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Consolidated Bio-Processing of Cellulosic Biomass for Efficient Biofuel Production Using Yeast Consortium

A Thesis submitted in partial satisfaction of
the requirements for the degree of

Master of Science

in

Chemical and Environmental Engineering

by

Garima Goyal

March 2011

Thesis Committee:

Dr. Wilfred Chen, Chairperson

Dr. Ashok Mulchandani

Dr. David Cweritny

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The Thesis of Garima Goyal is approved:

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Committee Chairperson

University of California, Riverside

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prominence would have been possible without their continuous support. This thesis is dedicated to them.

ABSTRACT OF THE THESIS

Consolidated Bio-Processing of Cellulosic Biomass for Efficient Biofuel Production Using Yeast Consortium

by

Garima Goyal

Master of Science
Chemical and Environmental Engineering Graduate Program
University of California, Riverside, March 2011
Dr. Wilfred Chen, Chairperson

Fossil fuels have been the major source for liquid transportation fuels for ages. However, decline in oil reserves and environmental concerns have raised a lot of interest in alternative and renewable energy sources. One promising alternative is the conversion of plant biomass into ethanol. The primary biomass feed stocks currently being used for the ethanol industry have been food based biomass (corn and sugar cane). However, interest has recently shifted to replace these traditional feed-stocks with more abundant, non-food based cellulosic biomass such as agriculture wastes (corn stover) or crops (switch grass). The use of cellulosic biomass as feed stock for the production of ethanol via bio-chemical routes presents many technical challenges not faced with the use of corn or sugar-cane as feed-stock. Recently, a new process called consolidated Bio-processing (CBP) has been proposed. This process combines simultaneous saccharification of lignocellulose with fermentation of the resulting sugars into a single process step mediated by a single microorganism or microbial consortium. Although there is no natural microorganism that possesses all properties of lignocellulose utilization and

ethanol production desired for CBP, some bacteria and fungi exhibit some of the essential traits.

The yeast *Saccharomyces cerevisiae* is the most attractive host organism for the usage of this strategy due to its high ethanol productivity at close to theoretical yields (0.51g ethanol/g glucose consumed), high osmo- and ethanol- tolerance, natural robustness in industrial processes, and ease of genetic manipulation. Introduction of the cellulosome, found naturally in microorganisms, has shown new directions to deal with recalcitrant biomass. In this case enzymes work in synergy in order to hydrolyze biomass more effectively than in case of free enzymes. A microbial consortium has been successfully developed, which ensures the functional assembly of minicellulosome on the yeast surface composed of four yeast populations. These yeast populations include: one displaying scaffoldin on its surface and three populations secreting three different cellulases in the medium to hydrolyze the cellulose. The modular nature of the consortium system allows for the fine-tuning of each population by changing their initial inoculum ratio, thereby optimizing the cellulose hydrolysis and hence ethanol production. When comparing the optimized consortium with equal ratio consortium, the optimized one produced almost double the amount of ethanol (1.87 g/l) with a yield of 0.475 g ethanol/g cellulose. To further evaluate the feasibility of using consortium for CBP, it was grown at very low optical density (OD) under anaerobic conditions. Under stressful conditions like low OD and no oxygen, the consortium system was proficient in assembling the cellulosome on its surface and growing on the PAS-avicel as sole carbon source and concomitantly producing ethanol with a yield of 87% of the theoretical value.

For the dynamic study of yeast consortium system, quantitative real time PCR was used to enumerate the individual yeast population in the mixed culture. At the end of the cultivation, ratios of each population in this consortium maintained similar number as the initial inoculums ratios, which further confirms the consortium system is suitable for the application of CBP.

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CHAPTER 1

Introduction

Lignocellulosic materials are the most abundant, renewable, and economical carbon sources that have the potential to substitute the food based biomass for large amounts of petroleum used for fuels and chemical production (Demain et al 2005; US DOE 2006; Wen et al 2009). Lignocellulosic biofuel is gaining attention as a potential future transportation fuel and significant research in this area is focused on developing eco-friendly and economical technologies (Perlack et al., 2005). Ethanol is generally expected to be the first major commercial product of this emerging cellulosic biofuels technology. Traditionally, for ethanol production corn starch and sugarcane were used as raw materials (Brown, 2003). These substrates are considerably different structurally and functionally from lignocellulosic biomass and are easily hydrolyzed to glucose. But, overuse of these dedicated agricultural crops for biofuel production has led to two major concerns, food crises and cost ineffectiveness. Therefore, the development of microbial platforms has been extensively pursued to achieve cost-competitive ethanol yield, titer and productivity.

Complete degradation of cellulose requires a complex of at least three classes of enzymes, working together to breakdown cellulose into simple sugars (Reese et al). Exo-1,4-β-D-glucanases, also called cellobiohydrolases (CBH) (EC 3.2.1.91) cleave off cellobiose units from the ends of cellulose chains. Endo-1,4-β-D-glucanas,es (EG)(EC 3.2.1.4) hydrolyze internal β-1,4-glucosidic bonds in the cellulose chain, presumably acting mainly on the amorphous or disordered regions of cellulose. Hydrolysis to the final product is accomplished by 1,4-β-D-glucosidases (BG) (EC 3.2.1.21), which hydrolyze cellobiose to glucose and cleave off glucose units from the various soluble cello-

oligosaccharides. Co-operative action, often designated ***synergy***, of the three cellulolytic enzyme classes is essential for efficient enzymatic hydrolysis process.

Consolidated bioprocessing, a recent breakthrough advancement in the production of biofuels as a low cost process, of lignocellulose to bioethanol refers to the combining of the all biological events required for this conversion process (production of sacchrolytic enzymes, hydrolysis of the polysaccharides present in pretreated biomass, fermentation of glucose to ethanol) in one reactor (Lynd et al 2005).*Saccharomyces cerevisiae* is a universally used strain for industrial ethanol production due to its high ethanol productivity and high inherent ethanol/osmo tolerance. However, it doesn't have the ability to hydrolyze cellulose. In the past few years, many research groups have been working on the engineering of the yeast *S. cerevisiae* in the direction of achieving the goal of CBP. A number of studies have expressed multiple cellulose enzymes in single host in attempts to create fully cellulolytic, fermentative system (Den Haan et al 2007; Fujita et al 2004; Fujita et al 2002). Many researchers have reported coexpression and surface display of cellualses in *S. cerevisiae* to directly convert PASC to ethanol with high yield under anaerobic conditions (Caspi et al 2008).

Many anaerobic bacteria and fungi exhibit an elaborate structured enzyme complex on the cell surface, called cellulosome, to maximize the catalytic efficiency of cellulose hydrolysis with only a limited amount of enzymes, which directs to highly ordered enzyme-substrate-microbe synergy (Fierobe et al 2002; 2005). Compared with the mixture of free cellulases, the resultant cellulosome chimeras exhibited enhanced

synergistic action on crystalline/amorphous cellulose. Advances in metabolic engineering and synthetic biology have provided new tools to better understand a way to create cells with desired phenotypes in order to produce economically viable biofuels. One such outcome of this advancement is synthetic consortia for the surface display of mini-cellulosome by intra-cellular complementation for efficient cellulose hydrolysis and ethanol production. The objective of my thesis is to evaluate the feasibility of yeast consortium to perform with proficiency as CBP under different conditions.

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CHAPTER 2

Literature Review

2.1 General Background

The global fossil fuel reserves, produced by natural processes over500 million years, have been consumed by mankind in just the last 50 years (Kerr, 1998).The rate of consumption of fuel continues to accelerate to meet the demands of increasing populations throughout the developing world. This growth in consumption has raised concern regarding the fossil fuel resources outpacing our ability to find alternatives (Kerr, 1998). The resulting scarce supply and increasing fuel prices may lead to economic and political turmoil in many parts of the world. Another major drawback of continuous consumption of fossil fuels is the emission of carbon dioxide to the atmosphere. The effect of these emissions is referred to as the "Greenhouse effect", where the earth's atmospheric temperature has been raised by 33^0C (Cline, 1992; Hansen et al., 2000). To attain a continuous supply of fuel and reduce the deterioration of the environment, it is essential to develop alternative, sustainable, and environmentally-friendly fuel sources.

Biomass, including all the organic material, is one of the most attractive alternatives among potential fuel sources. It is the most sustainable energy resource and is reproduced by the bioconversion of carbon dioxide (McKendry, 2002). As the carbon dioxide released by combustion of fuel is recycled into biomass through photosynthesis, the use of biofuels can significantly reduce the accumulation of greenhouse gas (McKendry, 2002). The following equation describes glucose production through photosynthesis.

$6\ CO_2 + 6\ H_2O + 2800$ KJ (energy from sunlight) $\longrightarrow C_6H_{12}O_6$ (glucose) $+ 6\ O_2$[1]

The ever increasing dearth of fossil fuels and environmental factors are forcing the development of alternative methods of fuel generation. Using plant biomass for sustainable fuel production seems to be suitable alternative due to various reasons. The time required for biomass generation is negligible as compared to million years required for the generation of fossil fuels (McKendry,2002).Traditional techniques like fermentation can be used to produce bio-ethanol from biomass as a substitute for conventional transportation fuels (Mielenz, 2001). Bio-ethanol can be produced by using a wide variety of biomass feedstocks, which can be divided into two major categories: (i) energy crops grown for fuel production (corn, sugarcane, beets, switchgrass), and (ii) agricultural wastes and agro-industrial by-products, mainly crop/plant residues (e.g. corn stover, sugarcane bagasse, wheat straw). Feedstocks high in starch or sugar (corn, sugarcane, beets) can be used to produce conventional "first generation" biofuels, while cellulosic crops require "second generation" technology that is currently in the early demonstration and commercialization phase.

Ethanol has been produced through fermentation using fruiting part (containing simple sugars and starch) as raw material since the dawn civilization and consumed as beverages. Many microbes, especially yeast *S.cerevisiae,* are used around the world to ferment 6-carbon sugars to ethanol under anaerobic conditions as shown by following equation.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy}$$

Enzymes are required to breakdown cellulose into simple sugars (glucose) and sugar molecules are broken down into pyruvate molecules by the process of glycolysis.

Pyruvate is then converted into ethanol and carbon dioxide under anaerobic conditions. Ethanol can be produced as a transportation fuel using grains as raw material via fermentation, but the amount of grains required to provide substantial transportation fuel is enormous. Some researchers suggest that competition between food and fuel will lead to demand surpassing supplies, causing food shortages and inflation in food prices (Pimentel and Patzek, 2005). According to the non-partisan Congressional Budget Office (2009), 10 to 15% of the steep rise in food prices between April 2007and April 2008 was due to corn demand for ethanol production, which consumed about 25% of the US corn crop during that year. Regardless of the short-term impact, lignocellulosic biomass is claimed to be a better long-term option to grain as a feedstock for ethanol, in terms of both economic and energy balances (Hill et al., 2006, Hettanhaus and Wooley, 2000).

2.2 Ligno-cellulosic biomass

The carbohydrate composition of the cell walls in ligno-cellulosic materials has a considerable effect on ethanol yields and varies significantly with various factors such as geographical location, growth conditions, and crop maturity (Pordesimo et al., 2005). The three main constituents of any lignocellulosic biomass are (i) cellulose, (ii) hemicellulose, and (iii) lignin as shown in Figure 2-1.

2.2.1 Cellulose

Cellulose is the most abundant polysaccharide on earth and a major polymeric chain of glucose molecules. The cellulose molecule is comprised of long chains of cellobiose molecules joined together by β-1,4-glucosidic bonds as shown in Figure 2-2.

The molecular weight of the cellulose ranges from 300,000 to 500,000 (1,800 to 3,000 glucose units). The digestive system of man and most other animals (except ruminants) don't contain the necessary enzymes (cellulases) for hydrolyzing β-glucosidic linkages. However, cellulases are found in ruminants, various insects, fungi, algae, and bacteria.

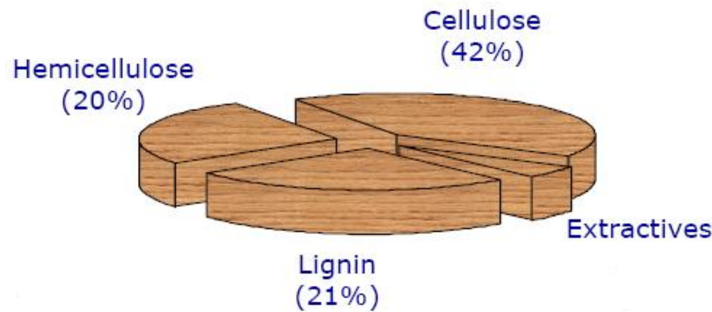


Figure 2-1:Composition of plant cell wall

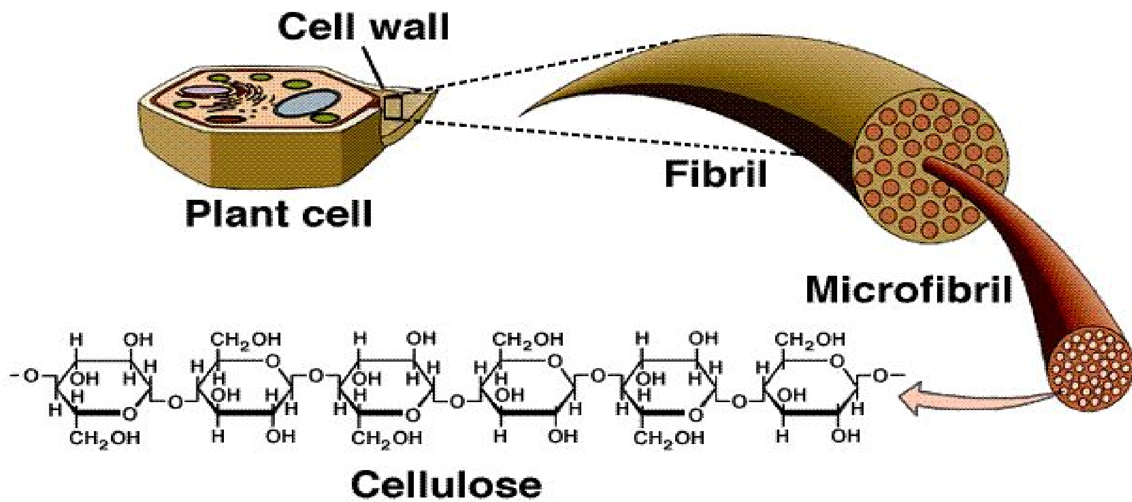


Figure 2-2: Structure of plant residues and long linear chains of glucose units (www.ualr.edu/botany/botimages.html)

2.2.2 Hemicellulose

Hemicellulose is primarily composed of xylan, a branched polymer composed of a five-carbon sugar, xylose. Degree of polymerization of hemi-cellulose is 50-200, which is shorter than cellulose. The acid hydrolysis of hemicellulose $(C_6H_{10}O_5)_n$ produces mainly xylose ($C_5H_{10}O_5$), which can be further converted to furfural (chemical feedstock) or ethanol. It accounts for up to 50% of the biomass from annual and perennial plants (Ebringerova et al., 2005). Mostly found in the primary cell wall of plant cells, hemicellulose molecules are hydrophobic in nature, often branched, and form a matrix to link fibrous cellulose and amorphous lignin. *S.cerevisiae* and many other commercially available types of yeast cannot ferment pentose sugars (sugars with 5 carbon atoms) into ethanol. Biochemical and metabolic engineering of yeast for the assimilation of xylose by yeast to ferment it to bio-ethanol has been studied for decades. Despite the efforts of several excellent research groups, development of yeast strains with sufficient activity and resilience to ferment hemi-cellulose hydrolysates remains elusive.

2.2.3 Lignin

Lignin is a complex aromatic biopolymer of high molecular weight and is formed by the polymerization of oxidatively formed radicals of p-hydroxy cinnamyl alcohols (Hira et al. 1978). It is present in the spaces between cellulose and hemicellulose in the plant cell wall and is interlocked with sugars like arabinose, xylose, galactose from hemicellulose (Northey, 2000). Carbon-carbon and ether linkages are the main bond within the lignin structure and therefore, breaking the carbon bonds without disrupting the lignin structure is unlikely. To ferment the sugars (cellulose and hemi-cellulose)

present in ligno-cellulosic material, the lignin matrix needs to be unlocked. Thus, any biochemical technique developed for the production of ethanol from ligno-cellulosic biomass should be efficient in breaking recalcitrant lignin and converting cellulose and hemi-cellulose into simple sugars and to ferment the simple sugars into ethanol (Wilke et al., 1981).

2.3 Cellulases

Degradation of cellulose requires a complex of enzymes, consisting of at least three classes of enzymes working together to breakdown cellulose into sugars (Reese et al). The cellulases include the large number of endo-(EC 3.2.1.91) and exo-glucanases (EC 3.2.1.4) respectively which hydrolyze β-1,4- glucosidic bonds present in the long cellulosic chains. In principle, the degradation of cellulose requires the cleavage of glucosidic bonds. Exo- 1,4-beta-D-glucanases, also called cellobiohydrolases (CBH), cleave off cellobiose units from the ends of cellulose chains. Endo-1,4-beta-D-glucanases (EG) hydrolyze internal beta-1,4-glucosidic bonds in the cellulose chain, presumably acting mainly on the amorphous or disordered regions of cellulose. In addition to endo- and exo-glucanases, β-glucosidases (EC 3.2.1.21) are also required to completely hydrolyze cellulose into glucose. This type of enzymes cleaves cellobiose – the major end product of cellulase digestion – to generate the simple glucose molecule. It may seem astonishing that a group of different enzymes is required to degrade chemically simplistic substrate. A highly crystalline structure of cellulose makes it very intricate substrate and this complexity reflects the difficulties an enzyme system encounters upon degrading the substrate (Bayer E.A et al 2006). Moreover, the degradation of crystalline cellulose

should be studied three dimensionally and in-situ, where the cellulose chains are packed within the micro-crystal, thus creating highly stable physical properties of a crystalline substrate.

2.4 Consolidated Bio-processing

Four major unit operations for biofuel production take place during conversion of lignocellulose to ethanol via processes featuring enzymatic hydrolysis: production of saccharolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars, and fermentation of pentose sugars. The hydrolysis and fermentation steps have been combined in simultaneous saccharification and fermentation (SSF) of hexoses and simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses to reduce the overall cost of biofuel production. Now a new process called “consolidated” bioprocessing (CBP)of lignocellulose to bioethanol has been introduced (Lynd et al 2005), where all four of these steps occur in one reactor and are mediated by a single microorganism or microbial consortium without the addition of enzyme able to ferment pretreated biomass to ethanol (Fig 2-3).The CBP process can dramatically reduce the cost of biological processing by four-fold and coverall processing by two-fold, when it is substituted for an advanced SSCF process featuring cellulase costing $0.10 per gallon ethanol (Lynd et al 2005), as shown in Figure 2-4.

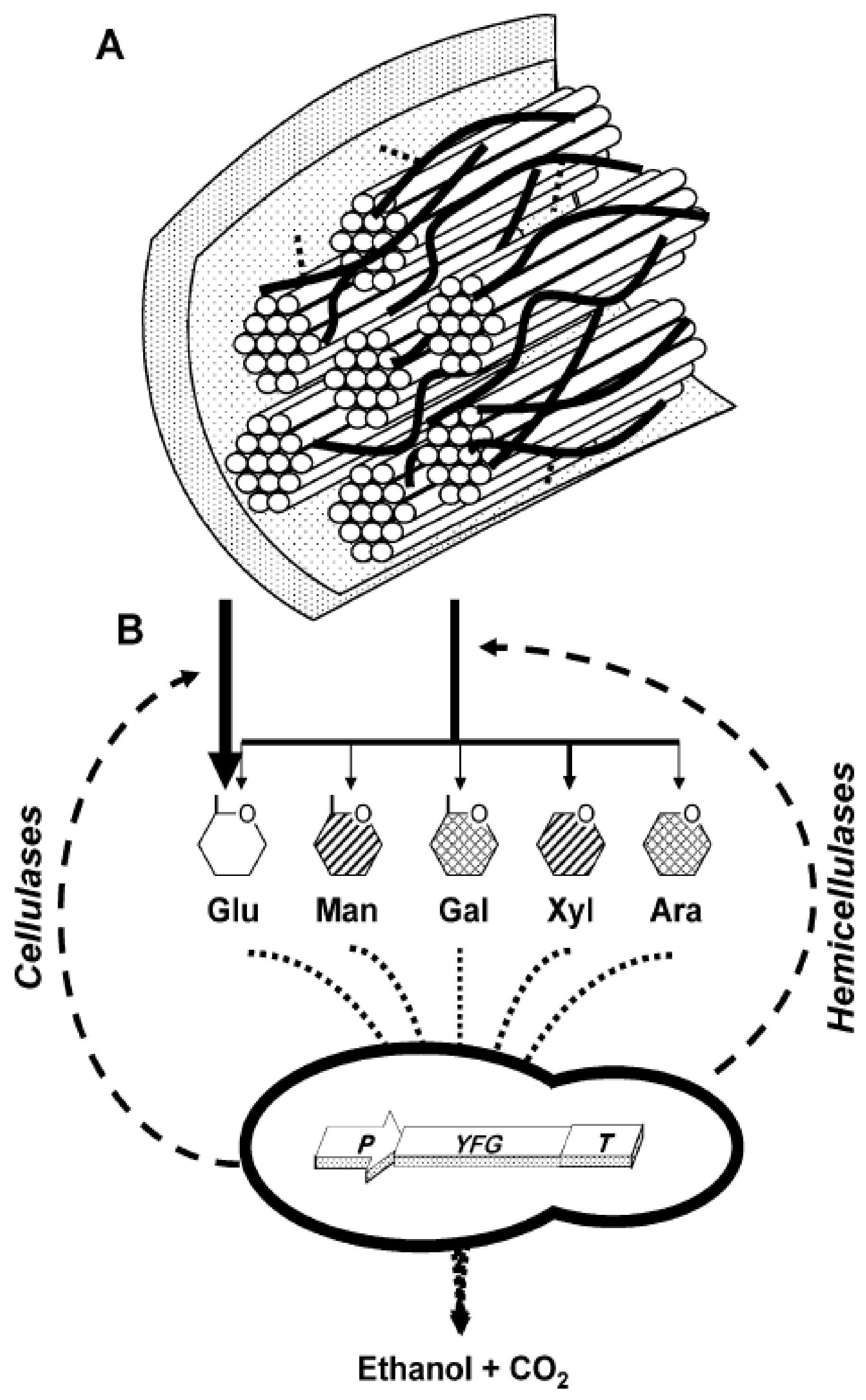

A
B
Cellulases
Hemicellulases
Glu
Man
Gal
Xyl
Ara
P
YFG
T
Ethanol + CO_2

Figure 2-3:Graphic illustration of **a).** Lignocellulose conversion to bioethanol in a single bioreactor by **b).**a CBP microorganism. The enzymatic hydrolysis of the cellulose and hemicellulose fractions to fermentable hexoses and pentoses requires the production of both cellulases and hemicellulases (*dashed lines*), and the subsequent conversion of the hexoses and pentoses to ethanol requires the introduction of pentose fermenting pathways. The thickness of the *arrows* imitates the relative amounts of hexoses and pentoses released during hydrolysis of plant material.(Adapted from Lynd et al 2007)

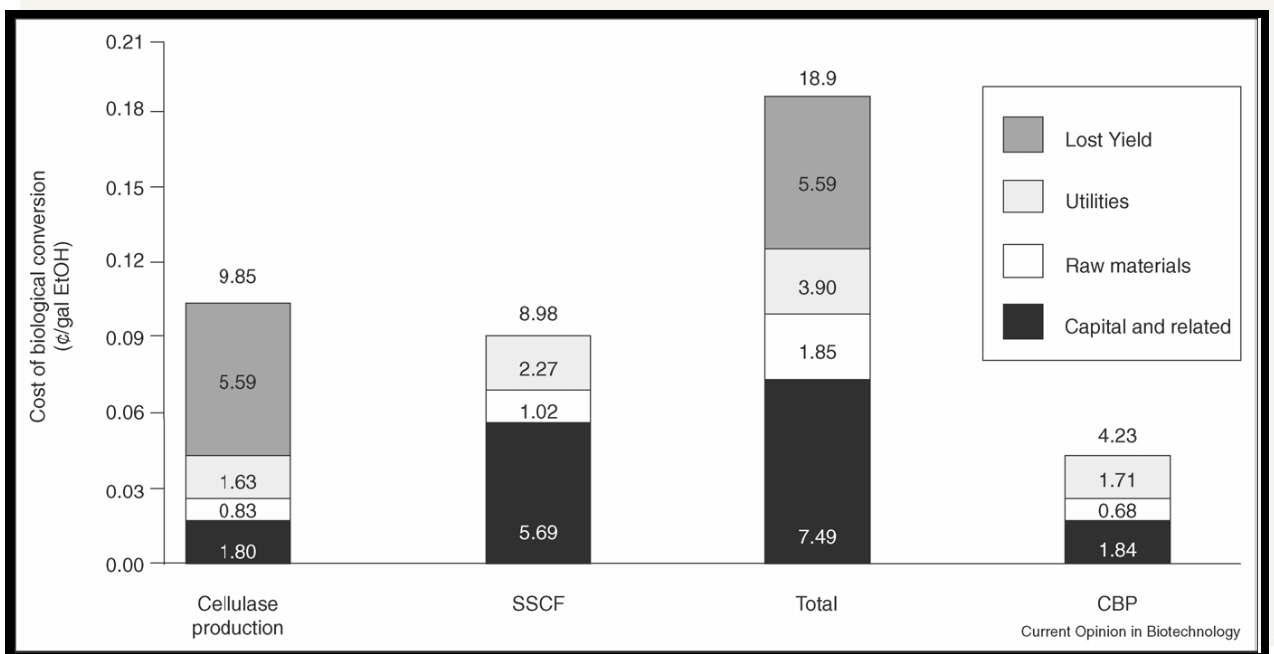


Figure 2-4:Cost comparison between CBP and other conventional processes for biofuel production. (From Lynd et al 2005)

An ideal microorganism for CBP should possess all the desired features such as, capability of simultaneous cellulose saccharification and ethanol fermentation. Although

there is no such microorganism present in nature that exhibits all the properties required for CBP, a number of bacteria and fungi have shown some of the desirable properties and can be divided into two categories. (1) Native cellulolytic microorganisms that possess superior saccharolytic capabilities, but not necessarily product formation and can be engineered for the high product yields and(2) recombinant cellulolytic microorganisms that naturally give high product rates, but into which sacchrolytic systems need to be engineered (Lynd et al 2005).One attractive candidate is *Saccharomyces cerevisiae* which has been widely used for industrial ethanol production due to its high ethanol productively and high inherent ethanol tolerance.

2.4.1 Baker's yeast as a CBP host

S. cerevisiae is a provenindustrial microorganism for ethanol production, but it does not have the sacchrolytic ability to rapidly convert pretreated cellulose to simple sugars (glucose). Apart from essential traits, such as high ethanol and osmotic tolerance and high product yields, industrial strains need to have the ability to concurrently ferment both hexosesand pentoses under robust industrial conditions that require minimum nutrient requirements and high ethanol and inhibitor tolerance. In addition, these strains must have the capability to produce the cellulases at high levels to hydrolyze cellulosics and their fermentation to ethanol.

Table 2-1 lists all the desirable features required for *S. cerevisiae* to act as a CBP host.

Required traits	Suitability of currently available strains of *S. cerevisiae*
Essential traits:	
Ability to ferment hexoses and pentoses	Only hexoses by native industrial strains. Partial pentose utilization has been engineered in some laboratory and industrial strains
High ethanol yield and productivity	Most industrial strains
High ethanol and inhibitor tolerance	Most industrial strains
General robustness for industrial processes	Most industrial strains
High level of heterologous gene expression	Primarily multicopy expression in laboratory strains
High levels of secreted heterologous proteins	Laboratory and some industrial strains
Desirable traits:	
Concurrent fermentation of sugars	Manipulated laboratory and some industrial strains (maltose and glucose utilization)
GRAS status	Most laboratory and industrial strains
Recyclable	Most industrial strains
Minimum nutrient supplementation	Some industrial strains, particularly wine strains
Amendable to DNA manipulation, particularly DNA transformation	Laboratory and some industrial strains

Table 2-1: Features required from *S. cerevisiae* as successful CBP microorganism (Van Zyl, et al 2007).

2.5 Heterologous expression of cellulases in *S.cerevisiae*

The major requirement for *S. cerevisiae* as CBP yeast would be sufficient expression and production of saccharolytic enzymes. In the last two decades, there have been many reports on the heterologous expression and secretion of cellulases in *S.*

cerevisiae. Van Zyl *et al.* provided a table summarizing reports of 74 attempts to express different cellulases in S.cerevisiae including CBH (exo-glucanses), EG (endo-glucanases) and BGL (beta-glucosidase) (Van Zyl et al 2007).Nature has provided us many excellent examples to emulate. Aerobic microbes of genus Trichoderma can produce high titers of extracellular enzymes of different types reaching up to 100g/l (Wilson, 2004; Bayer et al 2000). Mesophilic and thermophilic anaerobic bacteria also provide sources for cellulase production. However, due to energy limitations under anaerobic conditions, only a small amount of cellulases is secreted, resulting in relatively low rates of cellulose hydrolysis. Truly cellulolytic yeast must efficiently express multiple enzymes which act synergistically to hydrolyze cellulosic polymer into simple monomers. An alternative to secretion is to display the cellulytic enzymes on the yeast surface (Fujita et al., 2002, 2004).A yeast strain was developed capable of co-displaying three types of cellulolytic enzymes to achieve one step conversion of cellulose to ethanol. Although their effort of combining cellulose hydrolysis with ethanol producing ability advances toward the goal of achieving cost efficient CBP, the efficiency of cellulose hydrolysis still lags behind and needs to be improved before it can be used for commercial scale biofuel production.

During an enzymatic hydrolysis process, in which upto three enzymes are required to breakdown cellulose into sugars, glucose is released quickly in the beginning of the reaction. After glucose is released the hydrolysis rate slows down due to recalcitrant nature of substrate (Lynd et al 2002). The collaborative work between three cellulolytic enzymes, often designated as *synergy*, is very important for efficient enzymatic hydrolysis. The term *synergy* or “degree of synergy” (DS) is defined as the

ratio of overall degree of hydrolysis of a mixture of three different enzyme components, divided by the sum of the degrees of hydrolysis observed by individual enzymes. The DS can be calculated on the basis of product formation or the extent of substrate hydrolysis. Many studies have investigated and reported the synergism between cellulases to a great extent with main emphasis on binary/ternary enzyme mixtures (Fierobe et al 2004, 2002; Wen et al 2009; Lynd et al 2007).

Unfortunately, the problem associated with inefficient cellulose hydrolysis cannot be solved by simply increasing the amount of enzymes either surface displayed or secreted in the medium due to energy limitations under anaerobic conditions. The possible way to solve this hindrance could be to increase the catalytic efficiency of the cellulases, by maximizing the synergy with limited amount of enzymes. There have been several reports on the use of ternary cellulose-microbe-enzyme complexes yielding much higher cellulose hydrolysis rates than binary cellulose-enzyme complexes (Lu et al 2006). This enzyme-microbe-cellulose complex appears to be a surface phenomenon involving microbial adhesion onto cellulose via enzymes and thus requires the presence of metabolically active *Clostridium thermocellum* displaying cellulosome on its surface. The significant four-fold increase in the cellulose hydrolysis, observed due to synergistic effect, was noteworthy in decreasing the cost for cellulose hydrolysis (Zhang and Lynd, 2005).

2.5.1 Cellulosome for efficient cellulose hydrolysis

Anaerobic microbes can only produce limited amount of enzymes under anaerobic conditions due to energy limitations. In response to that they have developed an elaborately structured multi-enzyme complex, called cellulosome, to maximize the catalytic efficiency (Bayer et al 2004; Demain et al, 2005). Cellulosomes are extracellular self-assembled multi-enzyme complexes produced by and displayed on the cellulolytic anaerobic microbes that efficiently degrade cellulose. This macromolecular system brings multiple enzymes in close proximity to the substrate and provides a highly ordered structure that gives much higher catalytic efficiency than soluble enzymes present in the free state. The cellulosome system has exhibited much greater degradative potential as compared to non-complexed cellulase system, thus might act as a 'quantum leap' in development of biomass to biofuel technology.

The major components that distinguish the cellulosome from free enzyme systems are the scaffolding protein containing several cohesin domains, and enzymatic subunits containing dockerin domains as shown in Figure 2-5. Beside multiple cohesins, scaffoldin contains at least one cellulose binding domain (CBD), which acts as a targeting agent to facilitate the binding of substrate to the catalytic domains. Harsh treatments are required for complete dissociation of cellulosome into individual components, thus indicating the strength of cohesin-dockerin interaction. Interaction of cohesin and dockerin is Ca^{2+} dependent and of high affinity (Fierobe et al 1999 and 2001). Cohesin-dockerin interaction is highly species-specific which provides highly ordered and position-specific assembly of catalytic domains. However, within a given species,

cohesin can interact with any of the dockerin domains, suggesting a random incorporation of enzymes in the cellulosome (Pages et al 1999). Therefore, the relative abundance of enzymatic subunits is supposed to suggest the level of expression of corresponding genes, as described in the recent case of *C. cellulyticum* using a genetic approach (Fierobe et al 2004).

The functional presentation of cellulose-binding domain and catalytic subunits in the cellulosome offers upgraded cellulose hydrolysis over non-complexed cellulase system as a result of synergistic actions amongst different components (Bayer et al 1994). Recent studies have made "designer cellulosomes" due to modular nature of cellulosome assembly. A trifunctional miniscaffoldin has been created *in vitro* by incorporating different origin cohesins and each type of cohesin was shown to bind to the corresponding dockerin-borne cellulases (Fierobe et al 2001, 2005). The resulting recombinant mini-cellulosome showed 6-fold enhanced hydrolysis activity with cellulose over similar free enzyme system. This "designer cellulosome" concept was used for whole-cell hydrolysis of cellulose and ethanol production if displayed on to *S.cerevisiae* cell surface. Yeast capable of displaying mini-scaffoldin on its surface when tagged with three different dockerin-fused cellulases showed three-fold increase in cellulose degradation (Tsai et al 2009).

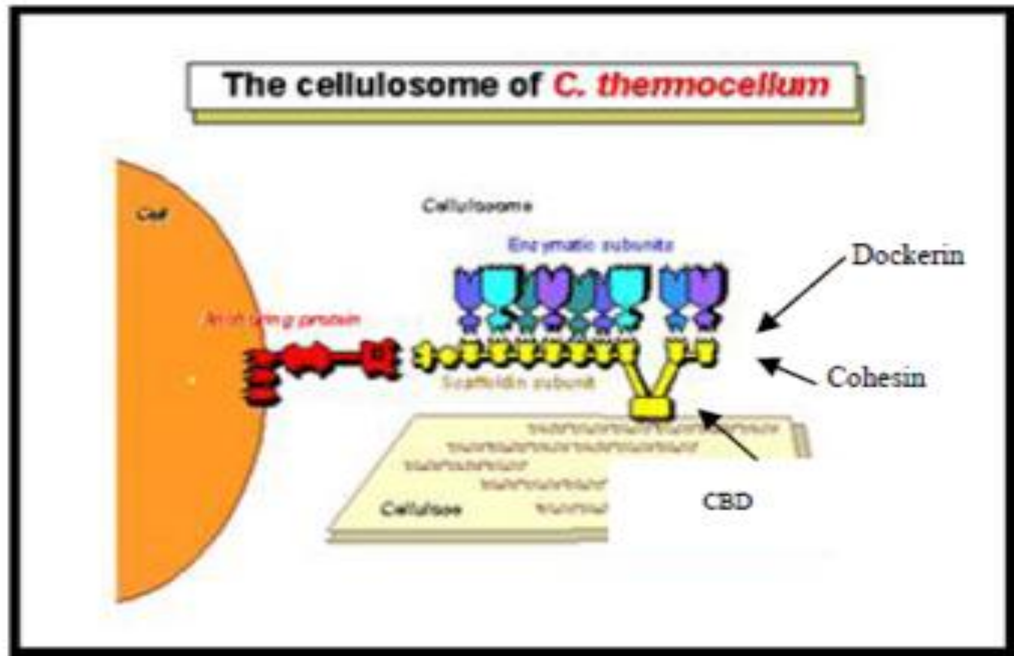


Figure 2-5:The cellulosome from C. thermocellum which consists of a scaffoldin domain with 9 cohesins and 1 CBD, 9 catalytic subunits tagged with dockerins and species specific cohesin - dockerin interaction. The cellulosome complex is attached to the cell surface through an anchoring protein. (Bayer et al., 2006)

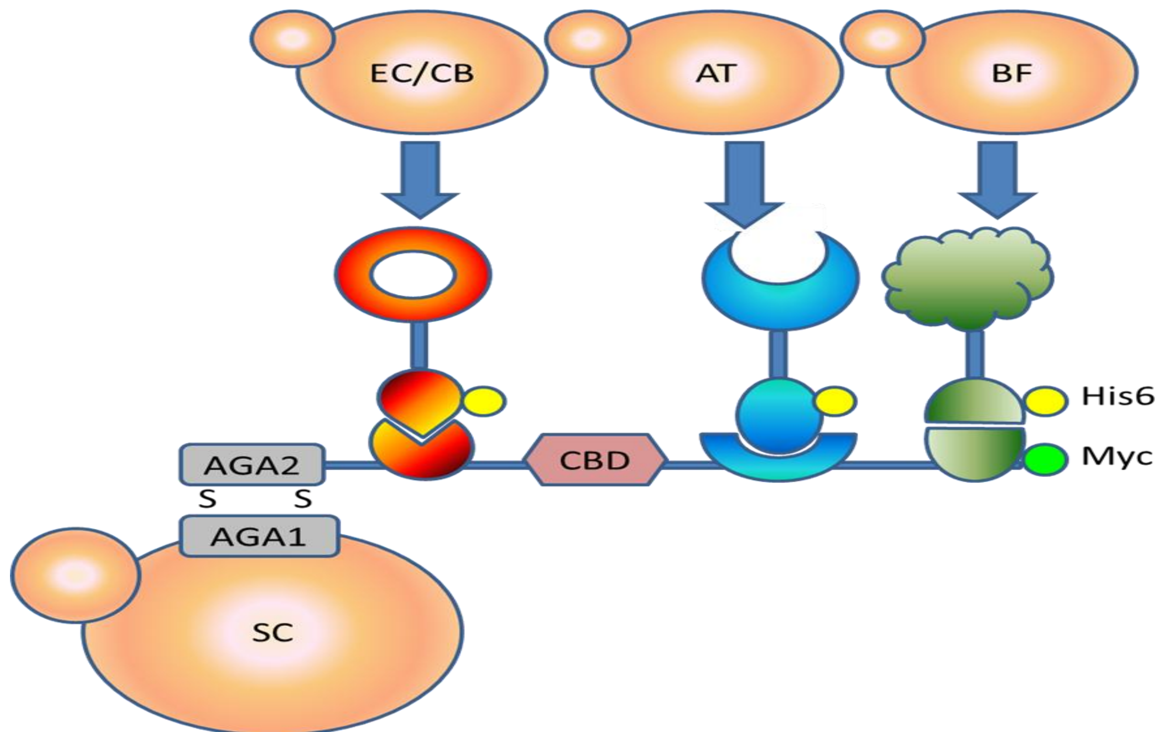
Another study reported the recombinant *S. cerevisiae* displaying trifunctional mini cellulosome. This minicellulosome consists of a chimeric miniscaffoldin containing a CBD and three cohesin modules. This was displayed on cell surface by a-agglutinin adhesion receptor and three cellulases tagged with three corresponding dockerin domains. A yeast strain capable of producing cell associated functional mini-cellulsome was able to produce ethanol directly from cellulose but with low productivity (Wen et al 2010). Low production of ethanol can be attributed to excessive metabolic burden due to heterologous expression of all the components in one strain.

2.5.2 Development of microbial consortium system

The moldular nature of the cellulosome, great diversity of cellulases, and the availability of gene fusion technology provide almost unlimited number of combinations of enzymes and ways to incorporate them into artificial cellulosome to approach or even beat the natural cellulosome. The use of a single strain for surface anchoring and cellulase secretion is unlikely to be successful due to energetic limitations. For the single host cell system, the inability to fine tune levels of the three cellulases resulted in a higly uneven distribution toward endoglucanase. In addition, the surface display of large, complex cellulosome structures may result in saturation of the translocation machinery and potentially cell death. To solve these problems, a recent report by our group has shown the site-specific display of a multi-functional enzyme complex on the yeast surface through cooperative intracellular complementation using a synthetic yeast consortium system (Tsai et al 2010).

It has been found in nature that for the degradation of polysaccharides, microbes communicate and work in synchrony at times or individually by sophisticated means called 'quorum sensing' with other bacterial or fungal species (Bayer and Lamed, 1992; Bayer et al., 1994). Feasibility of microbial consortium for biofuel production from agriculture waste has been suggested in literature (Ragauskas et al.2006). To develop this synthetic yeast consortium, engineered yeast capable of displaying miniscaffoldins on the cell surface was first created(Tsai et.al.2009), which consists of a cohesin from *C. cellulolyticum* followed by a CBD, a cohesin from *C. thermocellum*, and a cohesin from *R. flavefaciens*. A c-Myc tag was added to the C terminus of each scaffoldin to allow

detection with anti-c-Myc serum. In parallel, three engineered yeasts capable of secreting endoglucanase, cellobiohydrolase and β-glucosidase individually were also created for providing the enzymatic activity of the cellulosome structure. All the enzymatic subunits were fused with dockerin domains from same species as that of cohesins. A his6 tag was added at the c-terminus of the dockrin fused enzymatic subunit to allow the detection of cohesin-dockerin binding with anti-his6 antibody. Each population served as a building block for constructing the mini-cellulosome structure on the yeast surface. Added together, an engineered consortium capable of displaying cellulose-degrading cellulosome structures on *S. cerevisiae* surface was fabricated as shown in Figure 2-6.The yeast consortium system was able to distribute the metabolic burden of heterologous protein expression amongst four yeast strains and produce concomitant ethanol from cellulose with high yield and productivity.



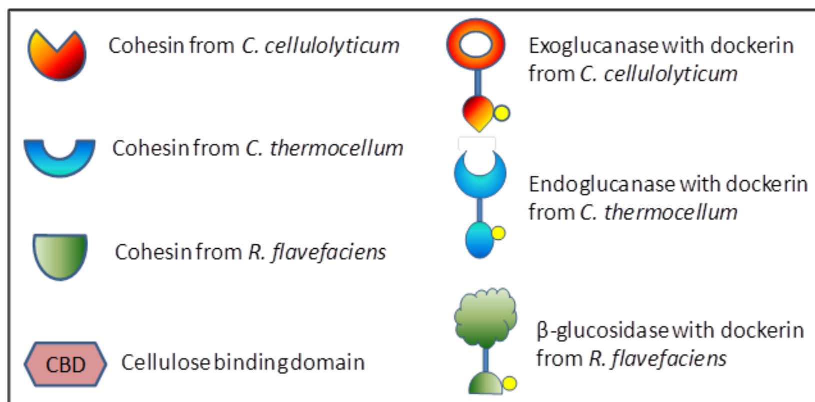


Figure 2-6:Surface assembly of a functional mini-cellulosome through intracellular complementation using a synthetic yeast consortium. The basic design consisted of four different engineered yeast strains capable of either displaying a trifunctional scaffoldin Scaf-ctf (SC) or secreting one of the three corresponding dockerin-tagged enzymes (endoglucanase (AT), exoglucanase (EC/CB) or β-glucosidase (BF) (Tsai et al 2010))

The overall objective of this thesis is consolidated bio-processing of cellulosic biomass for efficient biofuel production using yeast consortium. A yeast consortium system has been developed by our group to display functional mini-cellulosome on yeast surface for efficient biofuel production. Due to modular nature of consortium system, the overall cellulosome assembly, cellulose hydrolysis and ethanol production can be easily fine-tuned. The yeast consortium with its ability to hydrolyze recalcitrant cellulose efficiently and then its conversion to ethanol concomitantly is one step closer to act as CBP. To test the idea of yeast consortia as CBP, it's feasibility to perform under different conditions will be evaluated. The following chapters provide information and results for each of the studies that aim for the efficient cellulose hydrolysis and ethanol production by using yeast consortium system.

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CHAPTER 3

Optimization of synthetic yeast consortium system: Application for cellulose hydrolysis and ethanol production

Abstract

For the first time, we report the use of synthetic yeast consortium which is functionally displaying a mini-cellulosome on the yeast surface through intracellular complementation as CBP. Yeast consortium developed by our group was used in this study. The basic design of the consortium consisted of four different engineered yeast strains, one is capable of displaying a trifunctional scaffoldin Scaf-ctf (SC) and other three secreting each of three corresponding dockerin-tagged cellulases (endoglucanase (AT), exoglucanase (EC/CB) or β-glucosidase (BF)). The secreted cellulases tagged with dockerins were docked onto the displayed Scaf-ctf in a highly organized manner based on the specific interaction of cohesin-dockerin pair and this result in the formation of mini-cellulosome on the yeast surface. By exploiting the modular nature of each population to provide a unique building block for the mini-cellulosome structure, the overall cellulosome assembly, cellulose hydrolysis, and ethanol production were easily fine-tuned by regulating the ratio of different populations in the consortium. The optimized consortium consisting of a SC:AT:CB:BF ratio of 7:2:4:2 produced almost twice the level of ethanol (1.87 g/L) than a consortium with an equal ratio of the different populations. The final ethanol yield of 0.475 g ethanol/g cellulose consumed also corresponded to 93% of the theoretical value. This result confirms the use of a synthetic biology approach for the synergistic saccharification and fermentation of cellulose to ethanol using a yeast consortium displaying a functional mini-cellulosome.

3.1 Introduction

Cellulosic biomass is one of the most abundant and sustainable materials for biofuel production because of its high sugar content. It has been estimated that 1.4 billion tons of cellulosic biomass can be produced every year without affecting food supply, animal feed, and fiber use (Perlack et al 2005). According to the new Energy Policy Act, several billion gallons of renewable fuel must be produced by 2012 with most of those produced as biofuels using renewable biomass. Bioethanol is particularly attractive as an alternative transportation fuel and could lessen the Nation's dependence on foreign oil (Lynd et al 2005).

Unfortunately, cost-effective production of bioethanol from cellulosic biomass remains a major challenge, primarily due to its highly recalcitrant nature (Himmel et al 2007). The primary inhibiting factor is the high cost of the enzymes used in large quantities for efficient biomass conversion to fermentable sugars. The cost of bioethanol production has become more competitive by combining cellulose saccharification and fermentation (SSCF) in single reactor. A new method known as consolidated bioprocessing (CBP), which further combines enzyme production with SSCF into a single process, has gained increasing recognition as a potential solution for the low-cost production of bioethanol (Lynd et al 2008). However, a natural microorganism that possesses the capability for efficient enzyme production, cellulose saccharification, and

ethanol fermentation is difficult to find (Zhang et al 2005). In recent years, efforts have been made in engineering microorganisms toward the goal of consolidated bioprocessing. In particular, *Saccharomyces cerevisiae* is an attractive engineering candidate due to its high ethanol productivity and high inherent ethanol tolerance (Nevogit 2008). Although, many past attempts based on either secretion of cellulases or surface display of cellulases have resulted in relative low ethanol productivity (Cho et al 1999; Curry et al 1988; Fujita et al 2004; 2002).

In nature, many anaerobic microorganisms are found with an elaborate enzyme complex known as a cellulosome for efficient hydrolysis of cellulose. This highly ordered structure allows the assembly of multiple enzymes in close proximity to the substrate resulting in a high level of enzyme-substrate-microbe synergy (Fierobe et al 2005). Our group has recently reported the 3-fold increase in ethanol production from phosphoric acid swollen cellulose (PASC) as compared to non-complexed system by demonstrating the functional assembly of mini-cellulosome on the yeast surface (Tsai et al 2009). A similar enhancement in ethanol production was also reported by the Zhao group using an engineered yeast strain co-expressing a displayed mini-scaffoldin and three different cellulases (Wen et al 2010). However, co-expression of all four components in a single strain resulted in relative low levels of exoglucanase and β-glucosidase probably due to the heavy metabolic burden and potential jamming of the secretion machinery. To address these issues, we reported the use of a synthetic yeast consortium composed of one strain displaying the mini-scaffoldin and three strains secreting dockerin-tagged cellulases for the functional presentation of minicellulosomes onto the yeast surface(Fig.

2-6)for efficient bio-ethanol production (Tsai et al 2010). By exploiting the specific interaction of the modular nature of the consortium design, optimal performance can be obtained by fine-tuning the required ratio of cellulase-secreting cells and mini-scaffoldin-displaying cells. The resulting consortium is capable of producing ethanol directly from PASC.

3.2 Materials and methods

3.2.1 Strains and media conditions

All the strains used in this study are listed in Table 3-1. Except for the consortium experiments, yeast strains were either grown in YPD medium (2% dextrose, 1% yeast extract, 2% peptone) or SDC medium (2% glucose, 0.67% yeast nitrogen base, 0.5% casamino acids).

3.2.2 Development of synthetic consortia and fermentation

Yeast strains EBY100 harboring either pSctf or pBGLf (carrying a *Trp1* marker) and BY4742 harboring either pCEL15, pAt, or pCBH2c (carrying a *URA3* marker) were first pre-cultured in SDC medium at 30°C for 18 h. For co-culturing of the synthetic consortia, the initial strains were mixed to the desired ratio into 200 mL SGC medium (20.0 g/L galactose, 6.7 g/L yeast nitrogen base w/o amino acids, 5.0 g/L casamino acids) supplemented with 10 mM of $CaCl_2$to an optical density (OD_{600}) of 1 and grown for 48 h at 20°C.

3.2.3 Resting cell assays

For the resting cell assays, PASC was prepared from AvicelPH101 (Sigma,)according to the method of Walseth (Walseth 1952) and used as the substrate. Cells from the different consortia were first washed once with buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM $CaCl_2$ and resuspended in 20 mM Tris-HCl buffer (pH 6.0) supplemented with 10 mM $CaCl_2$ and 10g/L PASC to a final OD of 50. Samples were collected periodically and immediately mixed with 3 mL of DNS reagents (10 g/L dinitrosalicylic acid, 10 g/L sodium hydroxide, 2 g/L phenol, 0.5 g/L sodium sulfite) to determine the level of reducing sugar. After incubating at 95°C for 10 minutes, 1 mL of 40 % Rochelle salts was added to fix the color before measuring the absorbance of the supernatants at 575 nm. Glucose concentration was determined using a glucose HK assay kit from Sigma.

3.2.4 Fermentation

Cells from the different consortia were washed and resuspended in 10 ml SDC medium containing 6.7 g/L yeast nitrogen base without amino acids, 20 g/L casamino acids, and10 g/L PASC as the sole carbon source. Reducing sugars and glucose concentration were measured by the methods described above. The amount of residual cellulose was measured by the phenol-sulfuric acid method as described by Dubois et al (Dubois et al 1956). Ethanol concentration was measured by gas chromatography (model 6890, Hewlett Packard, USA) using a flame ionization detector and a HP-FFTP column.

3.2.5 Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Tsai et al 2009). Briefly, cells were washed with PBS and resuspended in PBS containing 1 mg/ml of BSA. Either anti-His6 or anti-Myc antibody was added and incubated for 1 h with occasional mixing. Alexa Fluor™488- conjugated anti-mouse IgG was added after washing and resuspending in PBS with 1 mg/ml of BSA. Images were acquired by a fluorescence microscope (Olympus BX51) after washing with PBS for three times. Whole cell fluorescence was measured using a fluorescent micro-plate reader (Synergy4, BioTek, VT) with an excitation wavelength at 485 nm and an emission wavelength at 535 nm.

3.3 Results

3.3.1 Systematic improvement of the synthetic consortia for producing cellulosic ethanol

All the consortia used in this study are listed in Table 3-2. Although the feasibility of using the yeast consortium for ethanol production has been demonstrated in our recently published report (Tsai et al 2010), the ethanol yield is lower than our previously published report using *in vitro* enzyme loading (Tsai et al 2009). This can be attributed to a lower percentage of cells displaying the mini-scaffoldin (18% to 60%) and the less efficient enzyme docking (less than 1:3 ratio between the Cmyc tag and His tag signal). To address these problems, a systematic improvement of the synthetic consortium was studied. By exploiting the specific interaction of the three separate cohesin-dockerin pairs employed, we can easily fine tune the ratio of different populations by regulating the

initial inoculation ratio. To investigate this possibility, we first optimized the level of reducing sugar production using this strategy. Initially, cultures with different inoculation ratios of SC and AT were harvested after 48 h of co-culturing and resuspended in buffer containing PASC. As shown in Fig. 3-1A, the reducing sugar concentration increased with the increasing SC:AT ratio reaching the highest level at a ratio of 3:1, while no further improvement was observed at higher ratios (data not shown). This rise in reducing sugar production was accompanied by a corresponding increase in both the Cmyc and His tag signals (Fig. 3-1B), consistent with the expected higher percentage of Scaf-ctf displaying cells. Moreover, the higher level of His tag signal suggests improved binding of the secreted AT onto the displayed Scaf-ctf due to the higher cohesin to dockerin ratio. This result clearly illustrates our ability to improve the performance of the consortium simply by adjusting the inoculation ratio.

Using a similar strategy, we attempted to modify the ratio of CBH and BGL in the consortium while trying to keep the ratio of SC to AT close to 3:1. As shown in Fig. 3-1C, glucose production can be further enhanced by increasing both the CB and BF ratios. The optimized consortium which released the highest level of glucose was found to consist of a SC:AT:CB:BF ratio of 7:2:4:2. This enhancement is a result of both a larger fraction of Scaf-ctf displaying cells (35%) and an increased amount of enzymes docked onto the mini-cellulosome (Fig. 3-1D). To investigate whether the increased glucose production in resting cell assay can be directly converted into ethanol production, anaerobic fermentations using PASC were conducted. As shown in Fig. 3-2, the optimized consortium showed an almost 2-fold increase in both cellulose hydrolysis and

ethanol production. The ethanol yield of 0.475 g ethanol/g sugar consumed corresponds to 93% of the theoretical value.

3.4 Discussion

Cellulose, a major component of the plant cell wall, is the most abundant renewable carbon source in nature that can be enzymatically degraded for bioethanol production. However, the high cost of cellulases needed for complete hydrolysis is still one of the major problems in the quest for an economically feasible cellulose-based bioethanol process (Mcbride et al 2005). Cellulosome is a multi-component enzyme complex that has been extensively investigated in recent years because of its intriguing structure and great ability in providing synergistic and highly efficient degradation of cellulose. Progress has been made in engineering yeast cells to display mini-cellulosome structures toward the goal of CBP (Tsai et al 2009; Wen et al 2010). However, the two previous studies based on either *in vitro* loading of enzymes or simultaneous display of scaffoldin and secretion of cellulases in a single host cell have problems. In the case of *in vitro* enzyme loading, the process cannot truly be CBP since separate *E. coli* cultivations are necessary. For the single host cell system, heavy metabolic burden and the inability to fine tune levels of the three cellulases resulted in a highly uneven distribution of enzymes.

To address these problems, a synthetic yeast consortium was engineered by our group (Tsai et al 2010) capable of surface assembly of a functional mini-cellulosome via intracellular complementation. The basic design consisted of four different engineered

yeast strains capable of either displaying the mini-scaffoldin or secretion of one of the three required dockerin-tagged enzymes (endoglucanase, exoglucanse or β-glucosidase). There are several unique features of our consortium system. First, the dockerin/cohesin pairs used in the consortium system are from three different species, which enable specific interactions between each dockerin-tagged enzyme and the displayed mini-scaffoldin, resulting in highly controllable ordering of each enzyme in the mini-cellulosome structure. Second, by exploiting the modular nature of each population to provide a unique building block for the mini-cellulosome structure, the overall cellulosome assembly, cellulose hydrolysis, and ethanol production can all be easily fine-tuned by adjusting the ratio of different populations in the consortium. As a result, the improved consortium consisting of a SC:AT:CB:BF ratio of 7:2:4:2 produced almost twice the level of ethanol than a consortium with an equal ratio of the different populations. To our knowledge, this is the first successful report of use of synthetic consortium system as CBP for efficient cellulose hydrolysis and ethanol fermentation.

3.5 Acknowledgement

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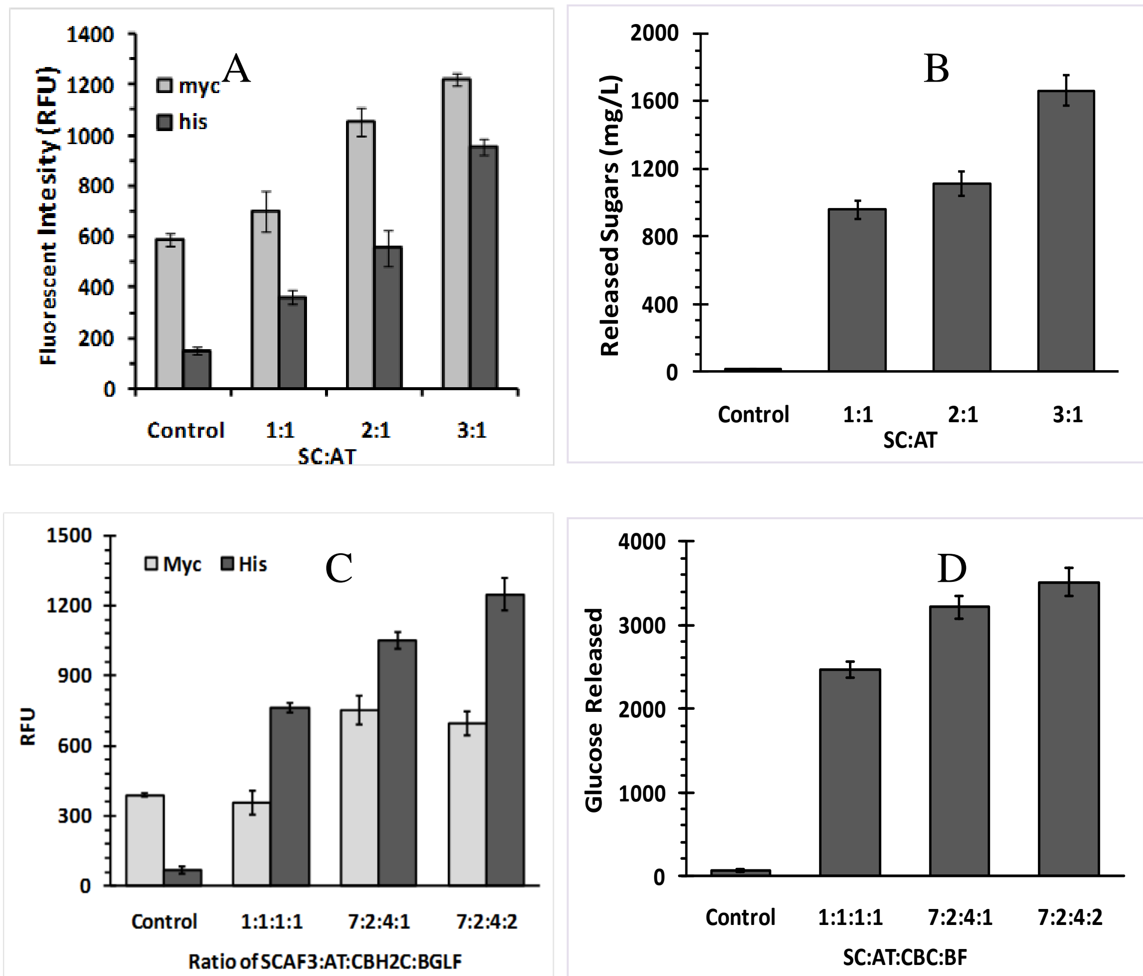

Figure 3-1: Systematic optimization of different cell populations in the synthetic consortium. Effects of SC to AT ratio on (A) reducing sugars production and (B) At binding. (C) Effects of SC:AT:CB:BF ratio on (C) glucose production and (D) enzyme binding. Enzyme binding was determined by immunofluorescence microscopy. Cells were probed with either anti-C-myc or anti-C-His6 sera and fluorescently stained with a goat anti-mouse IgG conjugated with Alexa Fluor 488. Whole cell fluorescence was determined using a fluorescent microplate reader.

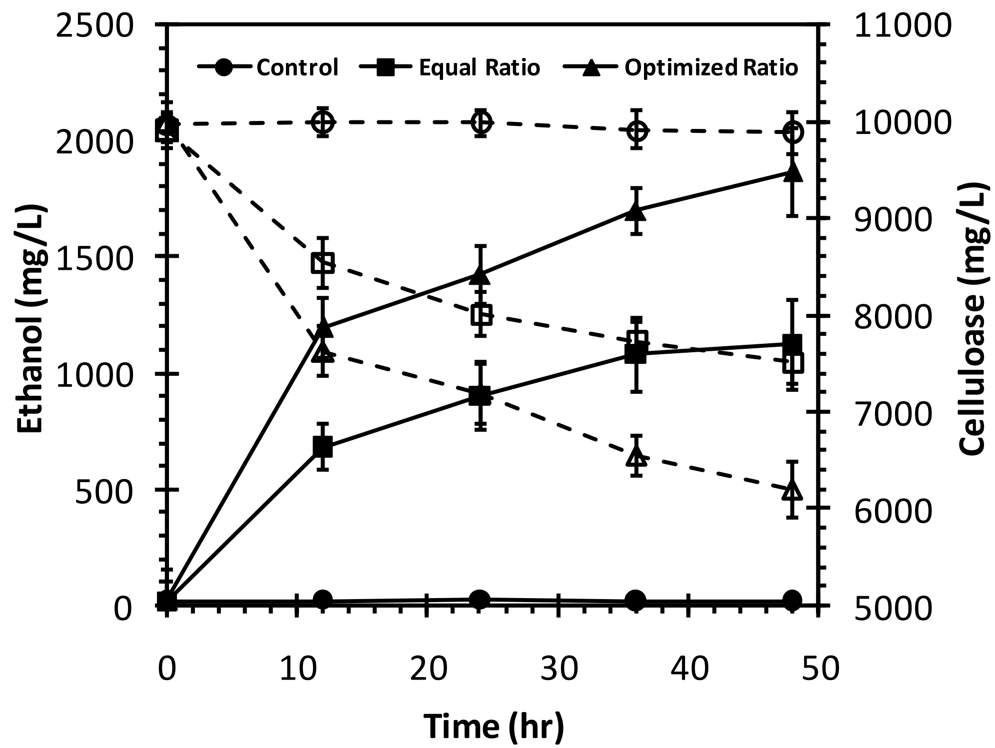

Figure 3-2:Cellulose hydrolysis (dashed line) and ethanol production (solid line) from PASC by the optimized consortium.

TABLE 3-1. Strains and plasmids used in this study

Strain	Plasmid	Phenotype
CE	pCEL15	Secrete a small peptide (negative control)
AT	pAt	Secrete endoglucanase At (CelA from *C. thermocellum*)
CB	pCBH2c	Secrete cellobiohydrolase CBHc (CBHII from *T. reesei*)
BF	pBGLf	Secrete β-glucosidase Bglf (Bg1I from *T.aurantiacus*)
SC	pScaf3	Display the mini-scaffoldin Sacf-ctf

TABLE 3-2. Consortia generated in this study

Consortium	Populations
C1	SC, CE, CE, CE
C2	SC, AT, CE, CE
C3	SC, AT, CB, CE
C4	SC, AT, CB, BF

CHAPTER 4

Synthetic Yeast Consortium for efficient biofuel production: A New Candidate for Consolidated Bioprocessing

Abstract

Early in our lab, a synthetic consortium capable of producing cellulase to hydrolyze cellulose and producing ethanol was created. However, to further demonstrate that the synthetic consortium is a suitable candidate for a Consolidated Bioprocessing (CBP), experiments were undertaken to show the ability of this synthetic consortium for hydrolyzing cellulose and using the resulting sugars for cell growth as well as for ethanol production. A cell surface display system based on alpha-agglutinin for surface assembly of a functional mini-cellulsome on yeast using a synthetic consortium system and three yeast strains capable of secreting three corresponding dockerin tagged enzymes were used as described by our previous study (Tsai et al 2010). The resulting synthetic consortium was proficient in growing on a media containing PASC (Phosphoric acid swollen cellulose) as the sole carbon source under anaerobic conditions. The final concomitant ethanol production of 1.25 g/L with 0.43 grams of ethanol produced per gram of cellulose consumed corresponded to an 87% of theoretical yield. Moreover, to demonstrate that none of the populstion in this engineered consortium take over the other one, quantitative real time PCR was used to enumerate the individual yeast population in the mixed culturefor the dynamic study of yeast consortium systems. To our knowledge, this is the first report of a dynamic study of mixed culture for biofuel production.

4.1 Introduction

Due to its renewable, abundant, and sustainable nature, ligno-cellulosic biomass is the only feedstock that has the potential to replace a significant amount of fossil fuels in the transportation sector. It has been estimated that 1.3 billion mega-tonnes (dry weight) of terrestrial plants are produced annually on a world-wide basis (Demain et al.,2005). Bioethanol, which is generally expected to be the first major commercial product of this emerging cellulosic biofuels technology, has great potential to lessen the dependency on foreign oil (Lynd et al, 2005).

Unfortunately, the recalcitrant nature of cellulosic material poses the major technological impediment to the more widespread exploitation of this resource. Lignocellulosic material naturally resists to chemical attack, solubilization, and bio-conversion due to its rigid and complex molecular polymeric structure (Himmel et. al. 2007). CBP (consolidated bioprocessing) is gaining increasing recognition as a potential breakthrough low-cost biomass processing method. When a mature CBP process is substituted for an advanced SSCF (simultaneous saccharification and co-fermentation) process, a four-fold reduction in the cost of biological processing and a two-fold reduction in the cost of overall processing can be achieved (Lynd et al., 2008). An ideal micro-organism for CBP should be capable of efficient enzyme production and have the ability to perform simultaneous cellulose saccharification and ethanol fermentation. *Saccharomyces cerevisiae* is an attractive candidate for the industrial ethanol production due to its high ethanol productivity and high inherent ethanol tolerance (Nevoigt 2008). In recent years, several attempts have been made to engineer S. cerevisiae to hydrolyze

cellulose by either secretion or surface display of cellulases. However, due to energy limitations under anaerobic conditions, only a small amount of cellulytic enzymes can be produced (Cho et al, 1999; Curry et al, 1988; Haan et al, 2007; Fujita et al. 2002; 2004).

In nature, many anaerobic bacteria and fungi produce a large extracellular enzyme complex on the cell surface to synergistically act on cellulose called a cellulosome. It's extremely organized structure permits the assembly of multiple cellulases in close proximity to substrate to maximize the catalytic efficiency of cellulose hydrolysis with only a limited amount of enzymes due to a high level of synergy between microbes, enzymes and substrate (Fierobe et al., 2005). In our previous report, we reported the successful display of a chimeric mini-cellulosome on the yeast surface which resulted in an increase in ethanol production due to synergy, which was later confirmed by other studies (Tsai et al., 2009; wen et al., 2009). In our recent work (Tsai et al., 2010), we have reported the use of a synthetic yeast consortium consisting of one yeast strain displaying scaffoldin and three yeast strains secreting cellulases fused with the dockerin domains to address issues such as excessive metabolic burden when all components are co-expressed in a single strain, as well as potential congestion of secretion machinery attributed to high cohesion-dockerin interaction. Ethanol production was further optimized by fine-tuning the ratio of the four yeast cells by changing their initial inoculum ratio.

The yeast Saccharomyces cerevisiae is considered to be a good host for heterologous gene expression and protein secretion. Cloned genes must be stably

maintained in the microbial populations for the process to be productive. High level cloned gene expression can result in drastic reduction in stability and copy number of 2 micron plasmids even under selective conditions (Elliott et al. 1989). To address this issue, we report here the use of CEN/ARS plasmids for expression of scaffoldin protein on the cell surface for stability and constant copy number. In this work, we used qPCR approach to study the dynamics of individual yeast population in the yeast consortium. qPCR is one of the most utilized tools to rapidly quantify individual yeast present in a mixed culture.

4.2 Materials and methods

4.2.1 Strains, plasmids, and media

Escherichia coli strain JM109 [recA1 end A1 supE44 hsdR17 gyrA96 thi, relA1, λ^{-1} Δ(*lac*-*pro*AB) F *tra*D36 *pro*AB*lac*IqZ DM15] was used as a host for genetic manipulations. Cells were grown in LB medium (5g/lyeast extract, 10g/l NaCl, 10g/l tryptone) supplemented with Ampicillin (100mg/l) when required. S.cerevisiae strain BY4742(*MAT***α***his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used for displaying the scaffoldin. The genotypes and sources of all the bacterial and yeast strains, as well as the plasmids that were constructed and used in this study are list in Table 4-1. The yeast strains were routinely cultured in SDC medium (20g/l Dextrose, 6.7g/l yeast nitrogen base, and 5g/l casamino acids) at 30^0C on a rotary shaker at 250 rpm.

4.2.2 Subcloning of Agalpha-scaffoldin into centromeric vector YCplac33

The PCR fragment (4049 bp) consisting of *Saccharomyces cerevisiae* 3-phosphoglycerate kinase (PGK1) promoter, C-myc tag, scaffoldin containing three cohesins from *Clostridium cellulolyticum*, *Clostridium thermocellum* and *Ruminococcus flavefaciens* and CBD domain, alpha-agglutinin structural gene- AG alpha1 and PGK1 terminator was amplified from the plasmid AGalpha-Scaf3. The primers used (i.e. forward primer (Pgkfp) and reverse primer (PgkRp)) contain underlined *NcoI* restriction site and pGKI promoter and terminator sequences respectively. The PCR product obtained was digested with *NcoI* and was ligated with both forward and revers linkers (sequences given in table 4-2) containing *Bam*HI compatible ends. The vector YCplac33 was digested with *Bam*HI and ligated to the insert. The ligated mixture was transformed in *E.coli* JM109. The transformants were initially screened by colony PCR and later confirmed by restriction digestion and named YCplac33-AGalpha-scaf3. The YCplac33-AGalpha-scaf3 plasmid was transformed in *S. cerevisiae*BY4741using standard lithium acetate procedure.

All the primers used in this study are given in Table 4-2.

4.2.3 Anaerobic fermentation

PASC was prepared as described by Walseth from Avicel PH101 (Sigma) and used as the carbon substrate. For anaerobic fermentation, yeast strains were grown in rubber stoppered glass serum bottles in SC-PASC medium containing 6.7g/l yeast nitrogen base without amino acids, 20 g/l casamino acids, and 10 g/l PASC as the sole carbon source supplemented with 10mM $CaCl_2$, 0.01g/l ergosterol and 0.42 g/l Tween

80. Precultures of yeast populations of all the consortia were grown separately in SDC media. For co-culturing of the synthetic consortia, initial strains were mixed in the optimized ratio to a total initial optical density of 1. Cultures were washed with sterile water to prevent media carry over. Samples were collected periodically through a capped syringe needle pierced through the bottle stopper. Yeast cells in fermentation media were counted on SDC plates by the plate count method in triplicates.

4.2.4 Resting cell assays

Reducing sugars were measured by DNS (Dinitro salicylic acid) method. Samples were collected periodically and mixed immediately with equal amount of DNS reagents (10g/l dinitrosalicylic acid, 10g/l sodium hydroxide, 2g/l phenol, 0.5 g/l sodium sulfite) and incubated for 5-15 minutes at 95^0C. 1 ml of 40% Rochelle salts was added to fix the color before measuring the absorbance at 575 nm using a spectrophotometer. Glucose concentration was determined by using a sigma HK assay kit. For measuring the amount of unhydrolyzed cellulose, a phenol-sulfuric acid described by Dubois et.al (1956) was used. Ethanol concentration was measured by gas chromatography (model 6890, Hewlett Packard, USA) with HP-FFTP column and FID detector.

4.2.5 Immunofluorescence Assay

The immune-fluorescence assay was performed as described previously (Tsai et.al 2009). In short, the cells were washed with PBS (Phosphate buffered saline) and re-suspended in PBS containing 1 mg/ml BSA (bovine serum albumin). Anti-His6 or anti-myc antibody was added to the cells to react with the surface displaying mini-

cellulosome and incubated at room temperature for 1 hour on a rotary shaker. AlexaFluor^{TM}488 – conjugated with anti-mouse secondary anti-body was added after washing and re-suspending the cells in PBS mixed with 1 mg/ml BSA. Cell fluorescence images were obtained by a fluorescence microscope (Olympus BX51) after washing the cells thrice with PBS.

4.2.6 Real time quantitative PCR

Yeast DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) and measured using Nano-drop at 260/280 nm. All the primers used for real time PCR are listed in Table I. Quantitative PCR assays were done in 25 µl final volumes containing 2 µl DNA template, 0.2 µM each respective primer, and 12.5 µl of SYBR Green Master Mix (Fisher Scientific). All the amplifications were carried out in optical grade 96 well plates from Bio-rad with an initial step at 95^{0}C for 3 min followed by 40 cycles of 95^{0}C for 15 s, 57^{0}C for 1 min, and 72^{0}C for 30 s. Melt curve analysis was performed at the end starting from 55^{0}C with 0.5^{0}C increment per second until 90^{0}C to avoid the formation of primer-dimer. The C_T value was determined automatically by the instrument, and all samples were analyzed in triplicates. To quantify the individual yeast population, standards were generated for each cell-line individually by ten-fold dilutions from 10^6 CFU/ml to 10^4 CFU/ml.

4.3 Results

4.3.1 Surface displayment of scaffoldin by centromeric system

Many studies have revealed that a high copy number of plasmids bearing heterologous genes causes high levels of transcription and therefore efficient production

of heterologous proteins (Bitter et al 2007). However, under nutrient-deficient conditions, microbes tend to lose plasmids due to excessive metabolic burden, which arises in order to overproduce the proteins. YCp plasmids containing yeast a centromeric sequence ensure mitotic stability and constant copy number. Structurally plasmid borne centromere sequences have the same distinctive chromatin structures that occur in the centromere region of yeast chromosomes (Bloom and carbon 1982). YCp functionally exhibits three characteristics in yeast cells including mitotic stability in the absence of selective pressure, segregation during meiosis in a Mandelian manner, and low copy number in the host cell. In our last report (Tsai et al 2010) heterologous genes encoding for cellulases and scaffoldin, were expressed by a 2 micron (multi-copy) plasmid. A new surface displaying vector was constructed in a CEN/ARS carrying plasmid YCplac33-Agα-scaf3 under transcriptional control of the constitutive promoter PGK1. This plasmid was subsequently transformed into *S. cerevisiae*BY4741.

To examine the functional displayment of scaffoldin and comparison between the 2 micron and centromeric plasmids expressing scaffoldin protein, immunofluorescence assays were carried out using the anti C-myc antibody (Fig. 4-1). While the fraction of yeast cells displaying scaffoldin in the case of the 2 micron based plasmid is approximately 65% (Fig. 4-1A), it is 20% higher in the case of the centromeric based plasmid (Fig. 4-1B), thus confirming the functionality and enhanced implementation of scaffoldin displayed by the centromeric based system.

4.3.2 Growth and ethanol production from PAS-avicel

The feasibility of surface display of a functional mini-cellulosome by intracellular complementation using a synthetic yeast consortium for efficient cellulose hydrolysis and ethanol production has already been demonstrated in our recent report (Tsai et al 2010). Thisyeast consortium containing four yeasts – one displaying scaffoldin on the surface, and the other three secreting three corresponding enzymes is capable of hydrolyzing cellulose into glucose. Four different consortia with the yeast populations- a strain displaying scaf-ctf (SCL) expressed by a low copy plasmid, a strain displaying scaf-ctf (SC) expressed by high copy plasmid, a strain carrying pCEL15 as a negative control, an At-secretion strain (AT), an CBH II-secretion strain (CB), and a Bglf-secretion strain (BG)- were used to investigate the ability of consortium system to grow on PAS-avicel and produce ethanol. All consortia used are mentioned in Table 4-3.

Different populations were initially grown separately in SDC medium and then mixed in the optimized ratio (7:2:4:2) (Tsai et al.2010) to a total OD of 0.8, and then incubated for half an hour for the assembly of mini-cellulosome on the surface of SCL strain. A small concentration of glucose, 0.2 g/L, was also added to the fermentation media to stimulate the fermentation. As shown in Fig 4-2A, maximum cell densities of 2.5×10^7 cells/mL were achieved by the C4 consortium. On the other hand, the control consortium C1 with no enzyme secretion showed an almost negligible increase in the cell density from the initial value 8×10^6 cells/mL. A small increase in C1 can be attributed to the fact that 0.2 g/L of glucose was added initially. This result clearly demonstrates the superior growth, and hence more stable expression of scaffoldin

protein on the yeast surface by consortium C4 than that of C3. Simultaneous ethanol fermentation and cellulose hydrolysis in the anaerobic fermentation was calculated by GC and phenol-sulfuric acid analysis, respectively (Fig 4-2B). The increase in cellulose hydrolysis is accompanied by a corresponding increase in ethanol formation. An ethanol production up to 1.25 g/L was observed after 192 hours of fermentation with a yield of 0.43 g ethanol/g cellulose which is equivalent to 87% of theoretical yield.

4.3.3 Dynamic study of synthetic yeast consortium system by real time qPCR

In this microbial consortium, all four yeast populations are contributing significantly to final ethanol production as the functional minicellulsome structure for hydrolyzing cellulose only formed when all the four populations coexisted. Due to plasmid stability issues and substrate proximity effects, the four yeast populations might lead to have different growth rates. To understand how the individual yeast population performs in a mixed culture and what the fraction of each yeast population is at the end of fermentation, quantitative real time PCR (qPCR) was conducted. A total of four individual standard curves were generated with different yeast cells grown in SDC media in independent experiments (Fig. 4-3). Ten-fold dilutions were carried out in duplicates from the pure yeast cultures with an initial concentration of 1×10^6. DNA obtained from each dilution was used as a template for the qPCR amplification. Similarly, DNA was also extracted from the fermentation samples taken periodically with 100 times dilution using a kit. This was then used as template for the quantitative real time PCR to acquire the individual cell growth rate and to study the dynamics of a mixed population. Fig. 4-4 shows the individual growth rates of all the four yeast

populations. Among them, the yeast strain carrying a centromeric plasmid and displaying scaffoldin on its surface (SCL) shows the highest of all. Besides, this result also confirms the presence of all strains through the end of fermentation, which again demonstrates the feasibility of using this consortium for a CBP.

4.3.4 Optimized synthetic consortia versus equal ratio synthetic consortia

Due to its modular nature, improvement to equal ratio synthetic consortium was accomplished by fine tuning the ratio of different populations through control of the initial inoculum ratio (Tsai et al. 2010). This approach was followed due to the lower ethanol yields that were observed when using equal ratio consortium as compared to *in vitro* enzyme loading for ethanol production (Tsai et al 2009). The optimized consortium showed almost two-fold increase in the ethanol production over the equal ratio one when fermentation was performed at relatively high OD (50.0). Next, to investigate whether an optimized consortium performs similarly when the OD was lowered from 50 to 0.8, and 0.2g/L of glucose was added, fermentation experiments were conducted using consortia C4, C2, and C1 in order to compare both systems at low OD. DNA was extracted from all the samples as described previously for quantification of cell density by qPCR. By plate count method, we evaluated the total cell density of all consortia (Fig. 4-5A), which was shown to be highest in the case of the optimized ratio consortium. Anaerobic growth of C4 optimized ratio consortium yielded 2.3×10^7 cells/ml which is approximately 25% higher than the equal ratio consortium. Whereas the free enzyme system C2 yielded very low levels of cell growth (1.2×10^7), the control C1 showed almost negligible change in cell density. To investigate the difference

between optimized and equal ratio consortia performance, ethanol and cellulose hydrolysis measurements were carried out. As shown in Fig 4-5 (B) and (C), the optimized consortium showed an almost 25% increase in the level of cellulose hydrolysis and hence ethanol production than the equal ratio consortium. However at high OD~ 50, an almost two-fold increase in ethanol production was observed in the optimized consortium (Tsai et al 2010). As higher enzyme density leads to higher synergy, one can imagine that the similar degree of synergy will show when cells are cultured to a high cell density. The ethanol yield of the C4 optimized consortium was 0.42 g ethanol/g sugar consumed, which is almost 83% of the theoretical value. These results clearly illustrate that manipulating the initial inoculum ratio to obtain an optimized ratio can provide an important advancement in improving the consortium system.

Furthermore, to study the changes in the progression of each cell line in both consortia, qPCR was conducted. As described previously, calibration curves were drawn (Fig 4-6). The comparison between the two consortia for each cell line was made (Fig. 4-7). Each cell-line grew faster in the optimized consortium than in the equal ratio one. As shown in Fig. 4-7A, the SCL strain displaying scaffoldin in the C4 optimized consortium exhibited the highest growth rate. This enhancement can be attributed to the higher stability of the SCL strain due to the presence of a centromeric plasmid and hydrolysis of PAS-avicel by C4 consortia. Yeast strains secreting enzymes (AT, CB, BF) in the media were also shown to exhibit different growth rates among each other (Fig 4-7 B, C and D). This difference could be a result of dissimilar levels of burden on

cellular machinery due to expression of different size proteins. Table 4-4 compares the ratio of all yeast strains before and after the fermentation in case of both consortia. Although all the populations showed growth, the ratio was diverting in both cases as compared to the original one.

4.4 Discussion

In this era of pollution and dwindling energy supplies, there is an urgent need to find new cost- and energy- effective methods to efficiently convert complex cellulosic materials into simple sugars and ethanol. For complete enzymatic hydrolysis of cellulose to glucose, three cellulases are essentially required namely exo-glucanase, endo-glucanase, and β-glucosidase (Zhang and Lynd, 2004). However, the high cost of commercially available cellulases has always imposed major limitations on the economical production of biofuel (McBride et al 2005). A lot of effort has been made toward the heterologous expression of enzymes by the yeast *S.cerevisiae* for one step conversion of cellulose to ethanol (CBP) (Fujita et al., 2005; Karlsson et al., 2002; Pu et al., 2006). In our last report we have tried to develop a chimeric mini-cellulosome on the yeast surface by in-vitro loading of enzymes, meaning that the process cannot be called CBP (Tsai et al. 2009). In addition to this, many other studies have tried to incorporate the use of a cellulosome enzymatic complex for cellulose degradation by co-expressing the scaffoldin and cellulases in a single host cell, leading to excessive metabolic burden on cellular machinery (Wen et al 2010). To address these problems, our group worked on the engineering of synthetic yeast consortium and reported the successful demonstration of a yeast consortium capable of surface assembly of a mini-cellulosome via intracellular

complementation. With the use of yeast consortium, we were able to overcome two major obstacles, namely distributing the metabolic burden amongst the four yeast populations, and avoiding the association between cohesin and dockerin before their actual translocation (Tsai et al 2010).

Stability of the yeast strain has been a major issue when it is genetically modified to express proteins heterologously. To address this issue we have genetically modified a yeast strain that is displaying scaffoldin on its surface by changing the plasmid from two micron to centromeric. As shown in Fig 4-1, despite the fact that centromeric based plasmids are low copy plasmids, the fraction of yeast cells carrying a centromeric plasmid for scaffoldin displayment is larger than cells with a two micron plasmid due to its more stable nature. Growth of consortia in liquid SDC media containing 10g/L PAS-avicel as the sole carbohydrate source led to a maximum of 1.25 g/L ethanol production, corresponding to a yield of 0.43g ethanol/g of glucose consumed. By quantitative real time PCR, we know that all of the yeast populations in the synthetic consortium were individually functional until the end of fermentation, though they exhibited different growth rates. With the comparison of optimized ratio and equal ratio consortia and analysis of qPCR data, it is probable that further improvement of engineered yeast consortium can most likely be accomplished by increasing the fraction of cells secreting enzymes in the medium.

In this report, we have shown the development of yeast consortium capable of growth on and one step conversion of PAS-Cellulose to ethanol concomitantly. The

developed consortium was able to assemble a functional cellulosome on the surface to hydrolyze cellulose, to grow, and produce ethanol from the resulting sugar without the need of growing the cells to high cell density on a soluble substrate prior to using them for conversion of cellulose to ethanol. To the best of our knowledge, this is the first successful report on the dynamic study of a yeast consortium system and on the demonstration of the feasibility of using a yeast consortium for CBP. This consortium system proves a noteworthy advancement toward understanding consolidated bioprocessing.

4.5 Acknowledgements

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TABLE 4-1: Primers used in this study

Oligonucleotides	sequence (5'-3')	Relevance
Primers		
pGKFP	CCGCCATGGTGTTTGCAAAAAGAACAAAACTG	Subcloningof Agα-Scaf
pGKRP	CCGCCATGGCCCTATGCGGTGTGAAATACC	Subcloningof Agα-Scaf
FL1A	GATCCGTTCAGCATC	Subcloningof Agα-Scaf
FL1B	GCAAGTCGTAGGTAC	Subcloningof Agα-Scaf
RL1A	CATGGATACAGTGCAG	Subcloning of Agα-Scaf
RL1B	CTATGTCACGTCCTAG	Subcloningof Agα-Scaf
ScafFP	GCGCCAAAAGCTCTTTTATCTCAACC	qPCR
ScafRP	CCACATCACTAATCACTTCTGATGTGGTG	qPCR
AtFP	GCAGAATGGGAAGACTGGAAGAGC	qPCR
AtRP	CCGCCGTCATGACTTGTAACATTGTTG	qPCR
CBHIIFP	CGCAAAGGTTCCCTCTTTTATGTGGC	qPCR
CBHIIRP	TCCGGATATCGGAATATTCCACGACAA	qPCR
BglfFP	ATCATGGCGGCCTTTTACAAGGTTG	qPCR
BglfRP	CCTCTCCAAAAACTCCGGTGAACTTTTC	qPCR

TABLE 4-2: Strains and plasmids used in this study

Strain	Plasmid	Phenotype	Source
CE	pCEL15	Secrete a small peptide (negative control)	Tsai *et al*, 2010
AT	pAt	Secrete endoglucanase At (CelA from *C. thermocellum*)	Tsai *et al*, 2010
CB	pCBH2c	Secrete cellobiohydrolase CBHc (CBHII from *T. reesei*)	Tsai *et al*, 2010
BF	pBGLf	Secrete β-glucosidase Bglf (Bg1I from *T.aurantiacus*)	Tsai *et al*, 2010
SC	pScaf3	Display the mini-scaffoldin Sacf-ctf in 2μ plasmid	This study
SCL	pAgα-scaf3	Display of Scaffoldin by AGα in centromeric plasmid	This study

TABLE 4-3: Consortia generated in this study

Consortium	Populations
C1	SC, CE, CE, CE
C2	CE, AT, CB, BF
C3	SC, AT, CB, BF
C4	SCL, AT, CB, BF

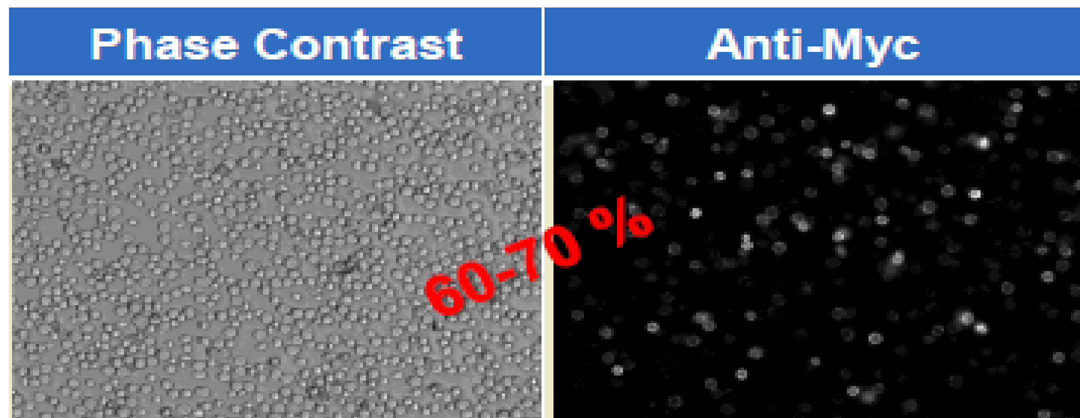
TABLE 4-4: Comparison of consortium ratio before and after fermentation

	initial	final
Optimied ratio	7:2:3.5:2	7:1.6:2.4:1.4
equal ratio	1:1:1:1	1:0.7:0.6:0.6

TABLE 4-5: Comparison of consortium ratio before and after cultivation under aerobic conditions

Mixed Culture under aerobic conditions				
	initial	final	std dev	
scaf III	3.47231	3.5422	0.42752	0.69499
At	1.10037	1.42837	0.1148	0.10096
CBH II	1.98402	1.71523	0.269	0.26572
BGLf	1.09205	0.96538	0.14265	0.10142
total OD	0.75	0.75		

A. 2μ based plasmid



B. CEN/ARS based plasmid

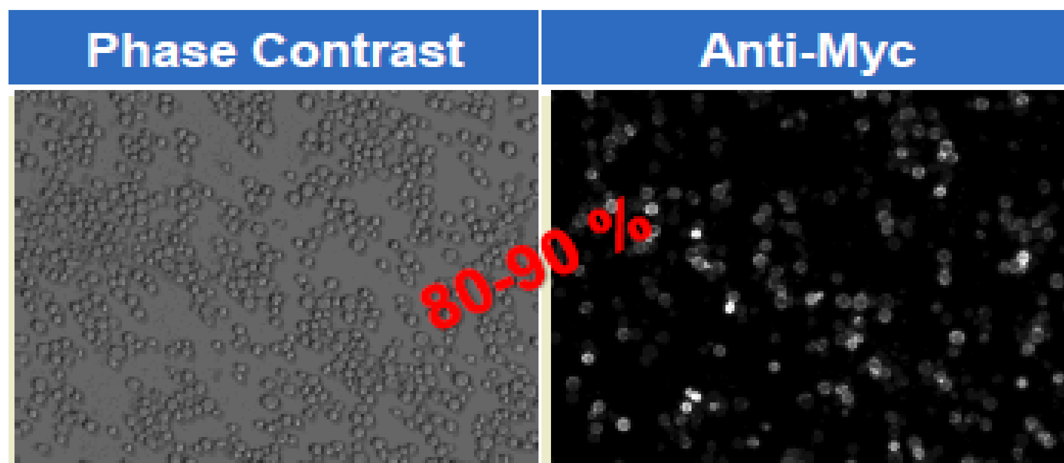
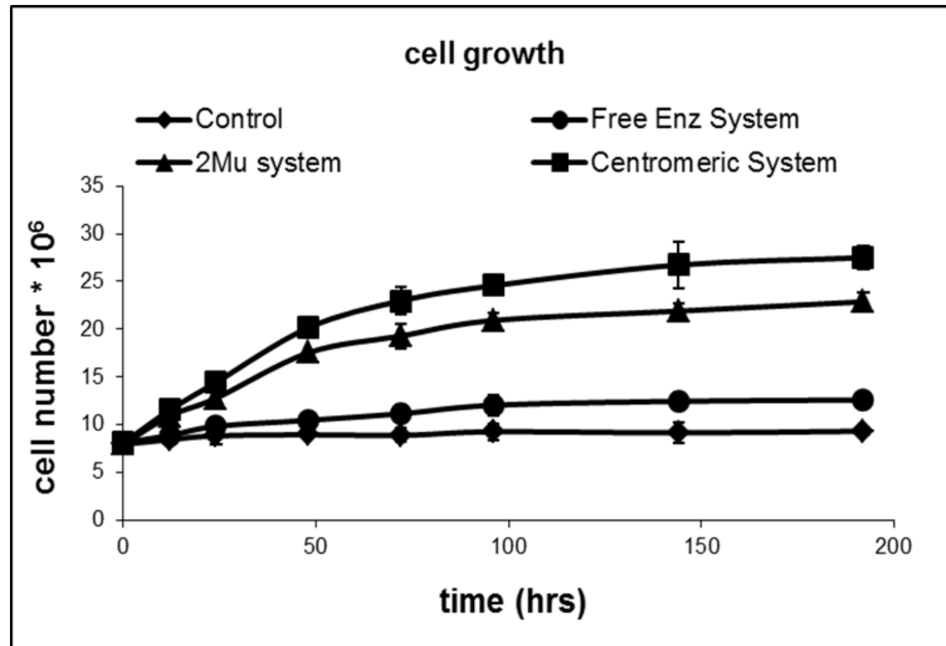


Figure 4-1:Results of the immune-fluorescence assay. Cells were probed with anti-C-Myc antibody and fluorescently stained with a goat anti-mouse IgG conjugated with Alexa Fluor 488. Figure **(A)** shows the phase contrast and immune-fluorescent image of 2-micron based plasmids, while Figure **(B)** shows the phase contrast and immune-fluorescent image of CEN/ARS based plasmids.

A.



B.

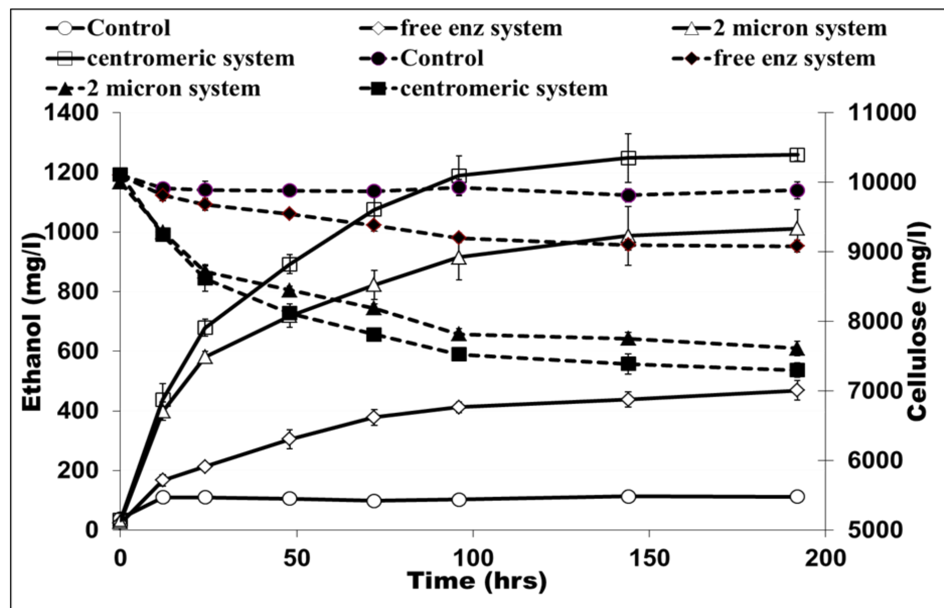


Figure 4-2:Results of a fermentation with four consortia. Figure **(A)** shows the growth curve for four consortia C1, C2, C3, and C4 by plate count method, while Figure **(B)** shows the cellulose hydrolysis and ethanol production for all four consortia C1, C2, C3, and C4.

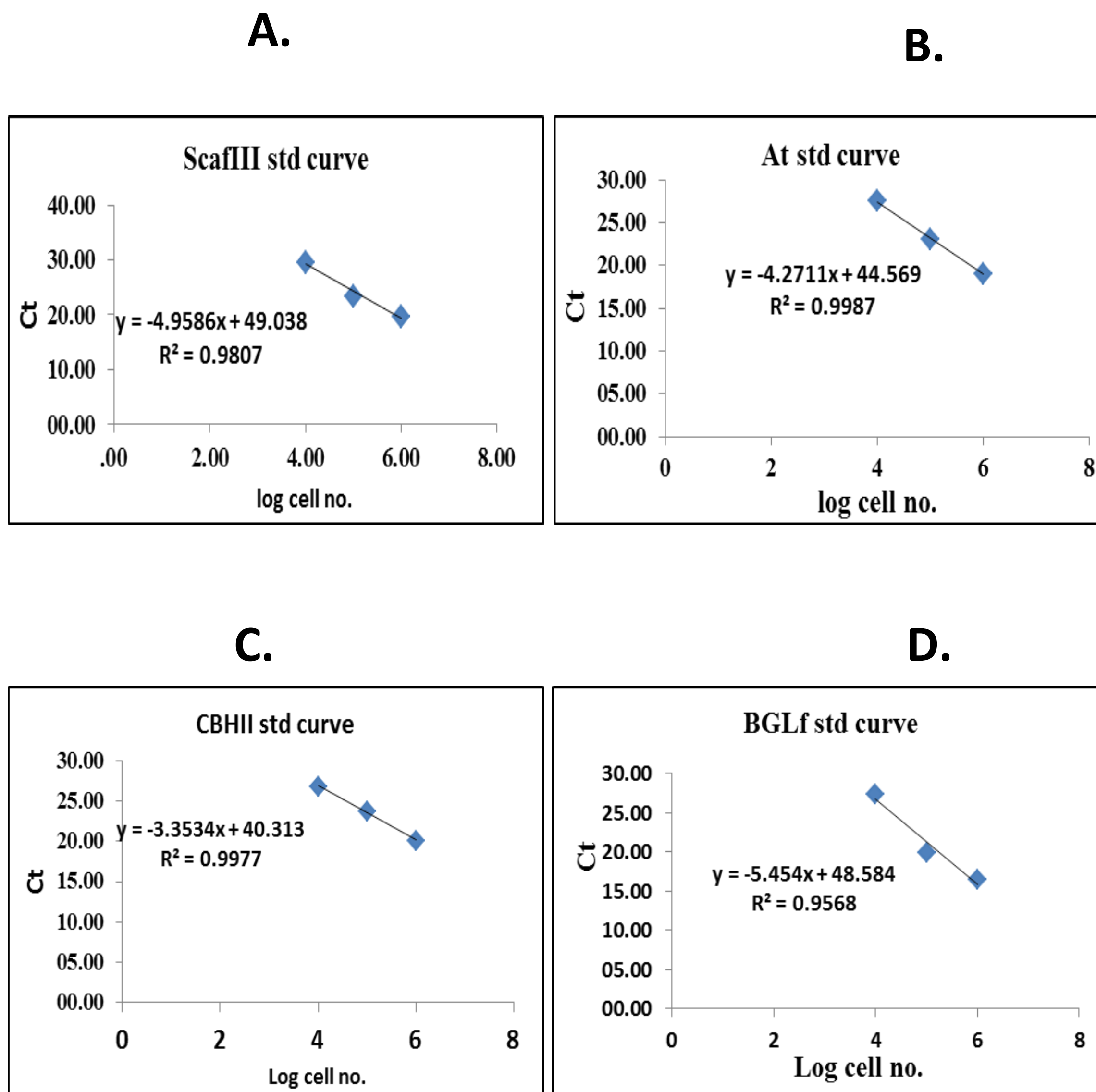


Figure 4-3: Plots of calibration curves for all four yeast populations **(A)** SC, **(B)** AT, **(C)** CB, and **(D)** BG that is required for the quantification of individual populations by qPCR.

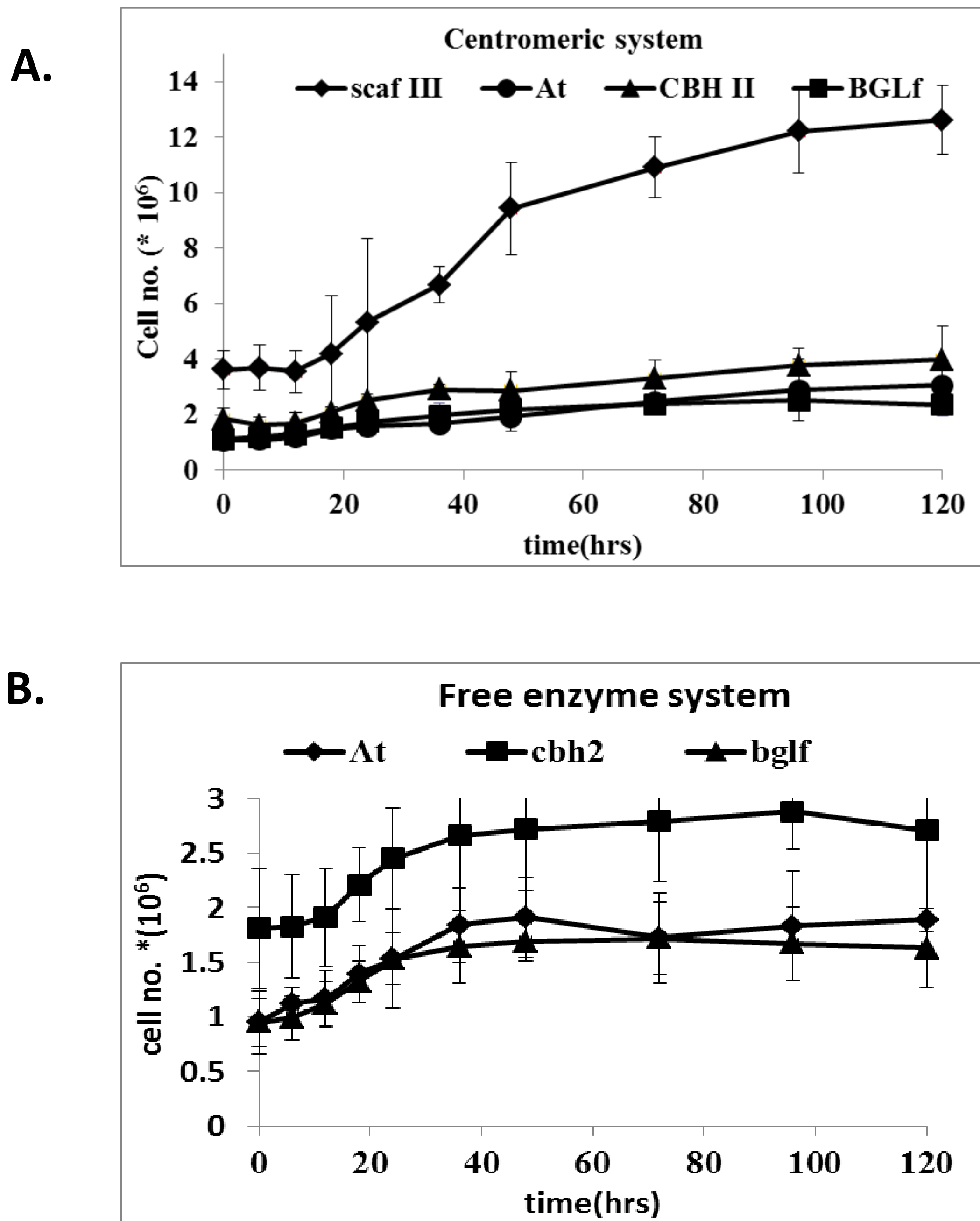
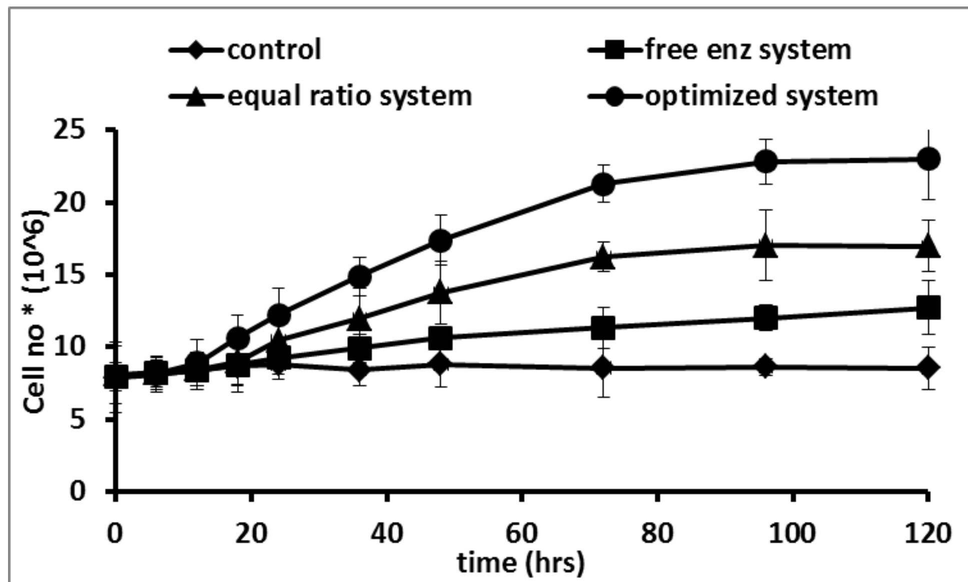
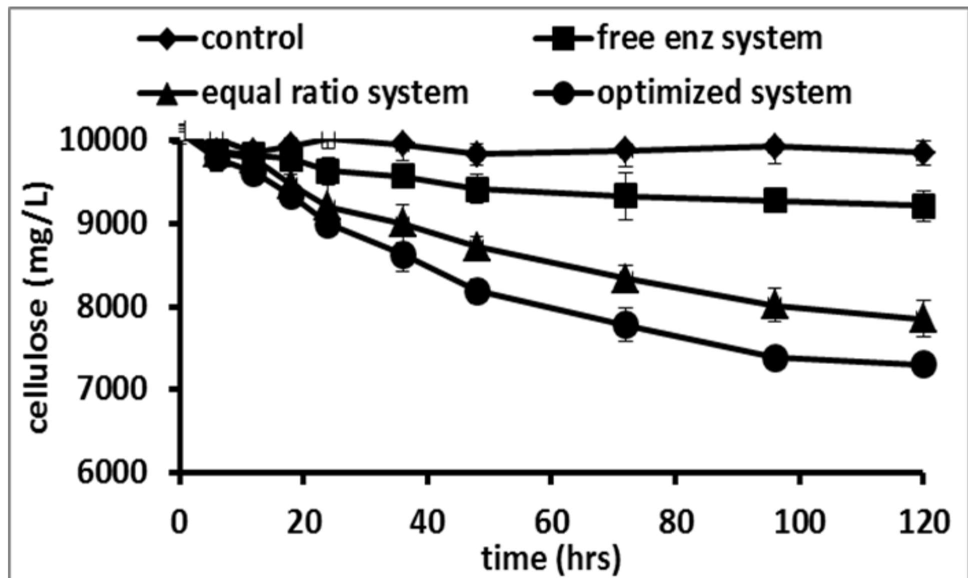


Figure 4-4:Dynamic study of yeast consortium system by qPCR. Calibration curves were used to enumerate the individual yeast population. Figure **(A)** shows the growth curves of all yeast populations as determined by using qPCR in the case of consortium C4.Figure **(B)** shows the growth curves of three yeast populations AT, CB, and BG in a free enzyme system i.e C2 consortium by qPCR**.**

A.



B.



Continued

C.

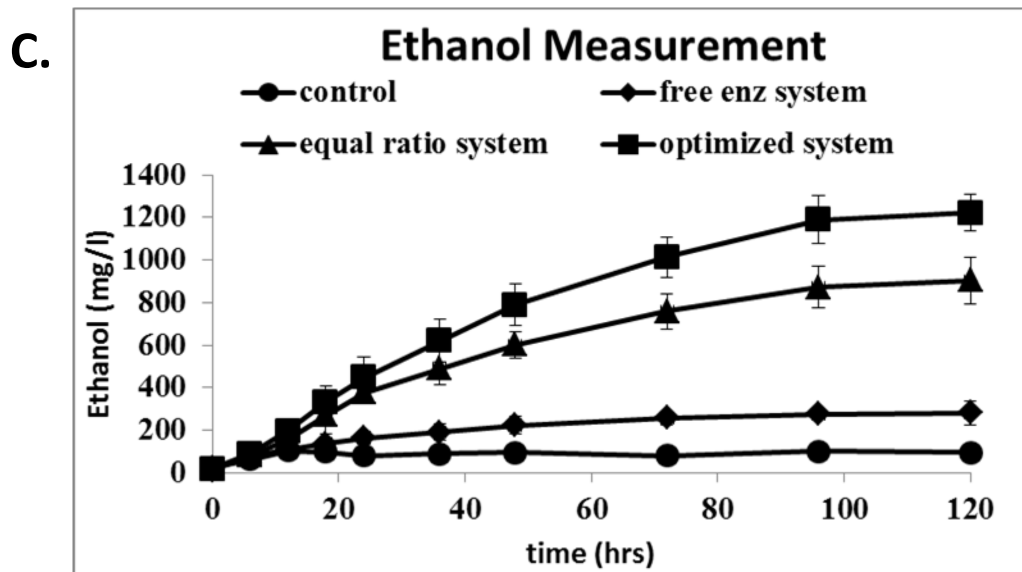


Figure 4-5:Fermentation results for three consortia C1, C2, and C4 in equal and optimized ratio. Figure **(A)** shows the cell growth results for all the consortia by plate count method. All the four yeast populations were mixed in a total initial OD of 0.8. Figure **(B)** shows the cellulose hydrolysis, while Figure **(C)** shows the ethanol production for all of the consortia**.**

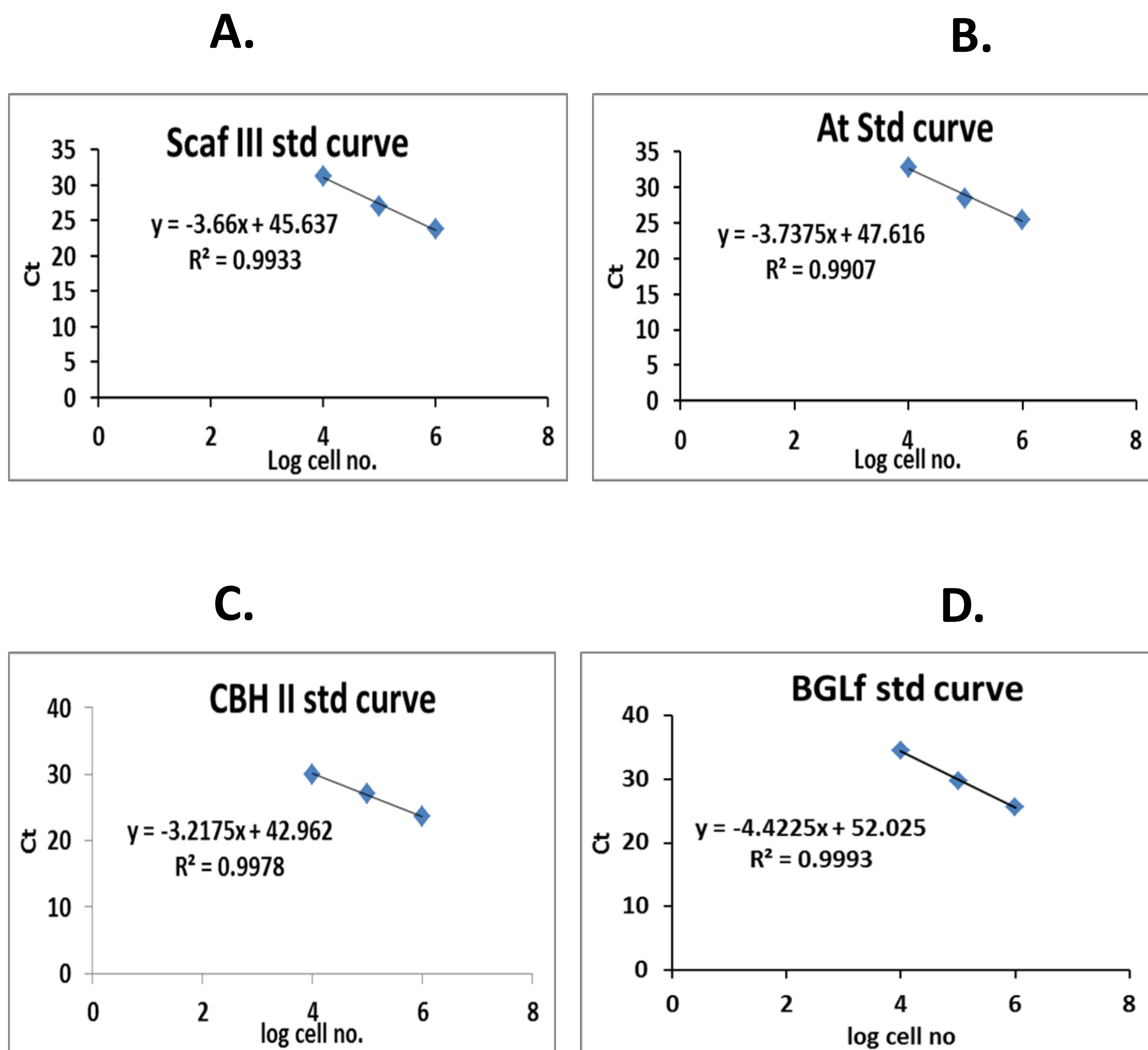


Figure 4-6:Calibration curves for all four yeast populations **(A)** SC, **(B)** AT, **(C)** CB, and **(D)** BG that is required for quantification of individual population by qPCR.

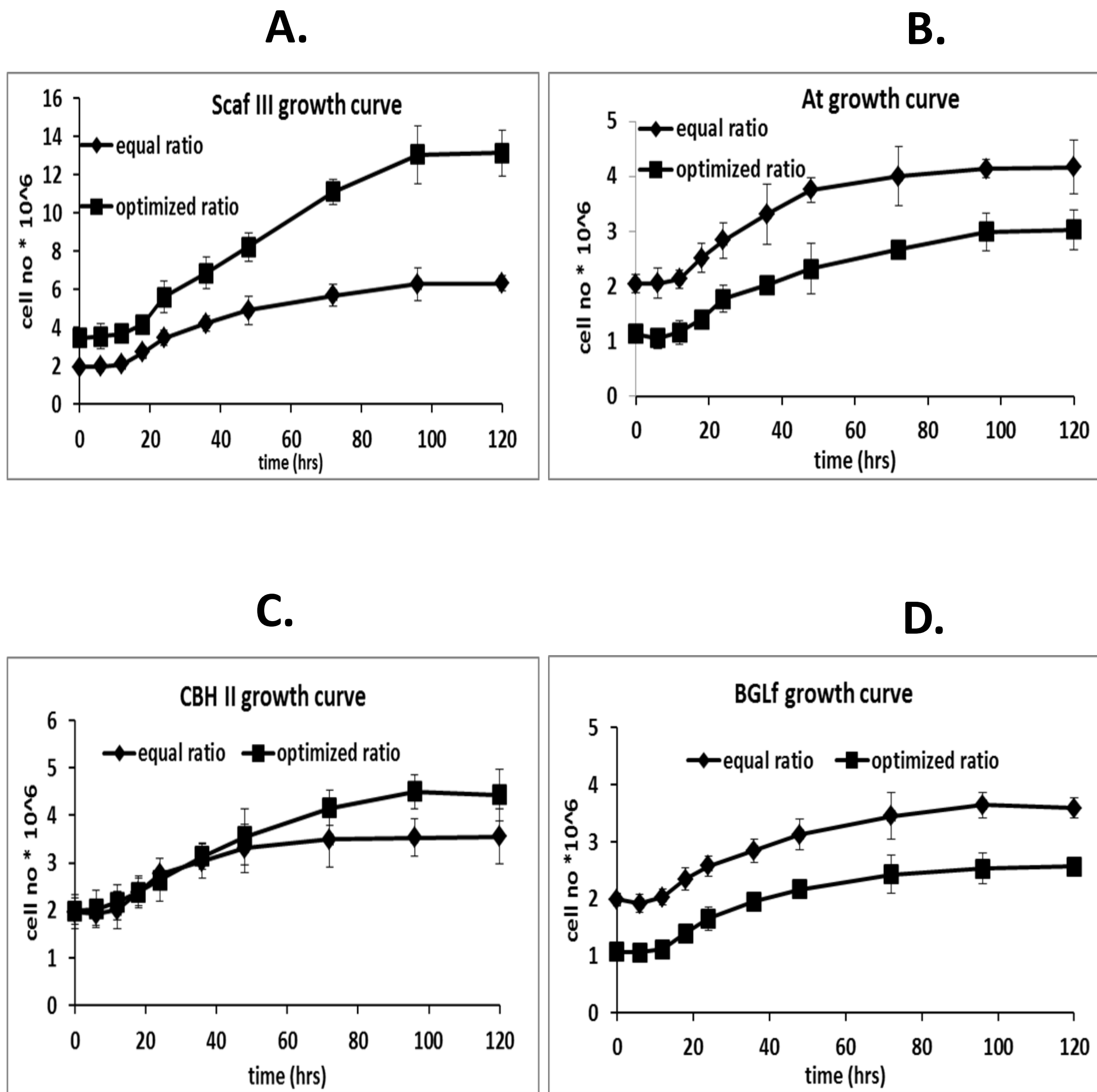


Figure 4-7:Growth curves of all yeast populations drawn individually by qPCR. Comparison of growth curves between optimized ratio consortium and equal ratio consortium for yeast population **(A)** SC, **(B)** AT, **(C)** CB, and **(D)** BG.

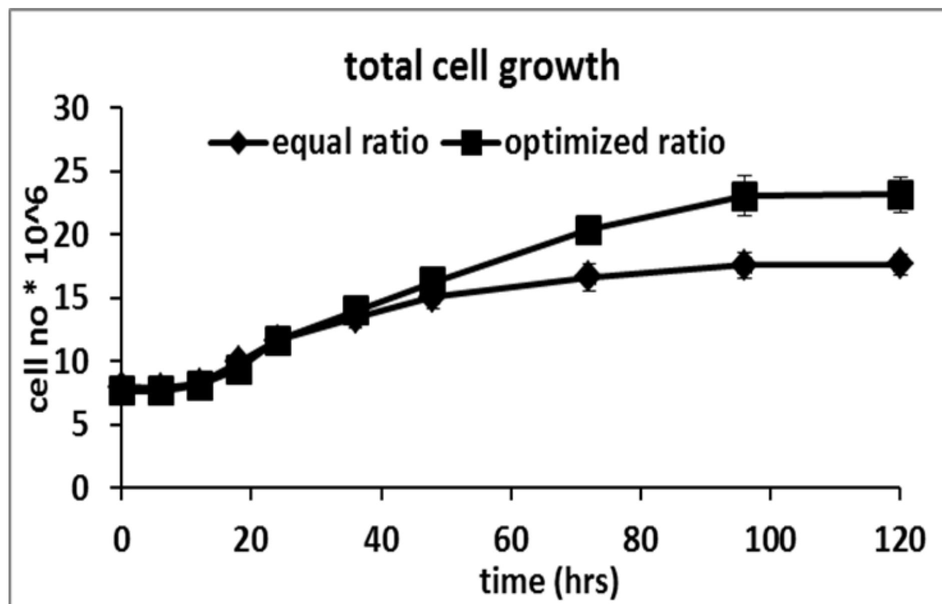


Figure 4-8:comparison of total cell growth of consortium C4 by equal ratio consortium and optimized ratio consortium.

CHAPTER 5

CONCLUSION

Conclusions and future directions

Consolidated bioprocessing (CBP) which combines saccharification of lignocellulose with fermentation of the resulting sugars into a single step is a promising technique, as it avoids a separate and dedicated process step for cellulase production. The present study is undertaken to characterize and optimize a microbial consortium system that can be used to directly produce fuel ethanol form cellulosic material and can act as CBP host. Several strategies were investigated that could potentially lead to an optimized level of ethanol production. Yeast *Saccharomyces cerevisiae* was chosen as microorganism of interest for all the genetic manipulations owing to its versatility. An inefficient production of heterologous cellulases by single yeast strain due to energy limitations led to the breakthrough of a highly ordered enzymatic complex called mini-cellulosome. A synthetic consortium which has been developed by our group was used in this study which is comprised of four different yeast strains. One is capable of displaying a trifunctional scaffoldin and other three are able of secreting each of the three corresponding dockerin-tagged enzymes (endoglucanase, exoglucanase, and β-glucosidase). This consortium system has several advantageous features such as distribution of metabolic burden amongst four yeast strains which arises due to heterologous protein expression, formation of highly ordered and controllable cellulosome structure leading to synergistic cellulose hydrolysis, and its modular nature leading to easy modification of four yeast populations for efficient cellulose hydrolysis and ethanol production.

The first study was aimed for the exploitation of modular nature of yeast consortium for the optimized ethanol production. The four yeast populations present in consortium system were easily fine-tuned by modifying the ratio of different populations in the yeast consortium. The final ratio resulted in the optimization of mini-cellulosome assembly, cellulose hydrolysis and ethanol production. The optimized consortium consisted of SC, AT, CB, and BG in the ratio 7:2:4:2 yielded almost twice the level of ethanol production than a consortium system consisted of all four populations in equal ratio. This is the first report on the optimization of consortium system for efficient bio-ethanol production. The results confirm the use of synthetic biology and metabolic engineering approach for synergistic saccharification and fermentation of ethanol from cellulose by utilizing yeast consortium capable of displaying mini-cellulosome.

In the second study, we have done further evaluation of consortium system for its feasibility to be used as CBP. Consortium with very low initial OD was grown under anaerobic conditions. The resulting synthetic consortium was proficient in functionally assembling minicellulosome on the cell surface and growing on a media containing phosphoric acid swollen cellulose (PASC) as a sole carbohydrate source and concomitantly producing ethanol with a yield corresponded to 87% of the theoretical value. When using a consortium approach, one of the critical concerns is the stability of each population in the system to avoid the dominance of one population over the other. For the dynamic study of yeast consortium system, quantitative real time PCR was used to enumerate the individual yeast population in the mixed culture. At the end of the cultivation, ratios of each population in this consortium maintained similar number as

the initial inoculums ratios, which further confirms the consortium system is suitable for the application of CBP. To the best of our knowledge, this is the first report of using a yeast consortium approach for CBP of cellulose.

Although synthetic yeast consortium is capable of producing ethanol from cellulose concomitantly and is the one of best methods available for efficient biofuel production, a lot of improvement is still required in the area of hydrolysis of recalcitrant ligno-cellulosic materials to simple sugars. For future experiments, highly ordered and complex mini-cellulosome can be engineered to further increase the cellulose hydrolysis efficiency. Cellulases which were used in this study can be replaced by other cellulases with high specific activity and secretion efficiency. Mini-cellulosome structure can be expanded by appending more cohesin-dockerin pairs to increase the amount of enzymes docking on the scaffoldin, which will possibly further enhance the cellulose hydrolysis and hence ethanol production. The present study provides important insights into several possible biological routes to combine eco-friendly and economical processes for ethanol production from cellulose.