times reduction and oxidation of the type I Cu atom (Rodgers *et.al.,* 2010).

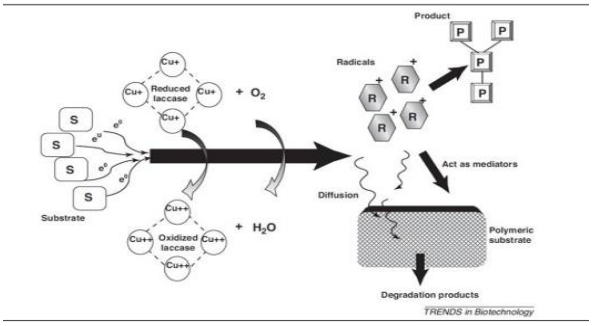

Figure 2: Mechanism of laccase catalysis. (Adapted from Santhanam *et al.,* 2011)

The degradation of lignin results in release of amorphous cellulose and hemicellulose polymer which are then needs to be hydrolyzes by cellulases and hemicellulose enzyme respectively for production of hexose and pentose monomer. Cellulase is the group of three enzymes which include endoglucanases (EC 3.2.1.4), exoglucanases (EC3.2.1.91) which acts synergistically to convert polymeric cellulose into cellobiose. β-glucosidases (EC3.2.1.21) convert cellobiose to glucose monomer. The simultaneous expression of Endoglucanase-II, cellbiohydrolase-II from *Trichoderma reesei* and β-glucosidase from *Aspergillus aculeatus* have been reported in saccharomyces cerevisiae. However, it unable to complete utilize amorphous cellulose (Fujita et. al. 2004). Comparatively, limited research has been intended towards the hydrolysis of hemicelluloses. Endo-β-xylanase and β-D-xylosidase are the two sets of enzyme catalyze the conversion of xylans into xylose. The xylanase encoding gene xyn-II from *Tichoderma reesei* and xylosidase encoding xylA from *Aspergillus oryzae* were simultaneously coexpressed in *S. cerevisiae* cell surface by fusing α-agglutinin gene encoding cell surface protein of saccharomyces cerevisiae (Chen *et. al*., 1995) to 5' end of xyn-II and xylA gene. The significantly high amount of xylose was reported in fermentative broth co-displaying xylanolytic enzyme (Katahira *et.* al.,2004).

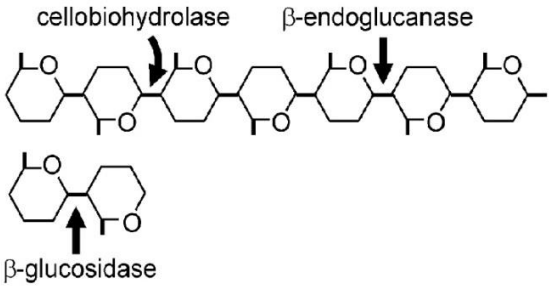

Figure 3: Mode of action of cellulases

The hydrolysis of hemicellulose produces pentose sugars such as D-xylose, L- arabinose along with hexose sugars namely glucose, mannose etc. In case of *S. cerevisiae,* hexose sugar can directly enter into central metabolism while pentose sugars cannot. To make bio-ethanol cost efficient, it is necessary to use wide range of substrate for ethanol production. In nature, two known xylose metabolism pathways exist but such metabolism is absent in *Saccharomyces*

cerevisiae. The first pathway is xylose isomerase pathway which is a bacterial pathway catalyze by xylose isomerase enzyme. And a second pathway is found in bacteria and in certain yeast species catalyzed by two set of enzymes; aldose (xylose) reductase and xylitol dehydrogenase (Nevoigt et.al 2008). The xylose isomerase pathway bypasses the use of reducing powers in the form of NADH or NADPH and improves the ethanol production (Alper and Stephanopoulos 2009).

The recombinant *Saccharomyces cerevisae* carrying XI (Xylose isomerase) and GXS1 (Glucose xylose symporter) gene can be employed for efficient fermentation of pentoses. Similarly, *S. cerevisiae* with XYL1 and XYL2 genes encoding XR (Xylose reductase) and XDH (Xylitol Dehydrogenase) respectively have been engineered to enhance the ethanol yield by co-fermentation of glucose and xylose (Brat *et al*., 2009).

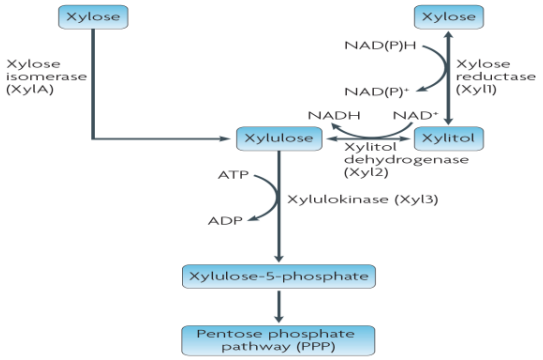

Figure 4: Schematic representation of two D-xylose metabolic pathway integrated in *Saccharomyces cerevisiae.* (Adapted from Alper and Stephanopoulos 2009)

CONCLUSION

The processing of lignocellulosic biomass into bio-ethanol is complex. Metabolic engineering along with molecular biological techniques is applied to manipulate the gene for heterologous production of desirable product. Now a day, metabolic engineering approach manipulates biochemical pathway at genetic level either by introducing new pathway or by inhibiting competitive pathway. The wild type *S. cerevisiae* is unable to utilize the lignocellulosic biomass for ethanol production (Fernandes & Murray, 2010) and introduction of new metabolic pathway is essential for utilization of most of the lignocellulosic biomass (Jouzani, 2015). Such consolidate bioprocessing may accomplished by recombinant *Saccharomyces cerevisiae* introducing the various new metabolic pathways. The development of such model *Saccharomyces cerevisiae* resolves the problem associated in processing of lignocellulosic biomass for biofuel production.

ACKNOWLEDGEMENT

I would like to thank Prof. Dr. Rajani Malla; Head of Department for giving precious suggestions. And also my thanks goes to; Mr. Kapil Adhikari, Ankit Jaishwal and other classmates for their support.

Reference

Bottcher, A., Cesarino, I., Santos, A. B., Vicentini, R., Mayer, J. L., Vanholme, R., Mazzafera, P. (2013). Lignification in Sugarcane: Biochemical Characterization, Gene Discovery, and Expression Analysis in Two Genotypes Contrasting for Lignin Content. Plant Physiology, 163(4), 1539-1557.

Brat, D., Boles, E., & Wiedemann, B. (2009). Functional Expression of a Bacterial Xylose Isomerase in Saccharomyces cerevisiae. Applied and Environmental Microbiology, 75(8), 2304-2311. doi:10.1128/aem.02522-08.

Canilha, L., Chandel, A. K., Milessi, T. S., Antunes, F. A., Freitas, W. L., Felipe, M. D., & Silva, S. S. (2012). Bioconversion of Sugarcane Biomass into Ethanol: An Overview about Composition, Pretreatment Methods, Detoxification of Hydrolysates, Enzymatic Saccharification,

and Ethanol Fermentation. Journal of Biomedicine and Biotechnology, 2012, 1

Chen, M., Shen, Z., Bobin, S., Kahn, P. C., & Lipke, P. N. (1995). Structure of Saccharomyces cerevisiae -Agglutinin: EVIDENCE FOR A YEAST CELL WALL PROTEIN WITH MULTIPLE IMMUNOGLOBULIN-LIKE DOMAINS WITH ATYPICAL DISULFIDES. Journal of Biological Chemistry, 270(44), 26168-26177. doi:10.1074/jbc.270.44.26168

EISA, Energy independence and security act, 2007

Fujita, Y., Ito, J., Ueda, M., Fukuda, H., & Kondo, A. (2004). Synergistic Saccharification, and Direct Fermentation to Ethanol, of Amorphous Cellulose by Use of an Engineered Yeast Strain Codisplaying Three Types of Cellulolytic Enzyme. Applied and Environmental Microbiology, 70(2), 1207-1212. doi:10.1128/aem.70.2.1207-1212.2004.

Jouzani, G. S., &Taherzadeh, M. J. (2015). Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: A comprehensive review. Biofuel Res. J. Biofuel Research Journal, 152-195.

Katahira, S., Fujita, Y., Mizuike, A., Fukuda, H., & Kondo, A. (2004). Construction of a Xylan-Fermenting Yeast Strain through Codisplay of Xylanolytic Enzymes on the Surface of Xylose-Utilizing Saccharomyces cerevisiae Cells. Applied and Environmental Microbiology, 70(9), 5407-5414. doi:10.1128/aem.70.9.5407-5414.2004

Knežević, A., Milovanović, I., Stajić, M., Lončar, N., Brčeski, I., Vukojević, J., &Ćilerdžić, J. 2013).Lignin degradation by selected fungal species.Bioresource Technology, 138, 117-123.

Kuhad, R. C., & Gupta, R. (2009).Biological Remediation of Petroleum Contaminants. Soil Biology Advances in Applied Bioremediation, 173-187.

Leandro, M. J., Spencer-Martins, I., &Goncalves, P. (2008). The expression in *Saccharomyces cerevisae*of a glucose/xylose symporter from Candida intermedia is affected by the presence of a glucose/xylose facilitator. Microbiology, 154(6), 1646-1655.

Limayem, A., &Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science, 38(4), 449-467.

Menon, V., &Rao, M. (2012). Trends in bioconversion of lignocellulose: Biofuels, platform chemicals &biorefinery concept. Progress in Energy and Combustion Science, 38(4), 522-550.

Nevoigt, E. (2008). Progress in Metabolic Engineering of Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews, 72(3), 379-412. doi:10.1128/mmbr.00025-07.

Rodgers, C. J., Blanford, C. F., Giddens, S. R., Skamnioti, P., Armstrong, F. A., & Gurr, S. J. (2010). Designer laccases: A vogue for high-potential fungal enzymes? Trends in Biotechnology, 28(2), 63-72. doi:10.1016/j.tibtech.2009.11.001

Santhanam, N., Vivanco, J. M., Decker, S. R., & Reardon, K. F. (2011). Expression of industrially relevant laccases: Prokaryotic style. Trends in Biotechnology, 29(10), 480-489.

Sun, Y. and cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review, 84(1/2), 123-129. doi:10.1023/a:1021045122126.