Ammonia Wet Biomass Storage-Pretreatment & Universal NH_3-EtOH Fractionation Platform

DOE Advanced Biofuels, Integrated Biorefinery & Joint USDA Biomass RFPs

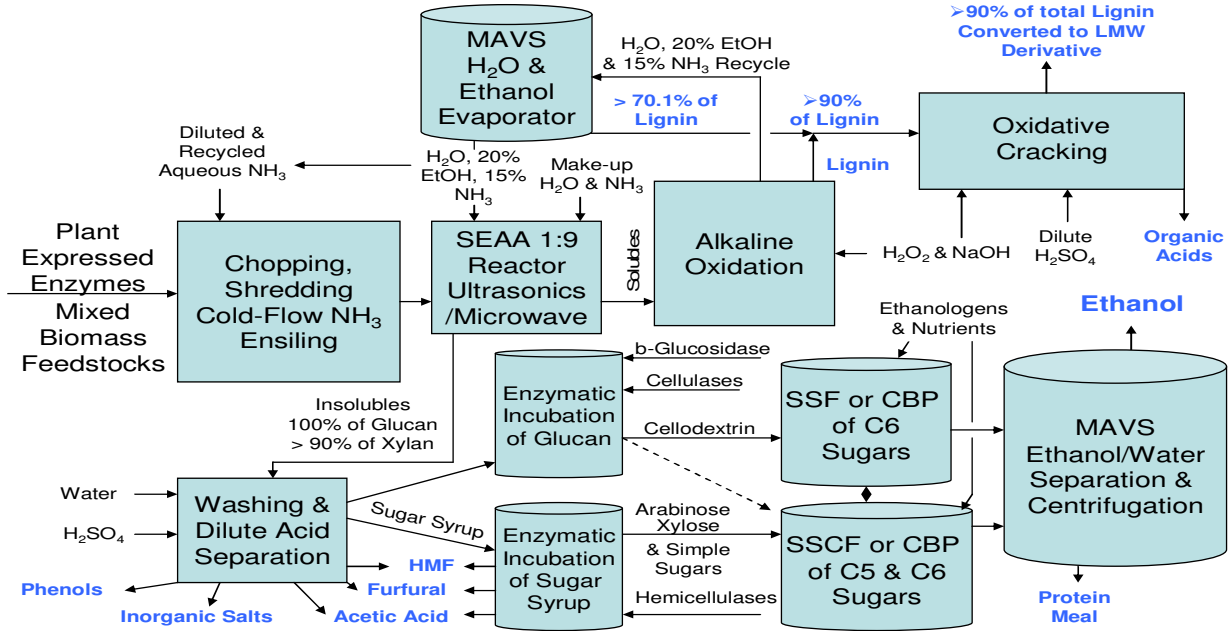


Figure 1. Integration of Ammonia Ensiling, Enzyme Inoculation, Aqueous Ammonia-Ethanol Pretreatment-Fractionation Platform, Alkaline Oxidation & Oxidative Cracking of Lignin

Introduction

Energy intensive pretreatments account for 19-22% of production costs for cellulosic ethanol and advanced cellulosic biofuels including hydrous ethanol, green gasoline and butanol. In addition, fermentation efficiency and yields are often hampered by toxic compounds released during pretreatment and hydrolysis due to severity of pretreatments. These factors and the cost of enzymes substantially reduce economic competiveness and require government subsidies in order for production of advanced cellulosic biofuels to be sustainable. However, costs of pretreatment and enzyme loading can be substantially reduced by modifying existing technology and incorporating a relatively simple and efficient clean fractionation process which allows for optimizing simultaneous saccharification and fermentation (SSF) of hexose and pentose sugars.

Low energy ammonia ensiling (and possibly enzyme inoculation) simultaneously stores wet biomass and provides pretreatment at ambient temperatures. Ammonia ensiling is followed by ammonia-ethanol soaking/steeping at < 60 °C for 10-24 hrs prior to fractionation into major carbohydrate components (lignin, cellulose and hemicellulose). This low energy approach using biological agents via closed loop systems reduces the release of toxic components and substantially enhances efficiency of biomass conversion processes by producing highly digestible cellulose and hemicellulose substrates. In addition to improving efficiency of hydrolysis and fermentation, fractionation of biomass provides flexibility via production of relatively high value coproducts from low cost feedstocks such as crop residues and food processing waste, etc. This provides sustainability via diversification and creation of additional

revenue streams from distribution of products which are not entirely subject to the volatility of commodity markets.

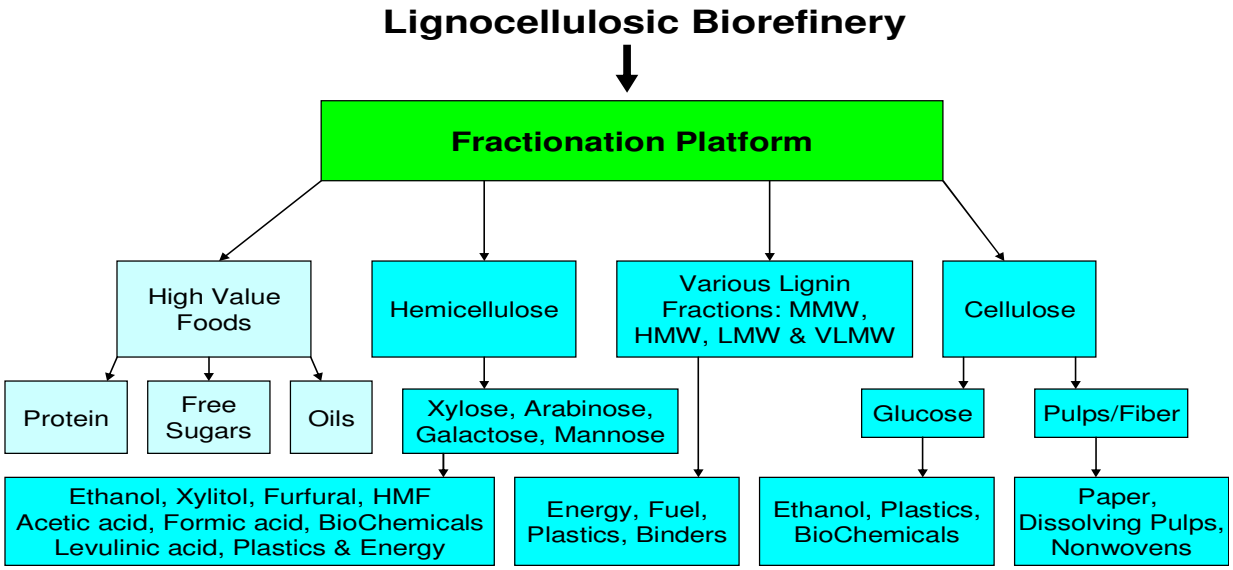


Figure 2. Lignocellulosic Biomass Fractionation Platform for an Integrated Biorefinery

Low-cost ammonia ensiling (using earthen or concrete bunkers with air-tight tarp covers) has been utilized in the livestock industry for increasing digestibility of fibrous feeds (lignocellulosic feedstuffs such as corn stover, corn silage, soy bean hulls, and low quality hay) since the early 1970's in the US. When utilized in conjunction with shredding and chopping a portion of corn stover during harvesting of corn, modified combines can maintain about 80-90% of their normal speed while harvesting both corn and stover. About 25% of corn stover is returned to the crop field to restore soil organic matter (SOM) along with incorporation of stubble and root ball residues which remain in the crop field. In total, about 45-50% of the corn plant is recycled as an organic fertilizer for subsequent rotation crops, thus insuring sustainability of agricultural production. The remaining 50% of crop residues which are non-food feedstocks are converted to special ingredients, industrial biochemicals and bioenergy. Bioenergy includes bioelectricity, biohydrogen, liquid biofuels, and biomethane which can be used as a feed for high temperature fuel cells which provide distributive generated cooling/heat/power (DG-CHP) for integrated operations including biorefineries and bioenergy complexes.

Shredding stover during harvesting requires 80-90% less energy than chopping. Shredding also increases surface area of feedstocks for enhancing efficiency of pretreatment, fractionation, hydrolysis and fermentation. During ensiling for periods of up to 12 months, ammonia pretreatment, plant expressed enzymes, and enzyme inoculation can substantially delignify biomass and reduce crystallinity of cellulose via enzyme inoculation and prehydrolysis. This is accomplished through the swelling effect that ammonia exerts upon cellulose, the result of which is an increase in surface area for enhancing enzymatic and microbial access. Enzymes designed to increase surface area of biomass particulates via cavitation, break lignin to cellulose bonds, and reduce crystallinity prior to fractionation and hydrolysis can be conveniently inoculated at the time of ensiling. Cellulosic crystallinity refers to the degree of structural order in biomass. In a crystal, the atoms or molecules are arranged in a regular, periodic manner. The degree of crystallinity in biomass feedstocks (which provides structural carbohydrates which increases

hardness and density of cellulosic biomass in living plants) substantially influences efficiency of fractionation and rate of conversion to fermentable sugars.

During soaking in ethanol-aqueous ammonia (SEAA) fractionation, hemicellulose is preserved in solid form and lignin components are liquefied. Treatment intensity and residence time for soaking/steeping may be substantially reduced due to ammonia ensiling treatment for extended periods of time. The removal of lignin components enhances subsequent enzymatic hydrolysis of the cellulose fraction. Removal and separation of hemicellulose and toxic substances during fractionation also enhances hydrolysis of hemicellulose, producing relatively pure xylose and arabinose sugars contained in the hemicellulose hydrolysate. These pentose sugars, particularly arabinose can be utilized to produce the red yeast *Phaffia rhodozyma* via fermentation (Nghiem et al., 2009) at integrated biorefineries. *P. rhodozyma* is rich in astaxanthin, a super antioxidant which is marketed as an animal feed supplement and also as a nutraceutical and pharmaceutical grade product with retail value of $1700/lb.

Typical pretreatment processes fail to capture as much as 50% or more of the hemicellulose component due to liquefaction of both lignin and hemicellulose during intensive thermochemical processes. In contrast, the low temperature aqueous ammonia-ethanol process substantially improves separation and capture of the hemicellulose fraction in solid form, allowing for preserving over 90% of the hemicellulose contained in feedstocks. Steeping is defined as soaking in liquid until saturated with a soluble ingredient, or soaking to remove or separate components such as lignin, hemicellulose and cellulose. Steeping also leaches nutrients from feedstocks, the steepwater from which provides a balance of essential nutrients when combined with hydrolysates prior to fermentations. Optimizing nutrient ratios for microbial populations allows for maximizing fermentation efficiency. Fermentation efficiency is also enhanced by increasing tolerance of microbes to toxic components and other synergistic stress factors such as temperature and ethanol concentration, etc., via genetic engineering of recombinant strains.

An example of how soaking in water assists with pretreatment and hydrolysis is steeping of maize (corn). This is the beginning step for wet mills and it is very similar to the SEAA process which adds ammonia and ethanol to the steep water. As described by the US Corn Refiners Association, harvested kernels of maize are cleaned and then steeped in water at a temperature of 50 °C for 30 to 40 hours. In the process moisture content of biomass rises from 15% to 45% and biomass volume more than doubles. The gluten bonds in the maize are weakened and starch is released. The maize is then ground to break free the germ and other components, and the steep water which contains essential nutrients, is recycled for use in animal feeds or used as a nutrient-rich component of fermentation slurries. As indicated above, a similar steeping process in terms of temperature, but shorter residence time is utilized by the SEAA process in which aqueous ammonia and ethanol are utilized for simultaneous pretreatment and fractionation of lignocellulosic feedstocks.

Not including the ammonia ensiling step, conventional “corn stover has been soaked/steeped with 15 w/w% ammonia at 1:9 solid–liquid ratio (by weight) at 60 °C for 24 h with ethanol utilized as an organic solvent added at 1, 5, 20, and 49 w/w% (balance was water). Hydrous ethanol can be utilized at integrated biorefineries without requiring intensive energy requirements for conventional water-ethanol separation (for starch fermentations, ethanol concentrations typically range from 17-20 w/w% in the broth upon completion). The extents at which xylan was solubilized with no ethanol and with ethanol added at 1, 5, 20, and 49 w/w% of the total liquid were 17.2%, 16.7%, 14.5%, 10.4%, and 6.3% of the original xylan, respectively.

Thus, at the highest ethanol concentration used, the loss of hemicellulose to the liquid phase was reduced by 63%. The digestibility of glucan and xylan in the pretreated corn stover samples by cellulase was not affected by ethanol addition of up to 20 w/w%. The enzymatic digestibility of the corn stover treated with 49 w/w% ethanol was lower than the digestibility of the sample treated with no ethanol addition. Thus, based on these results, 20 w/w% was found to be the optimum ethanol concentration for use in the SEAA process for pretreatment of corn stover." (Kim et al., 2009) Ammonia ensiling prior to aqueous ammonia-ethanol steeping could further improve efficiency of this process and potentially increase capture of hemicellulose to well over 90%. The SEAA process uses low temperatures to minimize degradation of fermentable sugars and prevent formation of potentially inhibitory compounds in addition to preventing solubilization of hemicellulose.

Subsequent to soaking/modified steeping in ammonia and ethanol, a solid/liquid separation process is conducted. The liquid enters an evaporator where ammonia is evaporated and recovered for recycling. The residual solids which are mostly forms of lignin, are recovered and either combusted as fuels via fluidized bed boilers or processed into high value low molecular weight (LMW) lignin derivatives. The LMW lignin chemicals are produced via optional alkaline oxidation and oxidative cracking processes and can be utilized in the bioplastics and biochemicals industries. The solids from the aforementioned solid/liquid separation process are washed with water to remove residual ammonia. Wash water is fed to the evaporator for ammonia recovery as described above, and then recycled as nutrient rich steep water. As indicated above, once cleansed of toxic components, the steep water can be mixed with hydrolyzates to provide a balance of essential nutrients for optimizing fermentation efficiency. The washed solids are then hydrolyzed with dilute acids, autohydrolysis or industrial cellulases and hemicellulases to produce fermentable sugars. These monomer sugars are utilized to produce high value coproducts and/or fermented to ethanol and other products in subsequent fermentations at integrated biorefineries.

Soaking in aqueous ammonia (Kim et al., 2003, 2005, 2007) and precipitation of hemicellulose with ethanol (Whistler and Richards, 1970; Sun and Tomkinson, 2001; Anderson et al., 1938) has previously been used for the separation and purification of polysaccharides contained in lignocellulosic biomass. The presence of ethanol in aqueous ammonia results in reprecipitation of the previously solubilized xylan onto the solid matrix and reduces the extent of hemicellulose solubilization. As a result more xylan, and possibly more arabinose, is available for hydrolysis to fermentable sugars and subsequently for production of biofuels and high value coproducts. The ethanol used in the pretreatment process is recovered and recycled (Kim et al., 2009).

With optimum ethanol concentration at 20 w/w%, the carbohydrate-rich solid sample obtained from SEAA treatment contained 51.4% glucan, 26.7% xylan, and 6.2% lignin. This essentially translates to 100% glucan retention, 89.6% xylan retention, and 70.1% lignin removal, respectively. The SEAA has been proven to be an effective method to produce carbohydrate-rich solid cake (fermentable cellulose and hemicellulose substrates) which can be used for production of fuels and chemicals (Kim et al., 2009).

Lignin removal could potentially be improved to over 90% by using a dilute aid process or alkaline oxidation subsequent to ammonia-ethanol steeping. An improved pretreatment method involving two steps has been developed (Cao et al., 1996; Dominguez et al., 1997; Gong et al., 1997; and Tsao et al., 1996): 1) steeping lignocellulosic biomass in aqueous ammonia at ambient temperature removes lignin, acetate, and extractives, followed by 2) dilute acid pretreatment that

hydrolyzes the hemicellulose fraction which is collected and washed. Up to 90% of lignin can be removed through the ammonia steeping step (Cheng, 2001). Integration of this sequential separation process with advanced dilute acid separation processes could capture 100% of cellulose, possibly over 95% of hemicellulose, and over 90% of lignins in addition to production of sugar syrup, furfural, HMF and a variety of acetic acid and other organic (carboxylic) acids from the hemicellulose fraction. Until enzymes become more effective and costs are reduced, dilute acid hydrolysis and fractionation could be utilized in conjunction with ammonia-ethanol pretreatment-fractionation to extract more lignin and hemicellulose. This would allow for increasing sugar yields while simultaneously reducing enzyme loading and fermentation times for SSF. Removing the majority of lignin prior to fermentation also enhances feed efficiency of the protein-rich distillers residues which are utilized as value added livestock feedstuffs. For example, distillers residues produced from processing corn stover containing 4% protein would consist of 20% protein and a balance of essential micro-nutrients. The actual protein component would be comprised of a complimentary amino acid profile in comparison with distillers grains, the concentrations for which are three times higher than those contained in whole corn. For biomass feedstocks containing 6-10% protein, the fermentation residue could consist of between 30-40% protein in addition to essential micronutrients. Subsequent processing of animal waste via anaerobic digestion is also improved since degradation of lignin occurs most efficiently in aerobic processes.

Unlike enzymatic incubation/pretreatment and wet fractionation of corn, plant expressed enzymes and enzyme inoculation during ensiling should reduce the time required for enzymatic incubation of lignocellulosic biomass during aqueous ammonia-ethanol steeping. In addition to mechanical shredding and chopping which increases enzymatic and microbial access to the polymers and monomer sugars contained in structural carbohydrates, quantum fracturing (electromagnetic pulse, ultrasonification, or microwave) can be utilized to further reduce particle size and promote cavitation, both of which increase surface area during fractionation in order to optimize nutrient use efficiency of microbes and maximize biomass yields. Sugar and ethanol yields can be increased by as much as 10-20% by utilizing shredding, preventing competition with bacteria growth via the disinfectant properties of ammonia, and utilizing quantum fracturing during the ammonia-ethanol steeping process in order to optimize nutrient use efficiency of microbes.

Utilizing a clean fractionation platform consisting of ammonia-ethanol steeping, toxic components of biomass such as acetic acid, furfural, HMF, inorganic salts and phenols released during pretreatment-fractionation and hydrolysis can be harvested as value added coproducts. Dilute acid and washing can be utilized on the hemicellulose fraction to enhance separation processes prior to enzyme incubation/steeping, hydrolysis and fermentation of sugar syrups/substrates. As indicated above, once toxic substances have been removed, the nutrient rich steep water can be mixed with hydrolysates to provide a balance of essential nutrients required for optimizing efficiency of microbial fermentations.

For the same volume of enzyme loading, amylase hydrolyzes starch about 100 times faster than current cellulase hydrolyzes cellulose (Sierra et al., 2008). Hence, SSF of glucan, cellodextrin and cellobiose requires considerably more time than starch and dextrin fermentations. Similarly, cellulose SSF are considerably less time consuming than xylose and arabinose fermentations. However, the efficiency of pentose fermentations can be increased by addition of glucose and/or simple sugars. Several enzyme companies including Genencor, Novozymes, Dyadic and others are continually developing new cellulose and hemicellulose enzyme complexes which are more

and more efficient at increasing the rate of hydrolysis and reducing costs of enzymes. Though utilizing dilute acid for hydrolysis is less expensive and considerably faster than current enzymes, acidification releases toxic compounds which inhibit subsequent fermentations.

Fractionation allows for separate hydrolysis and fermentation of hexose sugars and cofermentation of pentose and hexose sugars via split-stream feeds in order to optimize fermentation efficiency for both pentose and hexose sugars. This can be accomplished by using thin stillage from ultra high gravity starch fermentations as value added backsets for cellulose and pentose fermentations. Additionally, partial depolymerization of polysaccharides during low cost pretreatment and separate saccharification (actual extraction of sugars from lignocellulosic fractions) of cellulose (comprising polymers of hexose sugars, e.g. glucose) and hemicellulose (comprising primarily polymers of pentose sugars such as xylose and arabinose) substrates subsequent to fractionation would substantially improve SSF efficiencies. This integrated process for fractionation platforms and optimizing efficiency for high gravity (high sugar concentration) SSF also allows for leveraging existing corn ethanol infrastructures, lowering enzyme loading, and substantially lowering production costs for integrated biorefineries.

Low-cost ammonia ensiling simultaneously stores and pretreats any combination of wet biomass feedstocks regardless of hexose:pentose composition ratios, thus simplifying storage and providing an efficient pretreatment/prehydrolysis process. Treatment intensity of the subsequent aqueous ammonia-ethanol fractionation process can be varied to accommodate composition of specific or mixed biomass substrates while maintaining relatively low temperatures. Unlike conventional pretreatment processes, the low temperatures utilized for ammonia ensiling and ammonia-ethanol fractionation preserve the protein component of biomass feedstocks. Protein composition ranges from 8-12% for most biomass feedstocks, providing an opportunity to produce high value consumer food products and livestock feedstuffs as additional value added coproducts. The protein meal produced from fermentation residues is high in mineral nutrients and can be fed to ruminants with or without exogenous enzymes which further enhance efficiency of microbial digestion in the rumen.

Hydroponic greenhouses and algal bioreactors provide closed loop production of biomass and can be integrated with microbial fuel cells or microbial electrolysis cells for production of bioenergy (biomethane or biohydrogen) without affecting production of biomass. Lignin-free algal biomass contains limited cellulose in cell walls and is comprised primarily of starch. Hence, pretreatment is not required for algal biomass which can be produced 24/7 year round via controlled environment agriculture (CEA). Waste heat, CO_2, economical sugars from processing corn stover and other crop residues, and thin stillage can be utilized to reduce costs for either heterotrophic, phototrophic or hybrid photoheterotrophic production of algal biomass rich in either carbohydrates and/or lipids.

Renewable ammonia can be produced as a coproduct of hybrid anaerobic digesters (bacterial fermentation) equipped with an aerated-submerged biofilter for *in situ* ammonia removal. Removing ammonia enhances solids destruction and increases biomethane production by 26% during anaerobic digestion (Wang et al, 2003). For integrated biorefineries and municipal bioenergy complexes which process organic waste and/or algal biomass, ammonia is produced as a coproduct of methane and CO_2. Depending on the efficiency and volume of ammonia removal, this ammonia could be utilized as a value added disinfectant and source of nitrogen for microbial fermentations. This process could substantially reduce biorefinery production costs via closed loop systems architecture. However, since ammonia (gas) is in the form of ammonium (liquid)

during the anaerobic digestion process, the most efficient and practical way to remove ammonia/ammonium may be through the continual removal of effluent via high rate anaerobic digesters such as induced blanket reactors (IBRs) and modified high solids anaerobic digestion (HSAD). In this way, ammonium is utilized as a value added nutrient contained in fertigation streams for closed loop biomass production.

Renewable ammonia can also be produced from hydrogen using biomethane as a feedstock for steam reforming or using electrolysis (water electrolysis or microbial electrolysis cells [MECs]) as a source of hydrogen in conjunction with nitrogen derived from process air at integrated biorefineries (Wikipedia, Ammonia Production). High temperature fuel cells can coproduce hydrogen via proton exchange membrane technology. Electric efficiency is reduced to 37% for the same amount of fuel utilized. For MCFC-H_2 units, 7.21 MBTU/hr fuel feed would produce .8 MW of power generation, 1200 lbs/day of H_2, and 1.34 MBTU of heat recovery per MW of power generation for thermal integration.

Biogas Refining & Renewable Ammonia Production

A typical modern ammonia-producing plant first converts natural gas (i.e., methane) or LPG (liquified petroleum gases such as propane and butane) or petroleum naphtha into gaseous hydrogen. The method for producing hydrogen from hydrocarbons is referred to as "Steam Reforming." Methane-rich biogas can be utilized in place of natural gas. Additionally, methane feeds for high temperature fuel cells allow for cogeneration of 1200 lbs of H_2 per day per MW of DG-CHP produced. The hydrogen is then combined with nitrogen to produce ammonia. In the latter case, the production of hydrogen from biomethane can be skipped, but hydrogen sulfide still needs to be removed from biogas.

Starting with a natural gas or biogas feedstock, the processes used in removing sulfur and producing the hydrogen are:

The first step in the process is to remove sulfur compounds from the feedstock because sulfur deactivates the catalysts used in subsequent steps and also contaminates fuel cell stacks when biomethane is utilized as a feed for high temperature fuel cells. Sulfur removal requires catalytic hydrogenation to convert sulfur compounds in the feedstocks to gaseous hydrogen sulfide. In organic chemistry, R-SH is a thiol compound (R) that contains a functional group composed of a sulfur atom and a hydrogen atom (-SH). For refining methane-rich biogas, hydrogen sulfide is already in gaseous form, hence this step can be skipped:

$$\mathbf{H_2 + RSH \rightarrow RH + H_2S(gas)}$$

The gaseous hydrogen sulfide is then absorbed and removed by passing it through beds of zinc oxide where it is converted to solid zinc sulfide.

$$\mathbf{H_2S + ZnO \rightarrow ZnS + H_2O}$$

Catalytic steam reforming of the sulfur-free feedstock is then used to form hydrogen plus carbon monoxide. For H_2 cogeneration via high temperature fuel cells using methane-rich biogas feeds, the following hydrogen production and carbon dioxide removal steps are skipped:

$$\mathbf{CH_4 + H_2O \rightarrow CO + 3H_2}$$

The next step requires catalytic shift conversion or water-gas shift reaction to convert the carbon monoxide to carbon dioxide and more hydrogen:

$$CO + H_2O \rightarrow CO_2 + H_2$$

The carbon dioxide is then removed either by absorption in aqueous ethanolamine solutions or by adsorption in pressure swing adsorbers (PSA) using proprietary solid adsorption media. The final step in producing the hydrogen is to use catalytic methanation to remove residual amounts of carbon monoxide or carbon dioxide from the hydrogen gas:

$$CO + 3H_2 \rightarrow CH_4 + H_2O$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

To produce the desired end-product ammonia, hydrogen is catalytically reacted with inert nitrogen (N_2 derived from process air which is almost 80% nitrogen) to form anhydrous liquid ammonia. This step is known as the ammonia synthesis loop (also referred to as the Haber-Bosch process):

$$3H_2 + N_2 \rightarrow 2NH_3$$

The steam reforming, water-gas shift conversion, carbon dioxide removal and methanation steps each operate at absolute pressures of about 25 to 35 bar, and the ammonia synthesis loop operates at absolute pressures ranging from 60 to 180 bar depending upon which proprietary design is used. There are many engineering and construction companies that offer proprietary designs for ammonia synthesis plants. Haldor Topsoe of Denmark, Uhde GmbH of Germany, and Kellogg Brown & Root of the US are among the most experienced companies in this field.

Synthetic Biology: Integrated BioSystems & Future of Chemical Industry

The key to sustainability for the biofuels industry and successful transition to bioeconomies in general is value added production of high value coproducts including biochemicals. This will be accomplished by incorporating synthetic biology technology at integrated biorefineries and bioenergy complexes in order to demonstrate highly efficient biochemical conversion processes such as SSF and CBP which internally produce enzymes and eliminate entire unit processes. Synthetic biology and closed loop biosystems are ushering in a paradigm shift in world economies, providing unprecedented production efficiencies for simultaneous production of functional foods, special ingredients, biochemicals and bioenergy.

Synthetic biology is described as the convergence of molecular biology, information technology and nanotechnology, leading to the systematic design of biological systems. The aim of synthetic biologists is to create an organism from scratch. Microbiogists and teams of molecular biologists and geneticists utilize a computational biology approach similar to that of other types of engineering in the design and construction of biosystems that will support this new technology.

“Computational biology is an interdisciplinary field that applies the techniques of computer science, applied mathematics and statistics to address biological problems. The main focus lies on developing mathematical modeling and computational simulation techniques. By these means it addresses scientific research topics with their theoretical and experimental questions without a laboratory. It encompasses the fields of:

- Bioinformatics, which applies algorithms and statistical techniques to the interpretation, classification and understanding of biological datasets. These typically consist of large numbers of DNA, RNA, or protein sequences. Sequence alignment is used to assemble the datasets for analysis. Comparisons of homologous sequences, gene finding, and prediction of gene expression are the most common techniques used on assembled datasets; however, analysis of such datasets have many applications throughout all fields of biology.
- Computational biomodeling, a field within biocybernetics concerned with building computational models of biological systems.
- Computational genomics, a field within genomics which studies the genomes of cells and organisms. High-throughput genome sequencing produces lots of data, which requires extensive post-processing (genome assembly) and uses DNA microarray technologies to perform statistical analyses on the genes expressed in individual cell types. This can help find genes of interests for certain diseases or conditions. This field also studies the mathematical foundations of sequencing.
- Molecular modeling, which consists of modelling the behavior of molecules of biological importance.
- Protein structure prediction and structural genomics, which attempt to systematically produce accurate structural models for three-dimensional protein structures that have not been determined experimentally.
- Computational biochemistry and biophysics, which make extensive use of structural modeling and simulation methods such as molecular dynamics and Monte Carlo method-inspired Boltzmann sampling methods in an attempt to elucidate the kinetics and thermodynamics of protein functions." (Wikipedia, Computational Biology)

This new focus is referred to as systems biology. It is the key to learning how to design and engineer parts, devices and systems of a biological nature from standardized elements that are readily available (referred to as biobricks or bioparts).

Another sector of the field is experimenting with algae to produce oils that can be refined into diesel fuel. Algae cells are fed biomass made from natural substances such as sugar cane in the dark, bio-engineering that allows the algae to make oil without going through the process of photosynthesis. Further into the future, the hope is that the resultant oil can function as a triglyceride oil for the manufacture of other chemical products.

Research in synthetic biosystems and fermentation processes are resulting in revolutionary advances in production of ethanol via consolidated bioprocessing (CBP) and commercial heterotrophic and/or photoheterotrophic production of algal biomass (Solazyme; Amyris; General Atomics). These biosystems utilize symbiotic microbial cultures for development of clean and scalable solutions for production of bioenergy, industrial chemicals, and specialty ingredients markets for the food industry.

Synthetic biology will make great strides in improving chemical manufacturing processes when it can develop fermentation that results in the creation of industrial monomers. The value of these monomers will be in their lower cost and the ability to manufacture in smaller volume batches – making them a more valuable commodity than petrochemicals. As well, synthetic biology will allow the substances to be manipulated to provide a high degree of specialization to achieve

specific performance goals and product features. The future could become reality in this respect in just two years (Verdezyne; BioEnergy International; Boswell, 2009; Jenkinson, 2009).

Toxic Components & Characteristics of Lignocellulosic Biomass

In general, it would be more advantageous in terms of cost savings to modify pretreatment processes to reduce the formation of toxic compounds rather than add additional costs of conditioning steps. Though less toxic pretreatment methods typically release less sugar for fermentations, and therefore require additional hydrolytic enzymes, this may not appear to be the case for ammonia ensiling and enzyme inoculation followed by a low energy and relatively non-invasive aqueous ammonia-ethanol soaking/steeping pretreatment and fractionation process.

When lignocellulosic biomass is broken down collectively via pretreatment or into individual components via fractionation and hydrolysis, cellulose and hemicellulose are converted to mixtures of sugars (e.g., pentoses, hexoses and oligo/polysaccharides). Individually these sugars are most suitable for fermentation only when inhibitors (such as acetic acid produced during hydrolysis of hemicellulose) have been removed. Current acid or alkaline pretreatments are reasonably efficient at making the cellulose, hemicellulose, and lignin matrix amenable to enzymatic hydrolysis and fermentation. However, during intense thermochemical pretreatments a number of toxic compounds are released into the hydrolysate which inhibit downstream functioning of biocatalysts (microorganisms or microbes) which are responsible for producing ethanol and various coproducts. Conditioning methods designed to reduce the toxicity of hydrolysates are effective, but add to process costs and tend to reduce sugar yields, thus adding significantly to total production costs. Reducing the cost of cellulosic ethanol production requires a full understanding of the source and mode of action of hydrolysate toxic compounds, the means by which some organisms resist the actions of these compounds, and the methodology and mechanisms for conditioning hydrolysate to reduce toxicity (Pienkos and Zhang, 2009).

The inhibitory components of biomass include carboxylic acids, primarily acetic acid, the sugar degradation products furfural and hydroxymethylfurfural (HMF), phenolic compounds, and inorganic salts (Klinke et al, 2001, 2002, 2004). These toxic components degrade sugars and inhibit the growth of the fermentation organisms thereby reducing both the rate and yield of ethanol production. The sheer number of potentially toxic compounds in hydrolysates, the synergistic manner in which they can inhibit microbial fermentations, and the limited ability to identify all of them (Fenske et al. 1998; Chen et al. 2006) greatly complicate efforts to understand the contribution of individual compounds. This makes it difficult to determine overall toxicity and to detail the biological effect of these collective components. Because of this, the bulk of work has been done with individual or simple mixtures of model compounds, though some attempts have been made to draw inferences from hydrolysates by relating the inhibition of fermentation with the concentrations of known toxic compounds present (Pienkos and Zhang, 2009).

The following are specific examples of how some of the toxic substances contained in lignocellulosic feedstocks can inhibit fermentations:

- Furfural and HMF inhibit glycolysis, especially interfering with the activity of dehydrogenases, causing a reduction in growth rates and cell yields (Banerjee et al., 1981).
- Phenolics partition into membranes and lead to loss of integrity, interfering with cell growth and sugar transport (Heipieper et al., 1994).

- Acids disrupt cellular energy generation by collapsing pH gradients especially at low pH. The relative toxicity is a function of hydrophobicity because this characteristic determines the ability of the compound to pass through the membrane (Zaldivar et al., 1999).
- Aldehyde toxicity is also related to hydrophobicity, but aldehydes do not disrupt membrane integrity or cause a collapse of pH gradient (Zaldivar and Ingram, 1999).
- Alcohols are generally less toxic than related acids or aldehydes, but their toxicity is also related to hydrophobicity. They appear to cause a breakdown in membrane structure (Zaldivar and Ingram, 2000).

Pienkos and Zhang (2009) have conducted an extensive literature review pertaining to synergies of toxic substances contained in hydrolysates. This includes the various processes in which toxicity can be tolerated via relative resistance of two or more strains to hydrolysates or individual compounds in addition to enhanced resistance. Enhanced resistance refers to improvements in resistance in a single strain brought about by growth selection or through strain engineering efforts including CBP, one of the goals of which is to eliminate production of toxic byproducts. Efforts for improving conditioning (e.g. treating or removing toxic compounds) are also addressed via biological, chemical and physical methodologies. The biological and chemical approaches are designed to convert toxic compounds to less toxic products; the physical approach is designed to remove toxic compounds from the hydrolysate. Sequestration of the toxic compounds, followed by recovery will provide some insights into hydrolysate fractionation as well as open up possibilities for generation of biorefinery chemical feedstocks as previously discussed.

The presence of inhibitors in hydrolysates and the additional process costs of detoxification or conditioning steps added to improve the efficiency of fermentation are major impediments in the development of an economic process for the production of cellulosic ethanol (Pienkos and Zhang, 2009). However, these impediments can be overcome via low energy and non-invasive pretreatment processes in combination with fractionation platforms which separate and process toxic compounds along with other biomass components (including lignin) into value added coproducts. The relatively high value of the industrial biochemical, special ingredient, pharmaceutical and nutraceutical coproducts produced will offset the cost for fractionation while improving fermentation efficiency for separate hydrolysis and fermentation of hexose and pentose sugars contained in cellulose and hemicellulose fractions.

Need for Universal Low-Cost Wet Biomass Pretreatment & Fractionation Technology
Various biomass feedstocks and pretreatment processes generate different combinations of toxic compounds. Though this is due primarily to intense thermochemical pretreatment processes, it is also due partially to differences in composition (e.g. the pentose:hexose (C5:C6) ratio from different biomass substrates can range from ca. 70:30 to 15:85) (University of Georgia Research Foundation). In addition, various fermentation strains have different levels of natural and synthetic resistance to potentially toxic components and changes in the actual fermentation processes (such as temperature and concentration) can lead to different levels of resistance (Klinke et al. 2004; Palmqvist and Hahn-Ha¨gerdal 2000a, 2000b; Sun and Cheng 2002; Mussatto and Roberto 2004; Pienkos and Zhang, 2009).

Hence, several pretreatment processes ranging from base to acidic applications have been developed over the years. These various processes are designed to make the sugars more

available for subsequent hydrolysis and fermentation steps through the breakdown of the cell wall, and the degradation of the cellulose, hemicellulose and lignin matrix. A list of characteristics of the "ideal pretreatment process" was originally assembled by Mosier et al. (2005):

- Produces a highly digestible pretreated solid.
- Does not significantly degrade pentoses.
- Does not significantly inhibit subsequent fermentation steps.
- Requires little or no size reduction of biomass feedstock.
- Can work in reactors of reasonable size and moderate cost.
- Produces no solid-waste residues.
- Has a high degree of simplicity.
- Is effective at low moisture content.

Though the basic approach is the same today, some aspects of pretreatment (such as the ability to efficiently process wet biomass feedstocks) have recently received more emphasis than others. Reducing capital investment, energy costs of operation, and ecological impact for efficient pretreatment and economical storage of wet biomass feedstocks is essential. In addition to the above characteristics, an ideal pretreatment process would not adversely impact downstream processes including fractionation, fermentation and production of high value coproducts for integrated biorefineries. Upon completion of the pretreatment process, the severity of various conditioning methods vary widely. While it is true that the less severe methods often result in formation of less toxic hydrolysate, they also may result in reduced hydrolysis of hemicellulose to monosaccharides, requiring the use of additional enzymes during the saccharification process, and increasing the overall process costs (Pienkos and Zhang, 2009).

An exception to this may be extended periods of pretreatment at ambient temperatures via ammonia ensiling and incorporation of enzyme inoculation followed by a low temperature aqueous ammonia-ethanol pretreatment and fractionation platform. Additionally, as an alternative to conditioning in which toxic components are discarded, economical fractionation platforms and acid treatments of hemicellulose remove toxic components and process them into value added coproducts which are valued substantially higher than commodity biofuels. Eventually, synthetic biology will allow for provision of consolidated bioprocessing (CBP), thus eliminating the need for enzymes and acid separation processes for production of a variety of different bioproducts, nutraceuticals, pharmaceuticals, special ingredients, industrial biochemicals, and bioenergy (bioelectricity, biomethane, biohydrogen, and liquid biofuels).

Pretreatment, Fractionation & Separation Processes

Due to the complex structure of lignocellulosic biomass, it is considerably more difficult to efficiently process in comparison with starch (glucose) and simple sugars (sucrose and fructose). In order to overcome lignocellulosic recalcitrance, pretreatments are utilized in order to break the lignin bonds and hydrolyze/liquefy hemicellulosic sugars. This process allows for breaking up the structure of biomass sufficiently to allow efficient and effective enzymatic hydrolysis of the cellulose component which is protected by a sheath of lignin and hemicellulose. This is accomplished by altering the physical features and chemical composition of lignocellulose to make it more digestible. This subsequently increases digestibility of biomass by enhancing enzymatic processing for microbial populations, thus enhancing efficiency of fermentations.

Pretreatment also enhances digestibility of lignocellulosic biomass for animals, particularly ruminants such as cattle and sheep, thus reducing the amount of corn and other grains required for producing high quality protein foods such as meat, dairy products, and eggs. This allows for more corn to be processed into higher value food products. It also increases efficiency for production of energy crops on agricultural land. In addition, crop residues such as corn stover, wheat straw and soybean hulls can be processed into animal feeds, biofuels, and high value bioproducts and industrial biochemicals.

Though dedicated energy crops such as sweet sorghum, sugarcane, switchgrass and miscanthus differ in lignocellulosic composition in comparison with crop residues, they all contain relatively large amounts of lignocellulosic biomass similar to crop residues. "Lignocellulose provides structure to plants and is found in roots, stalks and leaves. In regards to actual composition, it is composed of three major components: cellulose (38–50%), hemicellulose (23–32%), and lignin (15–30%). Cellulose and hemicellulose are polysaccharides. Cellulose is a linear polymer of glucose linked by β-1,4 bonds. It is similar to starch, which is a polymer of glucose linked by α-1,4 bonds. However, this seemingly minor difference in linkages makes a major difference in chemical and biochemical reactivity - for the same enzyme loading, amylase hydrolyzes starch about 100 times faster than cellulase hydrolyzes cellulose. Hence, in order to optimize fermentation efficiency, particularly for SSF applications, cellulosic and starch substrates are usually hydrolyzed and fermented separately similar to hemicellulosic (primarily pentose) substrates.

"The reason that glucose hydrolyzes considerably faster than starch is due to the structural nature of lignocellulose. The same hydrogen bonds between adjacent cellulose polymers which form crystalline structures that give plants structural strength, also make them particularly difficult to breakdown and digest. In contrast to cellulose which is a six-carbon sugar, hemicellulose is a highly branched polymer composed primarily of five-carbon sugars (mostly xylose and arabinose). Hemicellulose is chemically bonded to lignin and serves as an interface between the lignin and cellulose. Hemicellulose is randomly acetylated which reduces its enzymatic reactivity. When hydrolyzed, the acetyl groups are converted to acetic acid which also inhibits fermentation. Lignin is a polymer of phenyl propane units linked primarily by ether bonds which act as glue. A plant can be compared to fiberglass, where the cellulose is analogous to the glass fibers and lignin serves as the epoxy resin." (Sierra et al., 2008) Ammonia ensiling allows for the cellulose and pentose fractions to be partially hydrolyzed prior to ammonia-ethanol steeping and fractionation. Enzyme inoculation during ensiling and/or enzyme incubation during or subsequent to steeping and fractionation could potentially allow for integration of cellulose with starch SSF processes at integrated biorefineries.

Current biorefinery processes hydrolyze about 63% of cellulosic biomass into hexose sugars and convert 76% of pentose sugars into ethanol. The industry goal is to improve both efficiencies to above 95% (Lynd, 2004), thereby increasing the total yield of ethanol from biomass to about 108 gal/metric tonne (119 gal/ton). This is in comparison with the average 60 gal/metric tonne (66 gal/ton) which is currently produced. According to the DOE, the theoretical maximum yield for producing ethanol from all of the sugars contained in corn stover is 113.8 gal/ton, while 124.4 gal/ton is produced from corn grain. This is equivalent to a theoretical maximum of 119.1 gal/ton resulting from processing the "entire corn plant" into ethanol. In addition, production of valuable coproducts such as special ingredients, sucrose (table sugar), vitamins, nutraceuticals, biochemicals and bioproducts will increase net energy gain (NEG) and subsequent revenues for biorefineries.

Factors Affecting Pretreatment
Cellulose crystallinity prevents rapid access of enzymes to hydrolyze polysaccharides. Pretreatments that selectively remove lignin enhance enzymatic digestibility, but also increase biomass crystallinity because lignin is amorphous. To properly assess the role of crystallinity it is necessary to understand how cellulose crystallinity, not biomass crystallinity, is affected by a particular pretreatment. Though this is difficult to determine since measuring the crystallinity of cellulose that is blended with other biomass components is problematic, the problem is resolved via clean fractionation platforms. (Sierra et al., 2008)

Degree of Polymerization
"Some enzymes that convert cellulose into sugars (endocellulases) cleave internal cellulose bonds, thereby creating reactive ends that can be attacked by other enzymes (exocellulases). Acidic pretreatments readily hydrolyze cellulose internally to create reactive ends, whereas alkaline pretreatments tend to protect internal cellulose bonds. Thus, if a pretreatment creates internal reactive ends, it should help the cellulase work more effectively.

Surface Area & Pore Volume
Altering the crystallinity, lignin content and acetyl content of lignocellulose often results in substantial changes in surface area and pore volume. Although these latter features likely play important roles in enzymatic digestibility, it is difficult to understand their relative importance because they are not easily isolated and controlled in experiments.

A study of three structural features (lignin content, acetyl content and crystallinity) resulted in the following conclusions:

- acetyl content has the least effect on digestibility
- lignin content determines the extent of digestion
- crystallinity affects the rate of digestion" (Zhu et al., 2008; Sierra et al., 2008)

Efficient Pretreatment
Specifically, pretreatments improve enzyme access and biomass digestibility by:

- removing or altering lignin
- removing hemicellulose
- decrystallizing cellulose
- removing acetyl groups from hemicellulose
- reducing the degree of polymerization in cellulose
- expanding the structure to increase pore volume and internal surface area.

A pretreatment that accomplishes all of these goals is likely to be very expensive, so most pretreatments focus on the most important characteristics realizing that various pretreatments affect biomass in different ways (Mosier et al, 2005; Wyman, et al., 2005).

Desirable characteristics of a pretreatment process include the following:

- preserving the cellulose and hemicellulose fractions
- limiting formation of degradation products that may inhibit fermentative microorganisms
- requires minimal energy
- effective on multiple lignocellulosic feedstocks
- minimize capital and operating costs (Sierra et al., 2008)

- conducive to promoting healthy microbial populations.

Table 1. Lignocellulosic Biomass Pretreatments

Primary Types	Res. Time	°C	Atm	Other Conditions
Acid-Catalyzed				
Water (H_2O)				
Autohydrolysis	~1 hour	~200	~15	
Steam Explosion	0.3–50 min	190–250	12–40	
Liquid Hot Water	2–15 min	190–220	13–25	
Liquid Hot Water (Neutral pH)	~15 min	160–220	6–25	
Dilute Acid (H_2SO_4, SO_2, HCl, HNO_3)	5–30 min	140–190	4–13	0.5–10% Acid
Concentrated Acid (H_3PO_4)	30–60 min	0	1	85% H_3PO_4
Peracetic Acid ($C_2H_4O_3$)	1–180 h	25–75	1	2–10% $C_2H_4O_3$, 0.2–1.0 g $C_2H_4O_3$/g biomass
Supercritical Carbon Dioxide	1 h	35–80	70–270	
Base-Catalyzed				
Sodium Hydroxide	24–96 h	25	1	1% NaOH, 0.1 g NaOH/g biomass
Lime ($Ca(OH)_2$)				
Low Lignin Content (12–18%)	1–2 h	100–120	1–2	0.10 g Ca(OH)2/g biomass
Medium Lignin Content (18–24%)	~30 days	~55	1	0.10–0.15 g $Ca(OH)_2$/g biomass
High Lignin Content (>24%)	~2 h	~150	15	0.15–0.20 g $Ca(OH)_2$/g biomass
Ammonia (NH_3)				
ARP	~15 min	~180	~20	15% NH_3
AFEX	~5 min	60–100	~20	1 g NH_3/g biomass
NH_3 Ensiling & NH_3-EtOH Steeping & Fractionation Platform	Variable for Feedstock (10-24 hr)	< 60	1	Solid to liquid ratio 1:9, 15 w/w% NH_3, 20 w/w% EtOH. Over 90% lignin removed using alkaline oxidation. Less than 2% of NH_3 remains in clean lignin, cellulose and hemicellulose fractions prior to incubation & SSF/SSCF/CBP. NH_3 and EtOH are recycled.

(Table Adapted from Sierra et al., 2008)

Development of pretreatment technologies for efficiently processing high moisture stover and other crop residues as wet biomass feedstocks would allow for optimizing agricultural production via relay cropping systems. According to the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI), the sulfur dioxide/dilute acid, AFEX and lime

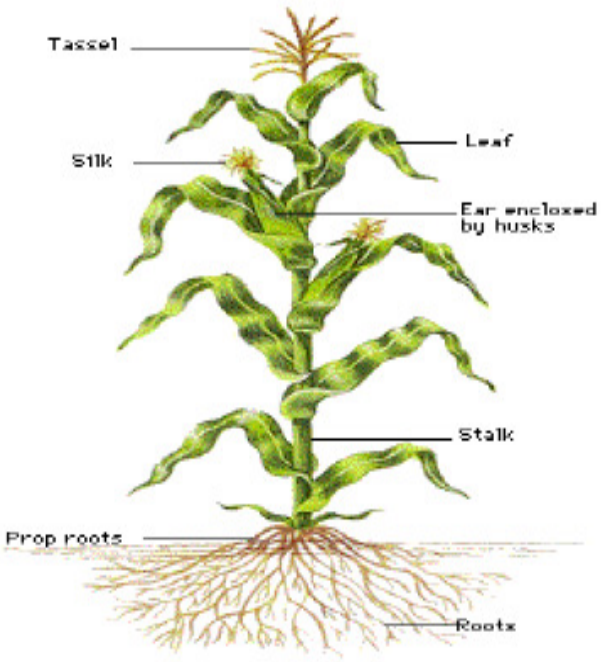
pretreatments are the least energy intensive and most efficient technologies for processing lignocellulosic biomass. Though the dilute acid flow-through provides the highest yields, it is not economical on a commercial basis due to lack of scalability (Biomass Refining CAFI-1, 2005; Biomass Refining CAFI-1, 2007).

The Biomass Refining CAFI is a multi-institutional effort funded by USDA Initiative for Future Agriculture and Food Systems Program for $1.2 million to develop comparative information on cellulosic biomass pretreatment by leading pretreatment options with A common source of cellulosic biomass (corn stover) and identical analytical methods:

- ARP (Aqueous ammonia recycle pretreatment) - YY Lee, Auburn University
- Water only and dilute acid hydrolysis by co-current and flow-through systems - Charles Wyman, University of California-Riverside
- Ammonia fiber explosion (AFEX) - Bruce Dale, Michigan State University
- Controlled pH pretreatment - Mike Ladisch, Purdue University
- Lime pretreatment - Mark Holtzapple, Texas A&M University
- Logistical support and economic analysis - Rick Elander/Tim Eggeman, NREL through DOE Biomass Program funding

Listed below is a table of values for a common corn stover feedstock which was utilized in comparisons of various pretreatment processes. Corn stover consisting of the majority of the corn plant minus the grain, 18" of the lower stalk, and roots were supplied by NREL c/o BioMass Agriproducts, Harlan, IA. The corn stover feedstock was washed, dried and knife milled to pass through a ¼" screen prior to water/chemical pretreatment processes. As is demonstrated by the data listed below, not all pretreatments provide the same biomass conversion efficiencies. In addition, as indicated above, different types of biomass feedstocks vary in composition. Thus, production of fermentable monosaccharides, oligosaccharides (hexose and pentose sugars), and polysaccharides will vary substantially from one lignocellulosic biomass feedstock to another.

Table 2. CAFI-I Corn Stover Feedstock Composition

Component	Biomass Percentage	Corn Plant
Glucan (C6)	36.1%	
Xylan (C5)	21.4%	
Arabinan (C5)	3.5%	
Mannan (C6)	1.8%	
Galactan (C6)	2.5%	
Lignin	17.2%	
Protein	4.0%	
Acetyl	3.2%	
Ash	7.1%	
Uronic Acid	3.6%	

Non-structural sugars	1.2%	

In regards to composition of biomass feedstocks and conversion to fermentable sugars, an oligosaccharide is a saccharide polymer containing a small number (typically three to ten) of component sugars, also known as simple sugars. The term glycan refers to a polysaccharide or oligosaccharide. Glycan may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, or a proteoglycan. Glycans usually consist solely of O-glycosidic linkages of monosaccharides. For example, cellulose is a glycan (or more specifically a glucan) composed of beta-1,4-linked D-glucose, and chitin is a glycan composed of beta-1,4-linked N-acetyl-D-glucosamine. Glycans can be homo or heteropolymers of monosaccharide residues, and can be linear or branched. A glucan molecule is a polysaccharide of D-glucose monomers linked by glycosidic bonds.

Table 3. CAFI-I Overall Yields for Corn Stover at 15 FPU (filter paper units)/g Glucan

	Pretreatment System	Xylose Yields*			Glucose Yields*			Total Sugars*		
		Stage 1	Stage 2	Total xylose	Stage 1	Stage 2	Total glucose	Stage 1	Stage 2	Combined Total
	Maximum Possible	**37.3**	**37.7**	**37.7**	**62.3**	**62.3**	**62.3**	**100.0**	**100.0**	**100.0**
Increasing pH ↓	Dilute Acid	32.1/31.2	3.2	35.3/34.4	3.9	53.2	57.1	36.0/35.1	56.4	92.4/91.5
	SO_2 Steam Explosion	14.7/1.0	20.0	34.7/21.0	2.5/0.8	56.7	59.2/57.5	17.2/1.8	76.7	93.9/78.5
	Flow-through	36.3/1.7	0.6/0.5	36.9/2.2	4.5/4.4	55.2	59.7/59.6	40.8/6.1	55.8/55.7	96.6/61.8
	Controlled pH	21.8/0.9	9.0	30.8/9.9	3.5/0.2	52.9	56.4/53.1	35.3/1.1	61.9	87.2/63.0
	AFEX		34.6/29.3	34.6/29.3		59.8	59.8		94.4/89.1	94.4/89.1
	ARP	17.8/0	15.5	33.3/15.5		56.1	56.1	17.8/0	71.6	89.4/71.6
	Lime	9.2/0.3	19.6	28.8/19.9	1.0/0.3	57.0	58.0/57.3	10.2/0.6	76.6	86.8/77.2
	NH_3 Ensiling & EtOH-NH_3 Steeping			33.93 (90% of xylan)			62.3 (100% of glucan)			96.23

*Cumulative soluble sugars as total/monomers. Single number = just monomers.
(Table adapted from Biomass Refining CAFI-1, 2007)

Though the flow-through pretreatment technology provides the highest conversion efficiency to sugars, it is not economical for commercial applications. Hence, the dilute acid/SO_2 steam explosion and AFEX technologies provide the highest conversion efficiencies at about 94%. This results in a maximum yield of 66 gal of ethanol per ton of corn stover that is pretreated and fermented. This can increase ethanol yields by 55-61% for processing the entire corn plant. Based on 200 bu/acre corn crop yields, this would be equivalent to producing 930 gal/acre ethanol (330 gal from corn stover for processing glucan and xylan). Based on 400 bu/acre corn crop yields via SDF and precision high density cropping systems, this would be equivalent to producing 1860 gal/acre ethanol (660 gal from corn stover). These figures are based on production of 3.0 gal of ethanol per bushel of corn which is achieved by processing cellulosic portions of corn grain, e.g. pericarp and endosperm fiber fractions along with corn stover via lignocellulosic biomass pretreatment. At 95% conversion efficiency, a yield of 3.3 gal/bu of ethanol could be achieved at integrated biorefineries which utilize SSF, SSCF or CBP.

Optimizing Sugar Production & Yield

After pretreatment, neutralization, and conditioning, fermentation can be initiated by addition of cellulase and beta-glucosidase enzymes in conjunction with a biocatalyst (ethanologens including synthetic microbes) in a process called simultaneous saccharification and fermentation (SSF) (Gauss et al, 1976; Philippidis et al, 1993; Olofsson et al, 2008). Commercial enzymes or dilute acid processes are used to complete the depolymerization process to simple sugars which are subsequently fermented to ethanol by microorganisms (Mielenz et al, 2009).

Because the unit value of chemical products (including ethanol) derived from biomass is generally low while the potential market is large, the economic viability of such processes depends on optimizing yield and productivity. Yield is the quantity of product produced per volume of biomass processed, while productivity is the rate at which the product is generated. Achieving high yield requires that all biomass components are efficiently converted, while high productivity requires that complex conversions occur as rapidly as possible. This can be accomplished via fractionation platforms which allow for separate SSF of relatively pure hexose and pentose sugars, or via simultaneous saccharification and cofermentation (SSCF) via optimized ratios of C5:C6 sugars which are obtained from various biomass feedstocks.

Several biomass pretreatment processes are currently being developed to convert lignocellulosic materials such as energy crops, crop residues and organic waste including sewage to fermentable sugars. Among these proposed technologies are autohydrolysis, alkaline, and acid based treatments. However, most of these pretreatment processes require energy intensive thermochemical processes which destroy the protein component of the biomass feedstocks.

In contrast to acid-based platforms, the universal process depicted above utilizes ammonia ensiling and enzyme inoculation followed by an aqueous ammonia-ethanol pretreatment-fractionation process in combination with dilute acid washing and additional separation processes for production of specific high value industrial biochemicals. Ammonia ensiling provides an economical storage and simultaneous pretreatment process for biorefining while preserving protein in fermentation residues for use as value added animal feeds.

In addition to preserving protein, the ammonia used in the wet biomass treatment and fractionation process provides a value added source of free amino nitrogen (FAN). Since many yeasts cannot utilize complex proteins such as peptides without protease enzymes, FAN is required for microbial metabolism and efficient fermentations of hydrolyzed and depolymerized substrates. Separate hydrolysis of glucan and xylan fractions optimizes sugar yields for subsequent fermentations including simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP). Similarly, very high gravity fermentations via SSF and CBP are most efficient when hexose and pentose sugars are fermented separately. A small amount of glucose increases fermentation efficiency for pentose sugars. Plant expressed enzymes, efficient pretreatment, and synthetic biocatalysts may eventually eliminate the need for enzymatic hydrolysis.

By converting organic waste, waste heat and CO_2 produced by biorefineries into value added products, closed loop biomass production and processing could reduce ethanol production costs to less than $0.50/gal via integrated biorefineries. An economical source of carbon via cellulosic hydrolysates could substantially reduce production costs for heterotrophic algal bioreactors. Hence, integration of algal-yeast fermentation with ethanol biorefineries via closed loop architecture could allow for production of biodiesel for less than $1/gal. Closed loop production of algal biomass could eventually allow for reducing production costs for biodiesel to $0.50/gal.

In addition, high value foods, nutraceuticals, pharmaceuticals, functional foods, biochemicals and bioenergy could also be produced with unprecedented efficiencies of operation.

Current & Future Technology Advances for Measuring, Predicting & Reducing Toxicity
According to Pienkos and Min (2009), "there are a number of process details [such as low energy ammonia ensiling, enzyme inoculation, and aqueous ammonia-ethanol steeping and fractionation platforms which reduce production of toxic compounds during pretreatment and fractionation in addition to separating them from fermentable substrates] that will require further improvements before lignocellulosic ethanol can be produced economically. Several of these relate to the costs of pretreatment and conditioning. These costs include not just process costs such as raw material costs and utility costs related to raising and lowering temperatures but also performance costs such as lower yields due to sugar losses as well as reduced yields and prolonged fermentations times due to toxic effects of hydrolysate.

All of the above process aspects have been explored to some degree with a goal of improving the overall economics, and significant strides have been made, but further progress is necessary, especially in the development of new methods to measure biological toxicity, new tools to analyze hydrolysates, and new approaches to understand the molecular targets for and metabolic responses to toxic compounds. The information obtained by the application of these new technologies could conceivably feed into a number of new research areas including [further] improvement of pretreatment and conditioning methods, identification of optimum biomass source/fermentation organism combinations, engineering of biomass feedstocks to reduce the toxic compound progenitors, [plant expressed enzymes] and genetic enhancement of resistance. As this review has demonstrated, these research areas are not new ideas. All of them have been examined and several of them have led to enhanced understanding as well as process improvements. These include:

- Optimization of pretreatment and conditioning processes to maximize elimination of toxic compounds and minimize sugar losses. In addition to the ammonia storage, pretreatments and organic solvent fractionation platforms described above, dilute acid hydrolysis with ammonium hydroxide conditioning is one of the leading process options. Other conditioning approaches, especially biological and physical (e.g., laccase catalyzed conversion of phenolics and ion exchange sequestration of acids) provide insights into the importance of specific classes of compounds in overall toxicity.
- Detailed analysis of hydrolysates and identification of scores of potentially toxic compounds including furans, aliphatic acids, phenolics, aliphatic and aromatic acids, aldehydes and ketones. Chromatographic methods are being developed to distinguish dozens of different components in hydrolysates and new detection methods (especially MALDI-TOF mass spectrometry) are being developed to expand the ability to identify additional compounds that may not be available as standards.
- Toxicity analysis is becoming increasingly detailed, moving from fermentor and shake flask measurement of inhibition of growth and productivity to identification of metabolic targets and mode of action. Toxicity analysis is also becoming increasingly complex as researchers explore inhibitor synergies, moving from single compounds to mixtures in an effort to model the complex mix of compounds found in hydrolysate. Systems biology approaches (i.e., transcriptomics) are being employed to analyze the response of fermentation organisms to challenge by toxic compounds found in hydrolysate.
- Evaluation of candidate fermentation strains for their ability to perform well in the presence of toxic compounds or hydrolysate has helped determine which strains are most

robust and has provided benchmarks for further improvements either in pretreatment and conditioning or in development of higher levels of resistance [primarily via recombinant microbial strains and responsible transgenic processes]. Strain improvement efforts include, selection of more robust strains through continuous culture; identification of specific detoxification enzymes and engineering of overproducing strains; over-expression of single genes in whole genomic libraries; and manipulation of specific genes whose expression levels change when the strain is exposed to toxic compounds. Some of these efforts have led to incremental improvements in strain robustness and have provided additional insights into the mode of action of toxic compounds as well as the relative importance of specific compounds in hydrolysates."

Optimizing Fermentation Efficiency for Cellulose & Glucose (Hexose Sugars)
There is considerable knowledge regarding SSF for starch (glucose) fermentations and optimizing fermentation efficiency for conventional distillers yeast. This knowledge base can be extrapolated for use with cellulosic fermentations which also process glucose sugars once cellulose is hydrolyzed. In regards to SEAA and UHG starch fermentations using E-Mill processes, both of which incorporate wet fractionation technology, these processes can be described as modified wet mill or hybrid steeping processes.

For example, an ethanol yield of 2.58 gal/bu is obtained by fermenting starch only (Singh et al., 2004), and 2.65 gal/bu when starch hydrolyzing enzymes are utilized via enzymatic milling and fractionation to remove non-fermentable components of corn (includes cellulosic fiber and corn germ) prior to fermentation (E-Mill process) (Wang et al., 2005). The modified steeping process utilized by the E-Mill fractionation process is similar in concept to soaking/steeping in ethanol and aqueous ammonia (SEAA). Integrated biorefineries which leverage existing corn ethanol infrastructures can process fiber and corn germ meal via cellulosic fermentations and recycle thin stillage from starch fermentations to increase ethanol yields by as much as 20%.

In addition to fractionation, improvements in development of proteases (such as GC 212 "FERMGEN") and ultrasonic cavitation effectually increases availability of essential "free amino acids" for growing yeast and reduces particle size to 200 microns. The smaller particle size and cavitation increases total surface area of grain fractions which enhances enzymatic and yeast processing. Installing new centrifuges or in-line decanters may be required to accommodate smaller sized particles. Smaller sized particles along with improving enzymes and starch content of corn hybrids, and utilizing the most efficient yeast strains, processing corn fiber, and recycling thin stillage could allow E-Mill processing and UHG SSF to exceed 3.2 gal of ethanol per bushel of corn processed. Processing corn stover in addition to corn at integrated biorefineries would allow for almost doubling sugars and other products produced from an acre of corn.

Comparison of Fermentation Profiles
(Adapted from Singh et al., 2005)

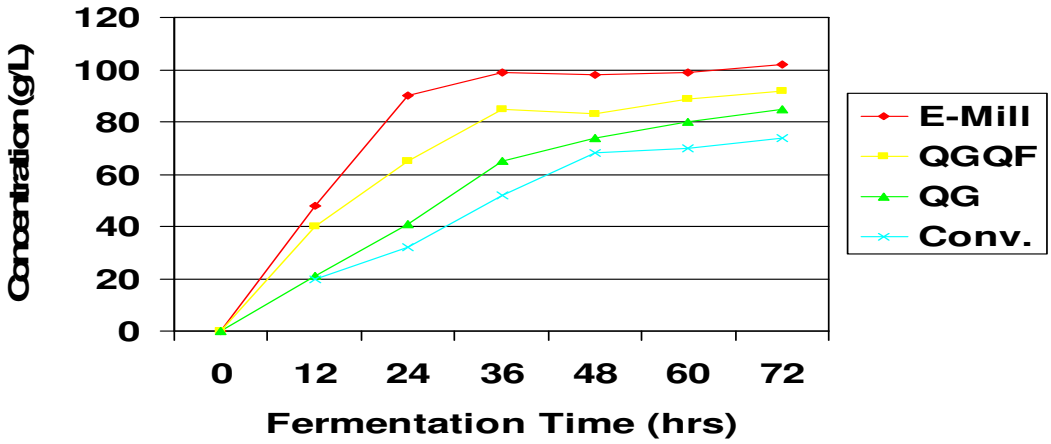


Figure 3. Comparison of Conventional, Fractionation & Enzymatic Technologies

An example of starch fermentations (Figure 3) illustrates how optimizing fermentation times can dramatically increase overall production. The E-mill process allows for recovery of the more uniformly distributed (and thus harder to separate) endosperm fiber. The subsequent increase in starch and nutrient concentrations in the fermentor results in substantially increasing the efficiency of yeast processing. Along with improved enzymatic processing and smaller particle size, this allows for reducing batch fermentation times from as high as 54 hours to as little as 24 hours depending on nutrient concentrations and optimizing fermentation efficiency for growing yeast. The following charts compare fermentation times with simple sugars (primarily glucose consisting of 88% of total sugars with the remainder being primarily fructose and sucrose) and other yeast nutrient concentrations. The data points utilized for concentration are measured in grams per liter (g/L) for this lab generated profile.

The above batch fermentation profiles for low sugar/nutrient concentrations used in lab demonstrations reveal that optimal efficiency for ethanol conversion occurs between 24 to 36 hours for VHG fermentations, i.e. once the ethanol concentration reaches about 98 g/L which is equivalent to 12.2 v/v%. Though commercial batch fermentations for the SEAA and E-Mill fractionation-enzymatic processing technologies could employ VHG/UHG concentrations approaching 21 v/v% at over 48 hours, the original lab scale profile has been extrapolated in order to illustrate that optimal conversion efficiency probably occurs between 26 and 30 hours of fermentation. Ethanol yields for optimal batch fermentation times is expected to be less than 20 v/v%. Ideally the goal would be to achieve around 18.18 v/v% (4.5 gal process water for each gal ethanol produced) for less than 30 hour fermentations in comparison to the industry average of about 17 v/v% for conventional batch fermentation times of 48-54 hours. Reductions in batch fermentation times from 48 hours to 27 hours results in increasing ethanol production and volume of coproducts by about 44%. This is accomplished by capitalizing on peak ethanol yields produced by “actively growing yeast” during the first 27 hours. When wet fractionation/modified steeping processes are combined with optimizing UHG fermentation efficiency for growing yeast, total biorefinery production volumes can be increased by over 72%.

Extrapolation of E-Mill Data
Lab Scale UHG Fermentation Profile

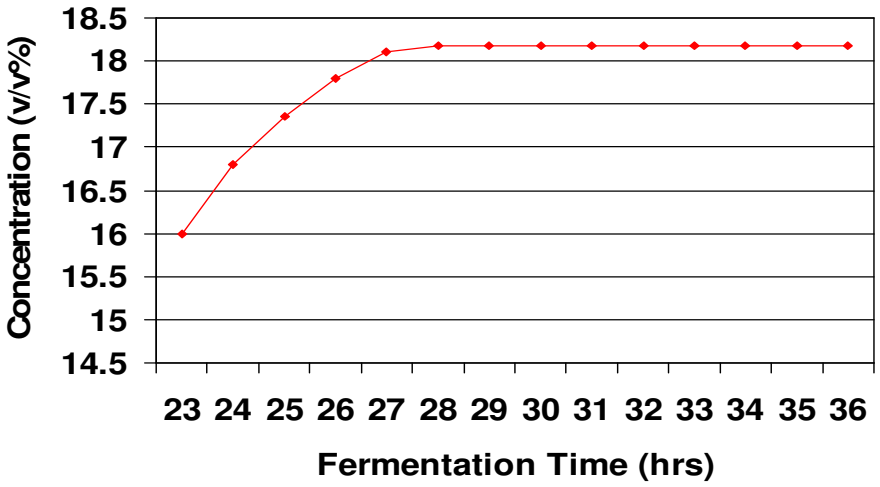


Figure 4. Determining Optimal Fermentation Time for E-Mill UHG Technology

Optimizing Batch Fermentation Times

The above slope, which is extrapolated from E-Mill fermentation data indicates that ethanol production can be optimized by starting new batches about every 27 hours. However, the original E-Mill data sets were calculated in a lab setting and are therefore subject to commercial verification. Smaller particle size, ultrasonic cavitation, improved yeast strains, improved protease enzymes, agitation, supplying oxygen, and kraeusening (method of yeast inoculation) will increase ethanol yields and reduce fermentation times.

Optimizing Fermentation Environment for Growing Yeast

Based on the above extrapolation, a commercial application of the E-Mill technology and aqueous ammonia and ethanol soaking/steeping would allow for processing the majority of glucose, xylose, arabinose and simple sugar concentrations contained in the mash in a relatively short period of time provided adequate cooling technology is employed for maximizing yeast vitality.

In an anaerobic environment, yeast metabolizes by fermentation and produces ethanol and CO_2 and produces much less energy than during respiration. There are only two molecules of ATP produced by individual yeast during anaerobic fermentation. Generally speaking, 1 bushel of corn (56 lb) provides 32 lb of starch which hydrolyzes to produce 36 lb of glucose. During fermentation, the glucose is transformed to 17.6 lb ethanol, 18.4 lb CO_2 and 6,120 BTU via an exothermic reaction. Theoretically, the release of energy could be reduced by an additional 18% to 286.36 BTU per lb of glucose or 5,040 BTU per bushel of corn by reducing yeast stress and optimizing efficiency of fermentation. The 17.6 lbs of ethanol represents 49% of the glucose, and 31% of the original bushel of corn. The bushel of corn also produces 17 lb of DDGS. Apart from these main fermentation products, glycerol, succinic acid, fesel alcohols and small quantities of organic acids are also produced. A well controlled fermentation produces around 90% of the theoretical alcohol yield, i.e. 15.84 lbs of ethanol per bushel of corn. However, resting yeast can only tolerate about 23-24 v/v% ethanol in the mash during fermentation. Hence, for practical purposes, 24 v/v% is considered to be the maximum ethanol yield for resting yeast fermentation. For actively growing yeast, the temperature/ethanol threshold is probably closer to 18 v/v%.

Fermentation strategies which harvest ethanol shortly after peak yeast production (once sugar concentrations are reduced and ethanol concentration reaches a certain threshold for budding

yeast) allow for actively growing/budding yeast to dominate microbial activity. This results in minimizing bacterial growth which is responsible for producing 90% of organic acids which inhibit yeast (Kelsall, 2008). Acetic acid, which is extremely potent for yeast in very minute concentrations (>0.05 w/v%), is produced and excreted in the presence of air by certain bacteria which consume ethanol, notably the *Acetobacter* genus and *Clostridium acetobutylicum*. Each molecule of lactic acid produced by competing bacteria during fermentation results in the loss of a molecule of ethanol! Some of the bacteria isolated from commercial sources are resistant to 10-15 v/v% ethanol and grow faster than yeast in commercial grain mashes at pH values as low as 3.6-3.8. The mixed acid bacteria including coliforms are only a problem in mash above pH 5.0 as bacteria are not tolerant to the lower pH values in fermenting mash. Butyric acid bacteria are found mostly in wet grain piles that have been allowed to 'incubate' in farmyards or processing plant areas (Ingledew, 1999, pp.52-53, 65)."

Though maintaining the fermentor pH below 3.0 would seem to be a logical solution to prevent bacterial growth, "at this pH level organic acids are covalent and can migrate across the yeast membrane. Once organic acids are inside the yeast nucleus where the pH is 5.4, the organic acids ionize and drop the nuclear pH to around 3.0 and subsequently cellular enzymes will stop producing alcohol. One molecule of ATP is required to pump out the proton and as anaerobic yeast only have 2 molecules, ethanol production stops. The yeast can function at low pH but the biochemical pathway for ethanol production is compromised when organic acids can migrate across the yeast membrane (Kelsall, 2008)."

Stress Factors for "Actively Growing Yeast" Are Synergistic
(Adapted for UHG fermentations from Ingledew, 1999, p.52)

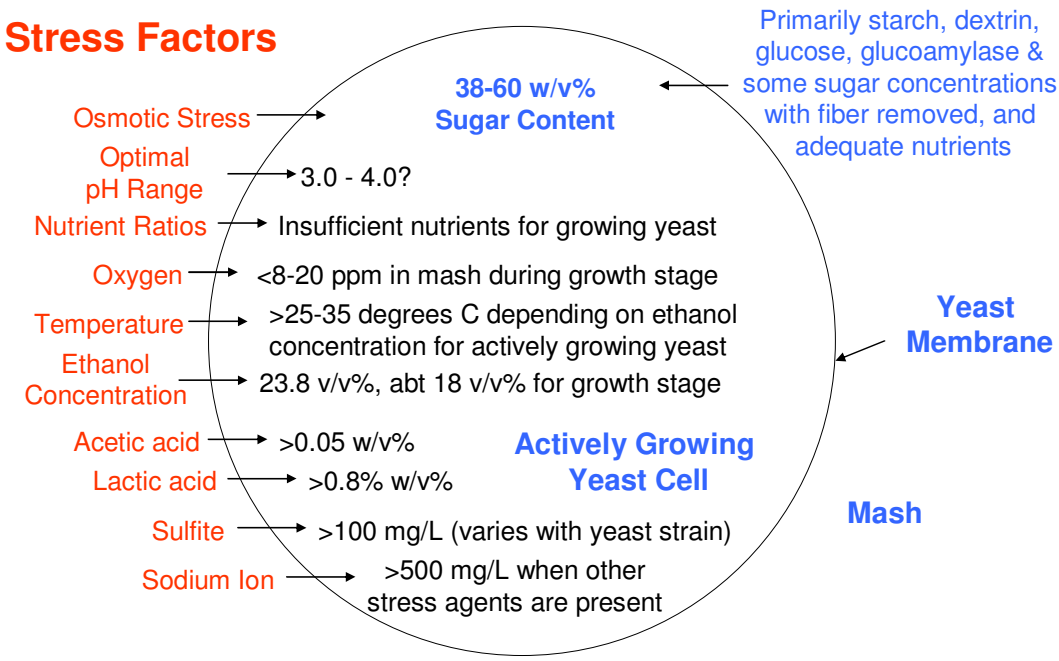


Figure 5. Typical Stress Factors Known to Affect Yeast Fermentations

Synergism

Synergism is defined as two or more nutrients or stress factors working together to produce a result not obtainable by any of those factors independently. For example, optimizing sugar concentration and nutrient ratios including oxygen for growing yeast can produce maximum ethanol yields provided temperature and ethanol concentrations are also optimal. If just one of the essential nutrients is insufficient, or one of the stress factors is not minimized, yeast vitality and normal functioning is impaired.

As nutrient ratios for yeast are optimized in conjunction with sugar concentrations, pH, temperature, and ethanol concentration, maximum ethanol yields will be achieved relative to fermentation time. In an optimal fermentation environment, actively growing yeast will literally overwhelm bacteria. Hence, with efficient antimicrobial and clean in place (CIP) systems employed, excretion of organic acids by bacteria will be minimized. Reducing volume of process water utilized as backset will reduce sulfite and sodium levels in fermentation mashes. Minimizing bacteria threats and contaminants in the fermentation mash allows biorefineries to focus on optimizing pH and temperature during fermentation and balancing essential nutrients for growing yeast.

Sugar Concentration & Osmotic Stress

Yeast can undergo osmotic stress when sugar concentration exceeds optimal levels. Osmosis is the diffusion of a solvent (frequently water) through a semi-permeable membrane, from a solution of low solute concentration (high water potential) to a solution with high solute concentration (low water potential), up a solute concentration gradient. It is a physical process in which a solvent moves, without input of energy, across a semi-permeable membrane (permeable to the solvent, but not the solute) separating two solutions of different concentrations. Osmosis releases energy and can be made to do work.

Net movement of solvent is from the less-concentrated (hypotonic) to the more-concentrated (hypertonic) solution, which tends to reduce the difference in concentrations. This effect can be countered by increasing the pressure of the hypertonic solution, with respect to the hypotonic. The osmotic pressure is defined to be the pressure required to maintain an equilibrium, with no net movement of solvent. Osmotic pressure is a colligative property, meaning that the property depends on the molar concentration of the solute but not on its identity.

Osmosis is important in yeast cells and other microbial cells during fermentation as many biological membranes are semi-permeable. In general, these membranes are impermeable to organic solutes with large molecules, such as polysaccharides, while permeable to water and small, uncharged solutes such as simple sugars or monosaccharides. Polysaccharides are relatively complex carbohydrates. They are polymers made up of many monosaccharides joined together by glycosidic bonds. They are therefore very large, often branched, macromolecules. They tend to be amorphous and insoluble in water. Examples include storage polysaccharides such as starch and glycogen and structural polysaccharides such as cellulose and chitin.

Permeability of monosaccharides through yeast cell walls depends on solubility properties, charge, or chemistry as well as solute size. Water molecules travel through the plasma cell wall, tonoplast (vacuole) or protoplast in two ways, either by diffusing across the phospholipid bilayer directly, or via aquaporins (small transmembrane proteins similar to those in facilitated diffusion and in creating ion channels). Osmosis provides the primary means by which water is transported into and out of yeast cells. The turgor pressure of a yeast cell is largely maintained by osmosis,

across the cell membrane, between the yeast cell interior and its relatively hypotonic environment.

It is enzymes within yeast cells that are responsible for fermentation. The only method that sugars can come in contact with cellular enzymes is by passing through the cell wall as a solvent. This is the process of osmosis, e.g. simple sugars (mono-, di-, and oligosaccharides) solvents passing through a semi-permeable cell wall or membrane. However, if the sugar concentration is too great, the diffusion through the cell wall cannot occur. This is due to the fact that the yeast cell is 65% water. When in a solution with a higher density than the yeast cell composition, osmosis occurs. The phenomenon of osmosis acts as a pressure, forcing the less dense contents within the yeast cell to diffuse through the semi-permeable cell wall to the other side of the cell in an attempt to equalize the differing densities. If sugar concentrations are too high, osmotic pressure literally sucks the yeast cell dry, causing osmotic stress.

Unlike starch in which polysaccharides are simultaneously saccharified into glucose during fermentation, adding simple sugars such as sucrose can dramatically increase sugar concentrations. Therefore, in order to avoid osmotic stress sucrose should be added as an adjunct to augment starch feedstocks. This is achieved by inserting sucrose incrementally to starch or to cellulose fermentations in order to avoid osmotic shock so as not to reduce the efficiency of fermentation. This is commonly done by companies which commercially manufacture brewers yeast. Regulating sugar concentration in conjunction with balancing microbial nutrition results in optimizing fermentation efficiency and increasing ethanol yields.

Yeast Nutritional Requirements

Nutrition is the answer to faster fermentations, increased yeast growth and catalytic conversion of glucose into ethanol, as well as increased tolerance of yeast to stress agents. To grow successfully, yeast requires an adequate supply of nutrients including fermentable carbohydrates, sources of nitrogen including free amino acids, vitamins, and minerals for healthy fermentation. Many of these nutrients are naturally present in grains or developed by yeast enzymes during the mashing process. Nutrients consist of carbohydrates, nitrogen, vitamins and minerals.

Carbohydrates

Only low-molecular-weight sugars such as the mono-, di-, and oligosaccharides are available for yeast growth. Polysaccharides are not used by the yeast. The sugars are, in order of concentration, maltose, maltotriose, glucose, sucrose, and fructose, which together constitute 72 to 85% of the total extract. The other 15 to 28% consists of nonfermentable products such as dextrins, beta-glucans, pentosans, and oligosaccharides. Regardless of concentration, fermentable carbohydrates are usually assimilated by yeast in the following order: sucrose, glucose, and fructose are consumed most rapidly (24-49 hrs); followed by maltose (70-72 hrs); then maltotriose (after 72 hrs) (Hough, 1982). Some overlap in assimilation does occur. In order to optimize fermentation efficiency for growing yeast, the primary goal is conversion of glucose and some sucrose and fructose in the first 27 to 30 hours. A majority of yeast strains leave maltotetraose and dextrins unfermented (Stewart and Russell, 1985). Assimilation is merely the conversion of nutrients into fluid or solid substances of the yeast by the processes of digestion and absorption.

Nitrogen

"Usable or assimilable nitrogen becomes problematic in mash, juice or mash fermentation media. Yeasts are only able to utilize low molecular weight nitrogenous materials such as inorganic ammonium ion, urea, amino acids and small peptides. Yeasts used in industrial and potable alcohol manufacture are not proteolytic. They cannot derive assimilable nitrogen for growth from protein or the breakdown of peptides much larger than tripeptides (Patterson and Ingledew, 1999; Ingledew and Patterson, 1999; Ingledew, 1999, p. 67)."

Nitrogen is available for yeast growth in mash primarily as amino acids and ammonium salts. Since yeast are non-proteolytic, large peptides and other unusable but soluble/insoluble proteins are difficult if not impossible for yeast to assimilate without protease enzymes (Ingledew, 1999, p. 69). Yeasts prefer to use ammonium salts, but these are present in mash only in very small amounts (Kunze, 1986). Amino acids, small peptides, and protease enzymes are therefore the most important mash constituents. Amino acids, collectively referred to as "free amino nitrogen (FAN)," are the principal nitrogen source in mash and are an essential component of yeast nutrition (Munroe, 1995). It is the amino acids that the yeast cells use to synthesize more amino acids and, in turn, to synthesize proteins. "Ammonium ion is a preferred nitrogen source for yeast cultivation in laboratory studies. It appears to be utilized by all genera of yeasts, and is usually supplied as ammonia or as the sulfate or phosphate salt (Suomalainen and Oura, 1971). Urea is also utilized by most yeasts although biotin and other growth factors may then be required. Urea, which is not a normal constituent of mashes, is easily broken down to two molecules of ammonium ion and one molecule of carbon dioxide (Ingeldew, 1999, p.68)."

Vitamins
Vitamins such as biotin, panthotenic acid, thiamin, and inositol are essential for enzyme function and yeast growth. Biotin is not freely available from corn or corn steep liqour, but can be supplemented or obtained from barley malt during mashing. Biotin is involved in carboxylation of pyruvic acid, nucleic synthesis, protein synthesis, and synthesis of fatty acids. Biotin deficiencies will result in yeast with high death rates. Panthotenic acid is required by many strains of fermentation yeast and is an essential factor in carbohydrate and lipid metabolism and in cell membrane function. Panthotenic acid deficiencies can lead to the accumulation of hydrogen sulfide. Thiamine is essential in oxoacid decarboxylation. Inositol is required for cell division; deficiencies will decrease the rate of carbohydrate metabolism.

Minerals
Yeasts are unable to grow unless provided with a number of minerals. These include phosphate, potassium, calcium, magnesium, sulfur, and trace elements. Phosphate is involved in energy conservation, is necessary for rapid yeast growth, and is part of many organic compounds in the yeast cell. Potassium ions are necessary for the uptake of phosphate. Calcium improves the flocculation properties of yeast and should be present in concentration greater than 50 mg/l (Henson and Reid, 1987). Magnesium is required for yeast growth and acts as an enzyme activator. Yeast requires sulfur for the synthesis of methionine and for cycteine, which is incorporated into protein, glutathione, coenzyme A, and thiamin. The elements zinc, copper, and manganese are required in trace amounts.

Optimizing Yeast Nutrient Ratios for Ultra High Gravity Fermentations
Similar to wet mill steeping processes which produce value added nutrients for optimizing fermentations, SEAA and E-Mill fractionation and enzymatic milling processes provide for substantially improving fermentation efficiency. "Custom yeast nutrient supplements provide yeast with the correct nutrient balance for growth and reproduction, and maintain specific yeast

strains in their rapid growth phase through more of the fermentation. Higher concentrations of more actively growing yeast produce alcohol faster, consume sugars more efficiently, and leave less residual sugar at the end of fermentation. Improved yeast health results in higher yeast counts, faster fermentations, lower glycerol production, and high alcohol yields (Lallemand Ethanol Technologies, 2008)."

"The fact that growing yeasts produce alcohol much faster than non-growing (resting) yeasts is probably because yeast cells do not take up and ferment sugars unless there is a need for energy. Non-growing (resting) cells ferment only enough sugar to produce energy for cell maintenance. As energy production, growth and ethanol production are tightly coupled, all efforts therefore should be made to keep yeast under conditions which do not lead to low growth rates or to death. Attention to the nutritive status of mash will prevent premature termination of fermentation. This is especially important when high carbohydrate (high gravity) fermentations are carried out. Whereas VHG [and UHG] technology may lead to production of alcohol at lower cost, ignorance of the role of assimilable nitrogen and the need for oxygen or 'oxygenated growth factors' for sterol and unsaturated fatty acid synthesis would compromise this scientific advance, leading to incomplete or inadequate fermentations. It is too late to provide nutrients or new yeast after fermentations become stuck or sluggish (Ingledew, 1999, pp. 68-9)."

As long as an optimal fermentation environment is provided, maximum ethanol yields can be obtained by optimizing nutrient ratios for actively growing yeast. Similar to plant physiology and nutrient requirements, as one yeast nutrient is increased, it has a synergistic affect on yeast requirements for other nutrients. Achieving an optimal balance of nutrients for ultra high gravity (UHG) fermentations is accomplished by analyzing the nutrients contained in the corn steeping/soaking and protease incubation processes utilized by E-Mill technology. Conventional corn steep liquor contains a valuable source of free amino acids which are available to yeast, and other essential nutrients required for optimizing yeast vitality and metabolism which subsequently increases ethanol yields (Ingledew, 1999, pp. 53-55).

Table 4. Proximate composition (g/100 g dry weight) of corn steep powder made from corn steep liquor and corresponding composition of slurry produced from lignocellulosic biomass via SEAA and from corn via E-Mill fractionation and enzymatic milling processes.[a]

	SEAA Wet Fractionation Dry Grind Process	E-Mill Wet Fractionation Dry Grind Process	Conventional Wet Mill Corn Steeping Process	
			Traders[b]	Marcor[b]
Dry matter (DM)			95	
Nitrogen[c]			7.5-7.7	
Protein			47-48	
Fat			0.4	
Carbohydrate			0	
Fiber			0	
Ash			17	
Calcium			0.06	0.3
Magnesium			1.5	1.5
Phosphorus			3.3	3.9
Available Phosphorus			1.1	

Potassium	4.5	5.2
Sulfur	0.58	
Biotin	0	
Chloline	0.00056	
Niacin	0.000016	
Pantothenate	0.000003	
Pyridoxine	0.000002	
Riboflavin	0.000001	
Thiamin	0.000001	
Arginine[d]	3.3	2.3
Cystine[d]	1.9	1.7
Glycine[d]	4.5-5.1	2.3
Histidine[d]	2.8	1.6
Lysine[d]	2.5-3.4	1.7
Methionine[d]	1.9-2.1	1.1
Phenylalanine[d]	3.2-4.4	1.7
Threonine[d]	3.7-4.0	1.9
Tryptophan[d]	0-0.2	0.1
Tyrosine[d]	2.2-3.4	1.1
Valine[d]	4.7-5.8	2.6
Alanine[d]	7.4	3.7
Aspartic acid[d]	5.7	2.9
Glutamic acid[d]	13.9	7.3
Proline[d]	7.8	4.2
Serine[d]	4.1	2.2
Leucine[d]	8.2-11.3	4.2
Isoleucine[d]	2.8-3.6	1.6

[a]Trader's Protein, Memphis, TN, and Marcor Development Corp., Hackensack NJ.

[b]Reproducibility of results and methods used are unknown.
[c]Total crude protein x 0.16 (a typical protein contains 16% N).
[d]Amino acids expressed as % of crude total protein (after hydrolysis), not as free amino acids.

(Adapted from Ingledew, 1999, p. 54)

Table 5. Free amino acid composition of steep powder for SEAA from lignocellulosic biomass and from corn for E-Mill and Wet Mill steeping processes.*

	SEAA Process		E-Mill Process		Wet Mill Process	
Component	µmoles/ g powder	g amino acid/ g powder	µmoles/ g powder	g amino acid/ g powder	µmoles/ g powder	g amino acid/ g powder
Arginine					11.04	.00198
Cystine					4.68	.00112
Glycine + asparagine[a]					44.43	.00334
Histidine					0.32	.00005

Lysine	9.89	.00145
Methionine	17.94	.00268
Phenylalanine	36.06	.00596
Threonine	32.41	.00386
Tryptophan	97.58	.01929
Tyrosine	33.21	.00602
Valine	60.57	.00710
Alanine	193.78	.01726
Aspartic acid	7.21	.00096
Glutamic acid	42.78	.00067
Proline	122.83	.01414
Serine	41.85	.00440
Leucine	112.10	.01470
Isoleucine	28.97	.00380
Total		.1150

*Thomas and Ingledew, unpublished data.
[a] Calculated from unresolved peak as glycine.
(Adapted from Ingledew, 1999, p. 55)

The use of protease enzymes and improved enzymatic processing provided by the E-Mill process enhances the quality and availability of free amino acids for enhancing yeast nutrition similar to corn steep liquor. Utilizing enriched corn slurries is followed by complimenting this nutrient stream with supplements designed to provide an optimal balance of essential nutrients for actively growing yeast.

In 2007, Bellissimi and Ingledew created a new yeast food that is now in the process of being made commercially available. It allows faster fermentations to occur over and above the rates normally seen, and the overall fermentation times are reduced significantly. The yeast food works under both normal gravity and VHG conditions. Although the composition of this yeast food is not published, concentrations of the ingredients were determined by experiments that optimized each ingredient in relation to the others. The concentrations of each ingredient seem at first glance to be high, but were based on the fact that in a 950,000 L fermentor, over 10,000 lbs of new yeast are formed. The nutrition is added to increase the rate of growth of these yeasts and the number of them such that the fermentation will carry on long enough to convert very large amounts of produced glucose to ethanol and carbon dioxide.

Similar custom yeast food supplements for the E-Mill process, which utilize UHG fermentations consisting of up to 600 g/L solids, will allow for optimizing functioning for actively growing yeast. This will result in maximizing ethanol yields. The rate of glucose production and optimizing subsequent sugar concentrations should be considered in conjunction with development of optimal yeast nutrient supplements. Some nutrients may also increase yeast tolerance to temperature and ethanol concentrations. The following is a commercial example of a yeast nutrient supplement formulated for typical VHG fermentations consisting of around 300 g/L solids.

AYF 1000™ is a yeast nutrient for use in fuel ethanol fermentations. It contains a proprietary blend of enzymes, inorganic nitrogen, organic nitrogen, and trace minerals to provide yeast with

the correct nutrient balance for growth and reproduction. AYF 1000 increases yeast budding and maintains yeast in their rapid growth phase through more of the fermentation. Higher concentrations of more actively growing yeast produce alcohol faster, consume sugars more efficiently, and leave less residual sugar at the end of the fermentation. AYF 1000 has no adverse effects of byproducts and may improve their production and composition by reducing residual sugars (Lallemand Ethanol Technologies, 2008)."

Supplying Oxygen to Growing Yeast

Small amounts of oxygen, usually in the form of air, should be used as a yeast nutrient during anaerobic fermentation. The reason for this is that stored saturated fatty acids in yeast cells need to be regenerated into unsaturated fatty acids and sterols to create strong membranes in both mother and daughter yeasts. This allows yeast to efficiently reproduce.

"Small volumes of oxygen are needed in order to supply approximately 8-20 ppm of O_2 to the mash as yeasts grow, but this does not lead to aerobic growth. Air addition can be carried out by "rousing" the cooled mash to give 8-20 ppm O_2 using air or bottled oxygen or helped by "splash filling" of the fermentor usually just prior to adding yeast. The recommended rate is ~ 20 ppm over 3-4 hours when the yeasts are actively growing. This amount of oxygen, if used aerobically, would only be enough to allow respiration of 0.008 w/v% glucose. It therefore has no effect on sugar utilization; and the oxygen is probably all used anaerobically due to the excessive amounts of glucose present in the mash. Oxygen will remain in the fermentor headspace until used or until it is replaced by metabolically-produced CO_2. Oxygenation at the above low levels is a useful practice and has been carried out for many years by the brewing industry. Laboratory research reveals that the most effective time to ensure oxygen availability is when the yeasts begin to actively grow (O'Connor-Cox and Ingledew, 1990; Yokoyama and Ingledew, 1997).

Yeasts require small amounts of oxygen to synthesize membrane sterols and unsaturated fatty acids they are unable to make in the complete absence of oxygen (Kirsop, 1982). In addition to the synthesis of sterols and unsaturated fatty acids, oxygen is required for a number of hitherto poorly understood functions (Thomas et al., 1998). When oxygen is unavailable, available sterols and unsaturated fatty acids are partitioned between mother and daughter yeast cells to ensure cell membrane integrity. In time, these compounds are diluted below a threshold value required for growth. Cell multiplication ceases, therefore reducing ability of the cells to mediate or complete fermentation and making them more susceptible to the wide range of external stresses (osmotic stress, temperature, ethanol, salts, acids, sugars) shown in Figure 5. It is not yet known if these ions and chemical stress agents act synergistically when more than one is present (Ingledew, 1999, pp.56-7)." However, as with livestock and other organisms, it certainly appears that increasing the stress factor for one aspect of yeast functioning would have a domino effect which would result in increasing stress factors for other aspects of yeast functioning. An example is the affect that increasing fermentor temperature has on tolerance to ethanol concentration.

An airlift fermentor capability could provide oxygen to yeast as well as provide agitation for releasing CO_2 during fermentation. Heat exchangers could also provide agitation/mixing action without utilizing airlift capability for fermentors. Excess CO_2 which is not immediately released is known to inhibit growing yeast. These improvements designed to optimize the environment of yeast could offset the relatively large increase in solids concentrations resulting from utilizing E-Mill technology (removal of non-fermentable portions of substrates) in commercial settings which would normally increase fermentation times.

Optimizing Fermentation Temperature

Increasing temperature is a major stress factor during fermentation for actively growing yeast. As the alcohol level increases, the fermentation temperature becomes increasingly stressful for growing yeast. As the fermentation rate naturally declines, the temperature is reduced. As a result of synergistic stress factors, as a normal and primarily stress free fermentation proceeds, ethanol increases and at about 10-13 v/v% it reduces further yeast growth. When this happens, the high temperature ranges utilized to produce ethanol become increasingly stressful to yeast. Poet Energy has successfully used temperature staging to decrease fermentation temperature and subsequently increase ethanol yields. Though this is not usually economical to maintain fermentation temperatures at 25 °C during the entire fermentation (as yeasts produce a good deal of heat during fermentation), staging provides a more economical approach to regulating fermentor temperatures.

Accurately regulating fermentation temperatures in conjunction with ethanol concentrations (temperature staging) could allow for reducing yeast stress and substantially increasing ethanol yields. Typically, initial fermentation temperatures begin around 34.44 °C (94 °F). In order to prolong the active conversion of sugars to ethanol by growing yeast (conversion rates typically drop after 20 hours into the fermentation), beginning at 18 hours into the fermentation the temperature could be ramped down to 29.44 °C (85 °F) by the 26th hour. Another option would be to maintain the fermentor temperature between 25-27 °C via thermally integrated heat exchangers at little or no additional cost other than the initial capital investment (Jones and Ingledew, 1994). The batch could then be terminated at about hour 27 and a new batch would begin after a thorough CIP (Kelsall, 2008).

Fermentation in most plants today takes place coincidently with the use of the industrial enzyme, glucoamylase. It is hypothesized that if glucose is produced quickly enough via enzymatic hydrolysis and SSF, the fermentation process could be speeded up by reducing fermentation temperatures as ethanol concentration increased. For less than 30 hour fermentations, this would require lower than normal solids and nearly perfect yeast nutrition. Actively fermenting yeast will reduce the volume of fermentation products such as diacetyl and acetaldehyde by more efficiently completing fermentations. In order to prevent bacterial contamination, improved sanitation and thorough cleaning between fermentation batches is essential, particularly for the kraeusening procedure and recycling relatively large volumes of thin stillage as process water in order to avoid continual build up of organic acids (Kelsall, 2008).

UHG Fermentor Temperature Staging

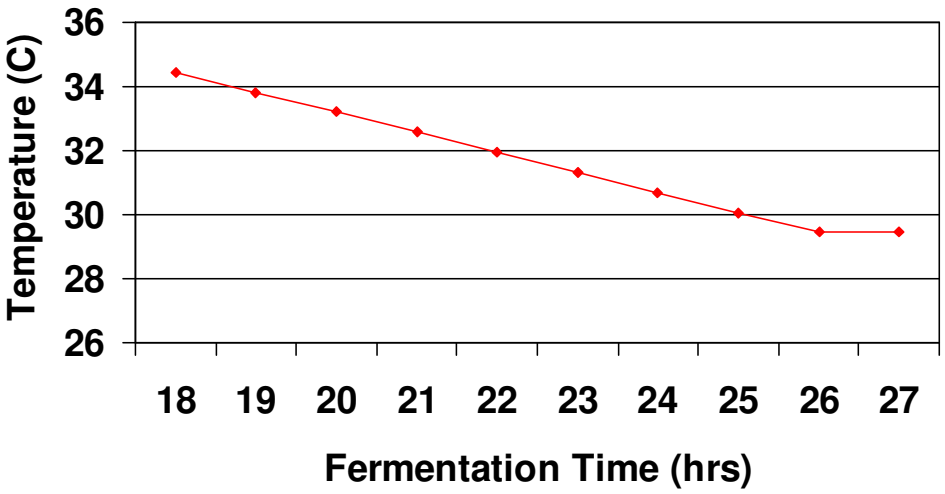


Figure 6. Reducing temperature stress for growing yeast.

Regulating fermentor temperature between 29-35 °C in conjunction with ethanol concentrations, maintaining pH between 3.0-4.0, and providing adequate oxygen will maximize production of actively growing yeast. Growing yeast produce ethanol and CO_2 33-35 times faster than mature yeast (Kirsop, 1982). By keeping cells longer in the growth phase, more carbohydrate is efficiently converted to alcohol, fermentation rates are maintained, and the ferments are more predictably completed (Ingledew, 1999). Thus, this scenario further illustrates how actively growing yeast will out compete bacteria, resulting in relatively high ethanol yields and low levels of organic acid production. Maintaining a relatively low pH between 3.0-3.5 can also reduce organic acid production by inhibiting growth of bacteria.

Inoculation via Kraeusening

"An alternate and effective method to increase fermentation rate is to increase the inoculation level of fermentations. When a very large number of cells in the range of 8 x 10^7 to 7 x 10^8/mL (O'Connor-Cox and Ingledew, 1991; Thomas and Ingledew, 1992a) are used, a vast amount of sugar is fermented even though little to no net growth is observed. In fact, the slower fermentation rate of these non-growing (resting) cells as carried out for cell maintenance purposes approaches the rate achieved at more normal yeasting levels (5 x 106/mL per ° Plato) where the smaller number of cells are actively growing and metabolizing at the much faster rate described by Kirsop (1982). For this reason, very high inoculation rates will often overcome stuck and sluggish fermentations and obviate the need for increased assimilable nitrogen (Ingledew, 1999, p.81)."

In addition to utilizing high producing yeast strains, kraeusening can be utilized as a method of yeast inoculation at about 18 hours into the fermentation and subsequently starting new batches about every 27 hours. This will help maintain an optimal level of yeast concentration and vitality. This results in maximizing conversion efficiencies and further reduces fermentation times by capitalizing on rapid production of ethanol and CO_2. Kraeusening, protease enzymes, supplementing with about 400 ppm urea or ammonia along with other essential nutrients for balancing yeast nutrition, smaller particle size and ultrasonic cavitation will offset longer fermentation times normally required for VHG and UHG fermentations.

A portion of unfermented sugars, nutrients, and enzymes remaining in the fermented mash after ending the batch at 27 hours, can be added to subsequent fermentation batches as backset for recycling process water and conversion of remaining sugars to ethanol. When actively fermenting beer is added to relatively new batch fermentations in addition to separate doses of mash and yeast, it's called "kraeusening" (occasionally spelled “kreusening”). This is accomplished by taking beer with actively fermenting yeast from other fermentors and inserting it directly into the mash/fermented mash, with some aeration, at about 18 hours into the fermentation cycle. No additional yeast propagation is required. As indicated above, this enhances the conversion rate of sugars to ethanol by increasing yeast population and vitality, thus reducing fermentation times.

Kraeusening has been utilized to enhance fermentation and beer quality for hundreds of years in the brewing industry. Kraeusening is routinely used by the brewing industry to induce a secondary fermentation into the nearly fermented beer. It adds condition and sparkle to a fermented beverage. In fuel alcohol production, Kraeusening gives the primary fermentation a boost since the primary fermentation will often begin to slow down at about the 20 hour stage. The introduction of newly fermenting yeast at 18 hours will spur the primary fermentation and keep the yeast dominant over bacteria.

Processing Corn Stover Biomass

Starch obtained from corn, also referred to as maize (*Zea mays* L. ssp. *mays*), is currently the predominant feedstock for producing ethanol in the US. However, less than half of total plant biomass is currently being utilized in the food and biorefining industries. In addition to processing starch in corn grain, processing corn stover (stalks, leaves and cobs) and corn kernel fiber (both pericarp and endosperm fiber) can almost double ethanol yields per tonne of corn biomass produced. This increase in ethanol production is possible by harvesting a portion of corn stover biomass from crops utilized directly for food (sweet corn); indirectly for food (animal production, nutraceuticals); and for biofuels (yellow dent corn) via production of ethanol and distiller’ grains (DGs) coproducts.

Nutrient-rich DGs, which are utilized as value added animal feedstuffs, are actually higher in nutrients than whole corn. Animal production using DG coproducts provides quality protein food products including beef, dairy, swine and poultry products in addition to biomethane, organic fertilizers and carbon dioxide (CO_2) processed from animal waste. This allows for closed loop production at integrated biorefineries which provides unprecedented efficiencies of operation, e.g. ushering in a revolutionary paradigm shift to distributed bioeconomies in which wholesome functional foods, nutraceuticals, pharmaceuticals, bioproducts (including bioplastics), biochemicals and bioenergy (including biofuels and bioelectricity) are produced simultaneously. This concept invalidates the food vs. fuel argument and illustrates how production of food and fuel are synergistic via integrated biorefineries which incorporate closed loop architecture.

According to US Department of Energy studies conducted by the Argonne Laboratories of the University of Chicago, one of the benefits of cellulosic ethanol is that it reduces greenhouse gas emissions (GHGEs) by 85% in comparison with reformulated gasoline. In addition to reducing GHGEs, recycling these valuable resources dramatically enhances industrial production, increases the efficiency of renewable energy technologies, and replenishes oxygen to the atmosphere via incorporation of closed loop biomass.

The “well to wheel” analysis referred to above reveals that conventional corn ethanol provides 20-30% reductions in GHGEs compared with gasoline. Cellulosic ethanol's favorable profile stems from using lignin, a coproduct used to either fuel the biomass conversion process or provide valuable biochemicals for production of bioplastics which displace petroleum products. Lignin is a renewable fuel with no net greenhouse gas emissions. Cellulosic biomass-based ethanol reduces fossil fuel use by 90% (Wang, 2005). A similar reduction in GHGEs for corn ethanol production is achieved by feeding nutrient-rich ethanol coproducts (DGs and fermentation residues) to animals. Animal waste is then processed into biomethane and organic fertilizers which displace natural gas and petroleum products. For integrated biorefineries which incorporate closed loop biomass and biorefinery production, pollution and GHGEs are essentially eliminated via efficient processing of corn stover and other second generation lignocellulosic feedstocks including an abundance of crop residues and organic waste. Third generation algal biomass feedstocks can be produced on site via controlled environment agriculture (CEA) and processed in similar fashion via integrated biorefineries. Vertically integrated production of feedstocks and bioenergy via integrated biorefineries and closed loop systems architecture is the best risk management strategy available.

With almost 86 million acres planted in corn in 2008, stover is currently the most abundant source of lignocellulosic biomass in the US and has been recognized as the most promising source of biomass for the bioenergy industry in the near-term. Harvesting 75% of corn stover associated with 200 bu/acre yields (approximately 50% corn and 50% stover as harvested) could result in processing 328 million tonnes of stover into 27.5 billion gallons per year (BGY). The actual grain-to-stover ratio for corn may vary up to 57.5:42.5% dry matter (DM). The above numbers are based on processing 50% of total corn biomass as stover DM, providing an ethanol yield of 84 gal/tonne of stover (67% conversion efficiency). At 100 gal/tonne of corn stover (80% conversion efficiency) for precision agriculture and mature biorefining, US ethanol production could increase to almost 66 BGY.

The goal of the ethanol industry is too eventually reach 95% conversion efficiency, which would provide an average yield of 119 gal per tonne of biomass. The US government target (Renewable Fuel Standard II) for the year 2022 is 35 BGY, 20 BGY of which is to be produced from non-food feedstocks. As illustrated from the above numbers, this can be most efficiently accomplished by producing ethanol from crop residues, e.g. non-food portions of crops such as corn stover, wheat/cereal straw (lignocellulosic feedstocks), other agricultural byproducts, municipal and industrial solid waste, sludge from paper manufacturing, and food processing waste. However, a portion of crop residues and organic waste must be utilized as organic fertilizers to replenish soil organic matter (SOM). This is necessary in order to maintain soil fertility and provide sustainable agriculture for bioenergy economies. This is accomplished via closed loop architecture which converts waste resources into value added bioproducts.

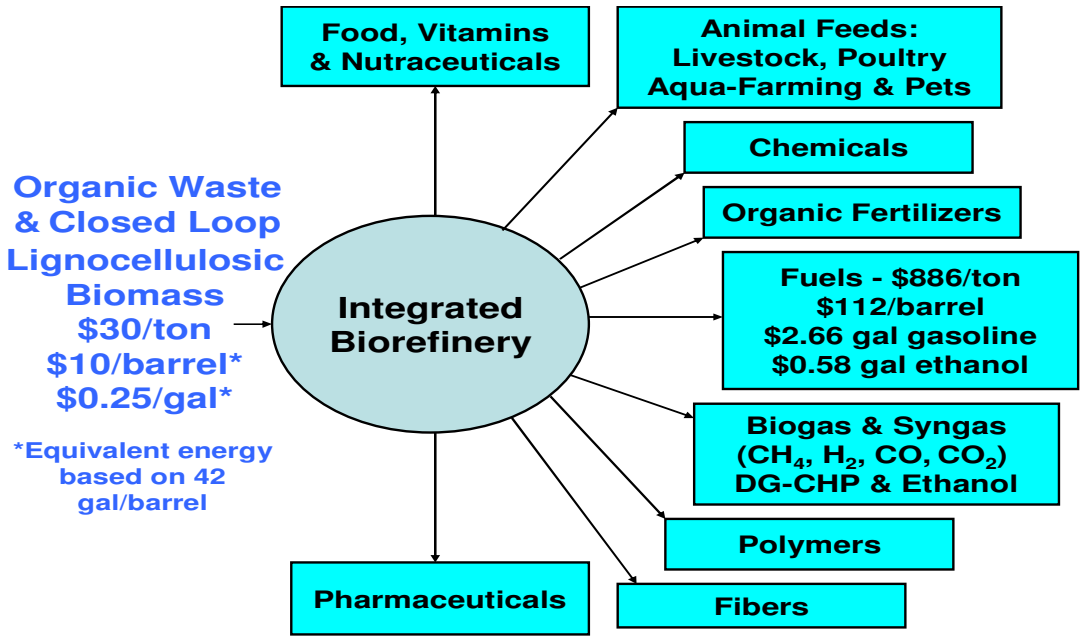


Figure 7. Integrated Biorefinery Providing Diversification, Flexibility & Sustainability

Derivative chemicals found in lignocellulosic biomass -- such as furfural and low molecular weight lignin -- give birth to new high value derivatives which can be utilized in food, medical and industrial manufacturing. For example, lignin derivatives can be utilized to produce high value bioplastics, and one of the biochemicals used to produce furfural can be used in the construction of artificial bones that the human body is unlikely to reject in the medical field. The biochemical derived from furfural retails for $1,000/lb, as opposed to ethanol which retails for about $0.20/lb. Similarly, omega-3 fatty acids obtained from algal biomass retail for over $4,500/gal ($600/lb). This is in comparison with biodiesel which retails for about $3-$4/gal ($0.40-$0.53/lb). The nutraceutical astaxanthin, a super antioxidant also obtained from potentially producing and processing green microalga *Haematococcus pluvialis* biomass or the red yeast *Phaffia rhodozyma* (Nghiem et al., 2009) at integrated biorefineries retails for over $1,700/lb. Production of omega-3s and astaxanthin (nutraceutical and pharmaceutical grade products) represent an exponential increase (150-850 orders of magnitude) in value in comparison with liquid biofuels. Obviously, there are strong economic and environmental advantages for integrating production of high value foods and other coproducts with production of commodity biofuels, all of which displace petroleum products and reduce economic dependence on fossil fuels. This is accomplished via integrated biorefineries which simultaneously reduce pollution and GHGEs.

Comprehensive nutrient-energy management via integrated biorefineries allows for comfortably reaching the US mandate for biofuels while simultaneously producing high quality foods and bioproducts including functional foods (aquaculture, poultry eggs, swine, beef and dairy products enriched with nutraceuticals), vitamins, nutraceuticals, pharmaceuticals, organic fertilizers, adhesives, surfactants, and bioplastics for displacing petroleum products, all of which are biodegradable. This is possible through development of economical separation technologies and biorefining platforms which are very similar to those employed at petroleum refineries, but which process an abundance of renewable agricultural byproducts and organic waste instead of limited supplies of crude oil.

In order for production of biofuels to be sustainable, high value functional foods, nutraceuticals, pharmaceuticals, and other high value coproducts must be simultaneously produced in order to offset costs of producing feedstocks and biorefining. In addition to providing diversification and flexibility for maximizing revenue streams, closed loop production at integrated biorefineries allows for achieving unprecedented efficiencies of operation which are not subject to the volatility of commodity markets, particularly regarding feedstocks and energy resources which comprise over 90% of production costs.

Producing High Value Added Coproducts via Integrated Biorefineries & Synthetic Biology

Astaxanthin

"Fuel ethanol is currently produced by either a dry-grind process or a wet-mill process. In both of these processes, the values added from the co-products are extremely important for sustainability. Without these co-products, fuel ethanol production is not economically viable.

Astaxanthin is one of the potentially new co-products of fuel ethanol production. This carotenoid probably is best known for its role in giving the flesh of salmonoids, shrimps, lobsters and crayfish the pinkish-red hue. In the marine environment, astaxanthin is acquired through ingestion of microalgae and phytoplankton, which are natural astaxanthin producers. However, since salmonids are unable to synthesize astaxanthin, the farm-raised fish need to be fed this carotenoid through their artificial diets.

Astaxanthin is a high-value specialty product. The selling price for astaxanthin in 2000 was estimated at ~$2,500/kg and that of 10% astaxanthin feed formulas in 2007 was listed at $250/kg. The world market for astaxanthin was predicted to reach over $250 million in 2009. Recently astaxanthin was discovered to provide many human health benefits. These discoveries could lead to development of nutraceutical applications and significant expansion of the market for astaxanthin.

Currently astaxanthin is produced commercially by either chemical synthesis or microbial fermentation. The market has been heavily dominated by the synthetic product mainly due to its much lower production cost. However, growing demand for products from natural sources may open up the market to astaxanthin produced by biological processes. There are only two microbial astaxanthin sources that may be able to compete economically with synthetic astaxanthin. These are the green microalga *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma.* The yeast *P. rhodozyma* is of particular interest since several strains can utilize various sugars, including glucose, xylose and arabinose, for growth and astaxanthin synthesis. Thus, *P. rhodozyma* can be used for astaxanthin production using fermentable sugars obtained from hydrolysis of lignocellulosic biomass as carbon sources.

Research has been conducted to develop a process for production of astaxanthin as a high value-added coproduct of corn and stover based fuel ethanol. Several strains of *P. rhodozyma* have been screened for their capability of utilizing fermentable sugars obtained from corn fiber (a ligno cellulosic byproduct from fuel ethanol production from corn) for astaxanthin production.

From the data obtained production of astaxathin by fermentation of ethanol byproducts appears to be very promising.

Five strains of the yeast *Phaffia rhodozyma*, NRRL Y-17268, NRRL Y-17270, ATCC 96594 (CBS 6938), ATCC 24202 (UCD 67-210), and ATCC 74219 (UBV-AX2) were tested for astaxanthin production using the major sugars derived from corn fiber (which could include stover), a byproduct from the wet milling of corn kernels that contains primarily cellulose and hemicellulosic polysaccharides. The sugars tested included glucose, xylose and arabinose. All five strains were able to utilize the three sugars for astaxanthin production.

Among them ATCC 74219 was the best astaxanthin producer. Kinetics of sugar utilization of this strain was studied, both with the individual sugars and with their mixtures. Arabinose was found to give the highest astaxanthin yield. It also was observed that glucose at high concentrations suppressed utilization of the other two sugars. Corn fiber hydrolysate obtained by dilute sulfuric acid pretreatment and subsequent enzyme hydrolysis was tested for astaxanthin production by strain ATCC 74219. Dilution of the hydrolysate was necessary to relieve the inhibition of the compounds formed during the acid pretreatment. All the sugars in the hydrolysate diluted with two volumes of water were completely consumed. Astaxanthin yield of 0.82 mg/g sugars consumed was observed." (Nghiem et al., 2009)

Artemisinin Combination Therapies (ACTs)

"Artemisinin derivatives are the key active ingredients in Artemisinin combination therapies (ACTs), the most effective therapies available for treatment of malaria. Because the raw material is extracted from plants with long growing seasons, artemisinin is often in short supply, and fermentation would be an attractive alternative production method to supplement the plant source. Previous work showed that high levels of amorpha-4,11-diene, an artemisinin precursor, can be made in Escherichia coli using a heterologous mevalonate pathway derived from yeast (Saccharomyces cerevisiae), though the reconstructed mevalonate pathway was limited at a particular enzymatic step.

By combining improvements in the heterologous mevalonate pathway with a superior fermentation process, commercially relevant titers were achieved in fed-batch fermentations. Yeast genes for HMG-CoA synthase and HMG-CoA reductase (the second and third enzymes in the pathway) were replaced with equivalent genes from Staphylococcus aureus, more than doubling production. Amorpha-4,11-diene titers were further increased by optimizing nitrogen delivery in the fermentation process. Successful cultivation of the improved strain under carbon and nitrogen restriction consistently yielded 90 g/L dry cell weight and an average titer of 27.4 g/L amorpha-4,11-diene.

Production of >25 g/L amorpha-4,11-diene by glucose fermentations followed by chemical conversion to artemisinin may allow for development of a process to provide an economical alternative for production of artemisinin to be incorporated into ACTs." (Tsuruta et al., 2009; Amyris)

Carbohydrate & Polymer Products

It is estimated that up to $250 billion of the $1 trillion global commodity, specialty, and fine chemical markets are either readily or potentially addressable by bioproduction processes. BioEnergy International is developing technologies for the next-generation of integrated biorefineries to produce many of these biochemicals. BioEnergy's unique patented

microorganisms and proprietary process technology are envisioned to transform the current hydrocarbon-based manufacturing processes across a variety of industries enabling the replacement of a barrel of crude with plant-based sugars to make everything from plastics and fabrics to transportation fuels.

Discoveries include butanol, butanediol, propanediol, adipic acid, HPA, succinic acid, and D (-) lactic acid. Production of D (-) lactic acid has already been commercialized and commercialization of succinic acid is currently under development for use in the bioplastics and other industries. Thus, BioEnergy is a good example of how private companies, universities, and federal research labs in the US are collaborating and becoming global leaders in the development and commercialization of next generation biorefineries for the production of high-value bio-based chemicals and fuels from renewable feedstock through the use of biocatalyst technology. The following are biochemicals which can currently be produced from lignocellulosic feedstocks.

Ethylene: Ethanol can replace fossil fuels in the production of ethylene, a basic chemical feedstock for making many types of plastics. From a plastics point of view, there is a growing appetite for sustainable polymers. Ethylene (IUPAC name: ethene) is the chemical compound with the formula C_2H_4. It is the simplest alkene. Because it contains a carbon-carbon double bond, ethylene is called an unsaturated hydrocarbon or an olefin. It is extremely important in industry and also has a role in biology as a hormone. Ethylene is the most produced organic compound in the world; global production of ethylene exceeded 107 million metric tonnes in 2005. To meet the ever increasing demand for ethylene, sharp increases in production facilities have been added globally, particularly in the Gulf countries. Currently the world's largest plant is situated in Asalouyeh, Iran. Jam Petrochemical Plant came online in 2008, with an annual production capacity of 1,321,000 tons, which is to be increased to 4.2 million in few years. (Ethylene, Wikipedia, 2009)

Xylitol: An organic compound with the formula $(CHOH)_3(CH_2OH)_2$. This achiral species is one of four isomers of 1,2,3,4,5-pentapentanol. This sugar alcohol is used as a naturally occurring sugar substitute found in the fibers of many fruits and vegetables, including various berries, corn husks, oats, and mushrooms. It can be extracted from corn fiber, birch, and corn. A hydrolyzed lignocellulose-containing material can be used as a starting material which is fermented with a yeast strain. The ethanol is recovered and a chromatographic separation is carried out on the fermented solution to obtain pure xylitol (United States Patent 6846657).

Xylitol is roughly as sweet as sucrose with only two-thirds the food energy. It is a sugar alcohol utilized in weight-loss products and multi-vitamin products. It does not contain any carbohydrates. It is useful as a sugar substitute for people with diabetes because it does not cause the significant rise in blood glucose (blood sugar) caused by real sugar. Nor does it seem to affect insulin levels in humans.

Xylitol may prevent dental cavities in several ways. It seems to decrease the level of certain cavity-causing bacteria in the saliva and dental plaque. It also decreases the acid production by the bacteria, decreases the stickiness of the bacteria, increases saliva flow, and increases calcium and phosphate in the saliva. All these actions can help to prevent cavities. Interestingly, habitually chewing gum with this sugar substitute can help mothers reduce the chance of transmitting cavity-causing bacteria to their young infants.

Xylitol may also inhibit bacterial growth (since most bacteria cannot use it as a source of energy), which might be why it may be useful for preventing ear infections (eMedTV).

***Furfural**:* When heated with sulfuric acid, hemicellulose undergoes hydrolysis to yield sugars, principally xylose. Under the same conditions of heat and acid, xylose and other five carbon sugars undergo dehydration, losing three water molecules to become furfural:

$$C_5H_{10}O_5 \rightarrow C_5H_4O_2 + 3\ H_2O$$

For crop residue feedstocks such as corn stover, about 10% of the mass of the original plant matter can be recovered as furfural. Furfural and water evaporate together from the reaction mixture, and separate upon condensation.

Derivative chemicals found in cellulosic biomass -- such as furfural -- give birth to new derivatives. One of these ancestors of furfural can be used in the construction of artificial bones that the recipient's body is unlikely to reject. That sells for $1,000 a pound, as opposed to ethanol, that sells for about 18 to 20 cents a pound.

Global total capacity of production is about 450,000 ton. China is the biggest supplier of this product and they have about a half of global capacity. China is currently the world's largest producer of furfural and there is "no significant furfural production" within the United States or European Union. Pure Energy has entered into partnerships with companies that use furfural, indicating that there are already buyers for this product. The planned process creates furfural at a 2-1 ratio of ethanol to furfural (Rands, 2009).

In the laboratory, synthesis of furfural from corn cobs takes place by reflux with dilute sulfuric acid. Furfural is used as a solvent in petrochemical refining to extract dienes (which are used to make synthetic rubber) from other hydrocarbons.

Furfural, as well as its derivative furfuryl alcohol, can be used either by themselves or together with phenol, acetone, or urea to make solid resins. Such resins are used in making fiberglass, some aircraft components, and automotive brakes.

Furfural is also used as a chemical intermediate in the production of the solvents furan and tetrahydrofuran.

HMF: Hydroxymethylfurfural has been identified in a wide variety of heat processed foods. Scientists with the U.S. DOE's Pacific Northwest National Laboratory have discovered a way to convert cellulose to 5-hydroxymethylfurfural (HMF) in one step using ionic liquid and chloride catalysts under low temperatures.

HMF, also 5-(Hydroxymethyl)furfural, is an organic compound derived from dehydration of sugars. This colorless solid is highly water soluble. The molecule is a derivative of furan containing both aldehyde and alcohol functional groups. HMF has been identified in a wide variety of heat-processed foods including milk, fruit juices, spirits, honey, etc. HMF, which is derived from cellulose without use of fermentation, is a potential "carbon-neutral" feedstock for fuels and chemicals.

HMF is an important component of biofuels, plastics and other materials. The team has researched the process for about three years and discovered that copper and chromium chlorides dissolved in the ionic solvent (1-ethyl-3-methylimidazolium chloride) at temperatures of 80 to 120 degrees Celsius (176 to 248 degrees Fahrenheit) breaks down the cellulose much faster and without many of the unwanted byproducts usually associated with the conversion. PNNL's process is 10 times faster than using acid and does not require mineral acid known to degrade the HMF, according to David King of Seattle PNNL.

The team recovered more than 90% of the HMF formed, which was 96% pure, and discovered that it could consistently achieve a high yield, as the method converted about 57% of the sugar content in the cellulose feedstock. In addition, the ionic liquid and chlorides can be reused without losing effectiveness.

Acetic Acid: Has chemical formula CH_3COOH and is also known as ethanoic acid. It is a byproduct of hydrolysis of hemicellulose and is toxic to yeast ethanolgens. Hence, it must be extracted from hydrolysates prior to fermentation. It is also produced via reactions of hemicellulose with sulfuric acid and by acetic acid bacteria of the genus *Acetobacter* which can be utilized to metabolize sugars in isolated fermentations.

Acetic acid is an organic acid which gives vinegar its sour taste and pungent smell. Pure, water-free acetic acid (*glacial acetic acid*) is a colorless liquid that absorbs water from the environment (hygroscopy), and freezes at 16.7 °C (62 °F) to a colorless crystalline solid. It is a weak acid, in that it is only partially dissociated acid in aqueous solution.

Acetic acid is one of the simplest carboxylic acids. It is an important chemical reagent and industrial chemical, used in the production of polyethylene terephthalate mainly used in soft drink bottles; cellulose acetate, mainly for photographic film; and polyvinyl acetate for wood glue, as well as synthetic fibres and fabrics. In households, diluted acetic acid is often used in descaling agents. In the food industry acetic acid is used under the food additive code E260 as an acidity regulator.

The global demand of acetic acid is around 6.5 million tonnes per year (Mt/a), of which approximately 1.5 Mt/a is met by recycling; the remainder is manufactured from petrochemical feedstocks or from biological sources.

Acetic acid is produced both synthetically and by bacterial fermentation. Today, the biological route accounts for only about 10% of world production, but it remains important for vinegar production, as many nations' food purity laws stipulate that vinegar used in foods must be of biological origin. About 75% of acetic acid made for use in the chemical industry is made by methanol carbonylation. Alternative methods account for the rest. Total worldwide production of virgin acetic acid is estimated at 5 Mt/a (million tonnes per year), approximately half of which is produced in the US. European production stands at approximately 1 Mt/a and is declining. About 0.7 Mt/a is produced in Japan. The two largest producers of virgin acetic acid are Celanese and BP Chemicals. Other major producers include Millennium Chemicals, Sterling Chemicals, Samsung, Eastman, and Svensk Etanolkemi.

Formic acid: Systematically called methanoic acid, it is the simplest carboxylic acid. Its formula is HCOOH or CH_2O_2. It is an important intermediate in chemical synthesis. A significant amount of formic acid is produced as a byproduct in the manufacture of other chemicals, especially acetic acid.

The principal use of formic acid is as a preservative and antibacterial agent in livestock feed. When sprayed on fresh hay or other silage, it arrests certain decay processes and causes the feed to retain its nutritive value longer, and so it is widely used to preserve winter feed for cattle. In the poultry industry, it is sometimes added to feed to kill salmonella bacteria. Other uses:

- It is used to process organic latex (sap) into raw rubber.
- Beekeepers use formic acid as a miticide against the Tracheal (*Acarapis woodi*) mite and the Varroa mite.
- It is of minor importance in the textile industry and for the tanning of leather.
- Some formate esters are artificial flavorings or perfumes.
- It is the active ingredient in some brands of household limescale remover.
- It is used in laboratories as a solvent modifier for HPLC separations of proteins and peptides, especially when the sample is being prepared for mass spectrometry analysis.
- It is used by clinical pathology laboratories to disinfect prion activity in brain samples

In synthetic organic chemistry, formic acid is often used as a source of hydride ion. The Eschweiler-Clarke reaction and the Leuckart-Wallach reaction are examples of this application. It is also used as a source of hydrogen in transfer hydrogenation.

In the laboratory formic acid is also used as source for carbon monoxide, which is set free by the addition of sulfuric acid. Formic acid is also a source for a formyl group for example in the formylation of methylaniline to N-methylformanilide in toluene.

Fuel cells that use modified formic acid are promising.

Levulinc acid: Levulinic acid, or 4-oxopentanoic acid, is a white crystalline keto acid prepared from levulose, inulin, starch, etc., by boiling them with dilute hydrochloric or sulfuric acids. It is soluble in water, ethanol, and diethyl ether, but essentially insoluble in aliphatic hydrocarbons.

Levulinic acid is used in the manufacture of nylons, synthetic rubbers, plastics, and pharmaceuticals. It is a precursor in the industrial production of other chemical commodities such as methyltetrahydrofuran, valerolactone, and ethyl levulinate. As well, levulinic acid is used in cigarettes to increase nicotine delivery in smoke and binding of nicotine to neural receptors.

D(-) lactic acid: Myriant/BioEnergy began commercial production of D(-) lactic acid in June 2008 for use in polylactic acid. D(-) lactic acid solves polylactic acid's thermal stability problem, unlocking the potential of polylactic acid by expanding its applications to engineering and high-performance plastics (BioEnergy International). The bioplastics industry is expected to grow to over $12 billion annually.

Succinic acid: In addition to D(-) lactic acid, Myriant is also developing biobased succinic acid. It is widely in demand at the right price for use as a plastic building block, and as a replacement for petroleum-based chemicals (BioEnergy International).

The same proprietary microbial platform used in the development and commercialization of D(-) lactic acid has yielded the successful development of a biocatalyst to manufacture succinic acid. Succinic acid is an important platform molecule, widely used as the intermediate for the production of numerous everyday consumer products, pharmaceuticals and adhesives, representing a total immediate addressable market in excess of $7.2 billion.

The US Department of Energy (DOE) has identified succinic acid as one of the top 12 chemicals with the largest market potential that can be derived from biomass. Additionally, BioEnergy's proprietary technology can be used to produce five of the remaining top 12 chemicals identified by the DOE. BioEnergy intends to develop a pilot plant for specialty biochemicals production and expects to generate its first commercial-scale production of succinic acid by early 2010.

Adipic acid: an organic compound with the formula $(CH_2)_4(CO_2H)_2$. From the industrial perspective, it is the most important dicarboxylic acid: About 2.5 billion kilograms of this white crystalline powder are produced annually, mainly as a precursor for the production of nylon.

A method has been reported that utilizes principles of green chemistry in that water is the only by product. Cyclohexene is oxidized with hydrogen peroxide using a tungstate -based catalyst and a phase transfer catalyst. The waste product is water.

By far the majority of the 2.5 billion kg of adipic acid produced annually is used as a monomer for the production of nylon by a polycondensation reaction with hexamethylene diamine forming 6,6-nylon. Other major applications also involve polymers: it is a monomer for production of Polyurethane and its esters are plasticizers, especially in PVC.

Small but significant amounts of adipic acid are used as a food ingredient as a flavorant and gelling aid. It is ironically used in some calcium carbonate antacids to make them tart.

HPA: heteropoly acid is a class of acid made up of a particular combination of hydrogen and oxygen with certain metals and non-metals. This type of acid is frequently used as a re-usable acid catalyst in chemical reactions.

Butanol: or butyl alcohol (sometimes also called *biobutanol* when produced biologically), is a primary alcohol with a 4 carbon structure and the molecular formula of C_4H_9OH. It belongs to the higher alcohols and branched-chain alcohols. It is primarily used as a solvent, as an intermediate in chemical synthesis, and as a fuel.

Butanediol: 1,4-Butanediol is used industrially as a solvent and in the manufacture of some types of plastics, elastic fibers and polyurethanes. In organic chemistry, 1,4-butanediol is used for the synthesis of γ-butyrolactone (GBL). In the presence of phosphoric acid and high temperature, it dehydrates to the important solvent tetrahydrofuran. At about 200 °C in the presence of soluble ruthenium catalysts, the diol undergoes dehydrogenation to form butyrolactone.

In its industrial synthesis, acetylene reacts with two equivalents of formaldehyde to form 1,4-butynediol, also known as but-2-yne-1,4-diol. This type of process is illustrative of what is known as Reppe Chemistry. Hydrogenation of 1,4-butynediol gives 1,4-butanediol.

It can also be manufactured on an industrial scale by the vapor phase hydrogenation of the esters and anhydrides of maleic acid and succinic acid.

Genomaticam (a San Diego-based company) has genetically engineered E.Coli to devour sugar and construct 1,4 butanediol. They expect to build and begin operating a pilot plant by the end of 2009. CEO Christopher Gann said it takes 32,000 BTU/per pound to make. This is much less than the process listed above, and does not have any by-products.

World production of 1,4-Butanediol is about one million metric tons per year and market price is about 2,000 USD (1,600 EUR) per ton (2005). Almost half of it is dehydrated to tetrahydrofuran to make fibers such as Spandex. The largest producer is BASF.

Propanediol: mainly used as a building block in the production of polymers. 1,3-Propanediol can be formulated into a variety of industrial products including composites, adhesives, laminates, coatings, moldings, aliphatic polyesters, copolyesters. It is also a solvent and used as an antifreeze and wood paint.

1,3-Propanediol may be chemically synthesized by the hydration of acrolein, or by the hydroformylation of ethylene oxide to afford 3-hydroxypropionaldehyde. The aldehyde is hydrogenated to give 1,3-propanediol.

Two other routes involve bioprocessing by certain micro-organisms:

- Conversion from corn syrup effected by a genetically modified strain of E. coli by Dupont Tate & Lyle Bioproducts (See: bioseparation of 1,3-propanediol). An estimated 120,000 tons were produced in 2007".[2]. According to DuPont, the BioPDO process uses 40% less energy than conventional processes,[3],[4] and greenhouse gas emissions by 20%.[3],[4] Because of Dupont and Tate & Lyle's success in developing a renewable BioPDO process, the American Chemical Society awarded the BioPDO research teams the "2007 Heroes of Chemistry" award [4].

- From glycerol, where the fatty acids are converted by yeasts to long chain dicarboxylic acids and then to propanediol (i.e. using Clostridium diolis bacteria). One of the byproducts obtained from the manufacture of dairy products is acid whey.

Lignin derivatives: High value low molecular weight lignin derivatives have substantial value as reactive chemicals for displacement of petroleum chemicals utilized in production of biodegradable plastics and binders. Lignol Innovations has developed proprietary methods for production of low and very low molecular weight lignin derivatives from lignocellulosic biomass feedstocks which they claim is the most valuable product which can be produced by biorefineries.

In 2008, a German company, Tecnaro, developed a process for turning lignin into a substance, called Arboform, which behaves identically to plastic for injection molding. Therefore, it can be used in place of plastic for several applications. When the item is discarded, it can be burned just like wood.

In Sep 2009, Chevron Technology Ventures in cooperation with Mascoma entered into a two year agreement to explore conversion of lignin coproducts from production of ethanol to hydrocarbon fuels.

Renewable Ammonia & Associated Nitrogen Products

Nitrogen fertilizers are essential for efficient agricultural production of food and energy crops. However, nutrient-use efficiency is low for synthetic fertilizers and organic waste resources such as food waste, animal waste and sewage which provide an abundance of nitrogen, phosphorus, potassium and other essential nutrients required for agricultural production, are currently underutilized. This is resulting in nutrient loading, nutrient leaching and nutrient run-off which contaminate soils, groundwater aquifers and water bodies (Erisman et al., 2008). However, nutrient-use efficiency can be vastly improved by improving waste management and processing organic waste resources into value added organic fertilizers which can displace a large portion of synthetic fertilizers. The recycling process incorporates comprehensive nutrient-energy management which results in dramatically increasing efficiency of agriculture and bioenergy for demonstration of sustainable bioeconomies while simultaneously reducing environmental impacts.

Renewable ammonia production is crucial for closed loop systems architecture at integrated biorefineries and Bioenergy Complexes. High temperature molten carbonate fuel cells utilized at integrated biorefineries are capable of producing over 47,000 tons of H_2 from biomethane via conversion of 60 MGY ethanol plants to 182 MGY Ag-Energy Parks and Bioenergy Complexes. Additional H_2 can be produced from integration of closed loop biomass via CEA and plant MECs. These Bioenergy Complexes can subsequently use H_2 to produce over 263,000 tons of anhydrous ammonia for use in:

- ammonia ensiling of biomass
- aqueous ammonia-ethanol pretreatment-fractionation platforms
- natural/biological disinfectants for biorefineries
- value added free amino nitrogen (FAN) nutrient required for microbial fermentations
- agricultural nitrogen fertilizers and mulitnutrient fertilizers for closed loop functional food and energy crop production
- production of a variety of high value consumer products including artificial fibers (nylon), rare dyes, bioplastics, and explosives, etc.
- direct wholesale and/or retail sales of either H_2, NH_3, or associated coproducts

Because nitrogen is essential to plant and animal health and nitrogen has no substitute, its use will continue to increase as the world's population continues to grow. Data that project the world nitrogen supply-demand balance, which was prepared by the Food and Agriculture Organization of the United Nations (2001), indicated that nitrogen demand will increase, on average, by more than 1.6% through the year 2006, and the potential supply was projected to increase by 1.3 percent per year during the same period. In 2009, annual demand will probably increase to as much as 4% per year through the 2025 crop year as the world produces biomass and transitions to bioeconomies.

Because the nitrogen supply has been in a surplus, the projected increase in demand, which is greater than that for supply, was expected to reduce the surplus somewhat. In 2009, those surpluses now appear to be exhausted. The gains will not be equivalent in all the world regions. Such regions as Asia, North America, and Western Europe were projected to have an internal

deficit in supply, whereas countries from the former U.S.S.R. and Central America and South America would have an internal surplus of supply. This regional supply imbalance will continue the trend of exports from these two regions to provide a significant share of the North American demand. (Kramer, 2004) However, the US and other North America countries simply cannot afford to increasingly become dependent upon exporting ammonia and associated nitrogen products which are responsible for production of food, bioproducts, industrial biochemicals and bioenergy.

Physical Properties of Ammonia & Associated Nitrogen Products
"Ammonia (NH_3) has a molecular weight of 17.03 and contains 82.2 percent nitrogen and 17.8 percent hydrogen. At standard temperature and pressure, ammonia is a colorless gas with a pungent, readily identifiable odor when it is present in concentrations of greater than 50 parts per million (ppm). Its boiling point is –33.35 °C, and its melting point is –77.7 °C.

Urea (NH_2CONH_2 or CH_4N_2O) has a molecular weight of 60.06 and typically contains 45.9 percent nitrogen. At room temperature, urea is colorless, odorless, and tasteless. When it is dissolved in water, it hydrolyzes very slowly to ammonium carbamate and eventually decomposes to ammonia and carbon dioxide (CO_2). This reaction is the basis for the use of urea as fertilizer. Commercially available urea-ammonium nitrate (UAN) solutions typically contain from 28 to 32 percent nitrogen.

Ammonium nitrate (NH_4NO_3) has a molecular weight of 80.04 and contains 33.9 percent nitrogen. It is a white, crystalline salt that is highly soluble in water. The solid salt picks up water from the air when the vapor pressure of water exceeds that of a saturated aqueous ammonium nitrate solution; solid ammonium nitrate does not occur in nature.

Ammonium sulfate [$(NH_4)2SO_4$] is a white, soluble, crystalline salt that has a molecular weight of 132.14, and contains about 21.2 percent nitrogen. The salt begins to decompose at 100 °C, and forms ammonia and ammonium bisulfate. Above 300 °C, ammonium sulfate decomposition becomes more extensive and forms nitrogen, sulfur dioxide, sulfur trioxide, and water in addition to ammonia.

Nitric acid (HNO_3), which has a molecular weight of 63.01, is a strong acid, a powerful oxidizing compound, and a nitrating agent that contains about 22.2 percent nitrogen. Crystals of pure nitric acid are colorless and stable. Above its melting point of –41.6 °C, nitric acid is a colorless liquid that fumes in moist air and has a tendency to decompose and forms oxides of nitrogen. The rate of decomposition is accelerated by exposure to light and increases in temperature. It is miscible with water in all proportions. It forms an azeotrope (constant-boiling mixture) with a composition of 68 percent nitric acid and 32 percent water that boils at 120.5 °C. Nitric acid is typically sold as a solution of from 52 to 68 percent nitric acid in water.

Any natural or manufactured material, which contains at least 5% of one or more of the three primary nutrients [nitrogen, phosphate (P_2O_5), potassium oxide (K_2O)], can be called fertilizer. Industrially manufactured fertilizers are called mineral fertilizers. Fertilizers that contain only one primary nutrient are called straight fertilizers. Those that contain two or three primary nutrients are called multinutrient fertilizers, sometimes they are also called binary (two-nutrient) or ternary (three-nutrient) fertilizers. Some of the most important (as well as the regionally important) straight fertilizers that contain nitrogen are as follows:

- Urea is the world's major source of nitrogen because of its high concentration and its usually attractive price per unit of nitrogen. Its application, however, requires exceptionally good agricultural practices to avoid evaporation losses of ammonia to the air. Urea should be applied only when it is possible to incorporate it into the soil either immediately after spreading or when rain is expected within the few hours following the application.

- Ammonium sulfate is not as concentrated as urea. In addition to nitrogen, however, it contains 23 percent sulfur, which is a plant nutrient that is of growing importance. It is used by preference on irrigated crops and where sulfur has to be applied. The same holds true for ammonium sulfate nitrate with 26 percent nitrogen (about ⅔ in the form of ammonia and ⅓ in the form of nitrate) and from 13 to 15 percent sulfur.

- Calcium ammonium nitrate with up to 27 percent nitrogen (equal parts of ammonia and nitrate nitrogen) is the preferred fertilizer on crops in semiarid regions of the subtropics.

In general, the three distinct types of multinutrient fertilizers are as follows:

- Complex fertilizers—manufactured through processes that involve a chemical reaction between the constituents that contain the primary plant nutrients (each granule contains the declared ratio of nutrients);

- Compound fertilizers—granulated straight fertilizers or intermediates; the granules containing the nutrients in varying ratios; and

- Mixed fertilizers or blends—simple mechanical mixtures of straight fertilizers (Food and Agriculture Organization of the United Nations and International Fertilizer Industry Association, 2000, p. 32–35).

Table 6. Ranges of nutrient contents in multinutrient fertilizers.

	Percentage		
Fertilizer type	Nitrogen	Phosphate	Potassium oxide
Nitrogen-phosphorous-potassium.....	5-26	5-35	5-26
Ammonium phosphates:			
Diammonium phosphate.....	16-18	42-48	—
Monoammonium phosphate.....	11	52	—
Nitrophosphates.....	20-26	6-34	—

(Food and Agriculture Organization of the United Nations and International Fertilizer Industry Association, 2000, p. 44 —)

Most nitrogen is used in the form of a nitrogen compound, most of which is derived from ammonia. Elemental nitrogen is used extensively by the aerospace, electronics, food, and metals industries because of its cryogenic and inert properties. Nitrogen can be used to prevent fires and explosions, as a purging agent for cleaning and processing equipment, and as a controlling

atmosphere for annealing and heat treating and other metal preparation processes in which oxygenation is a concern.

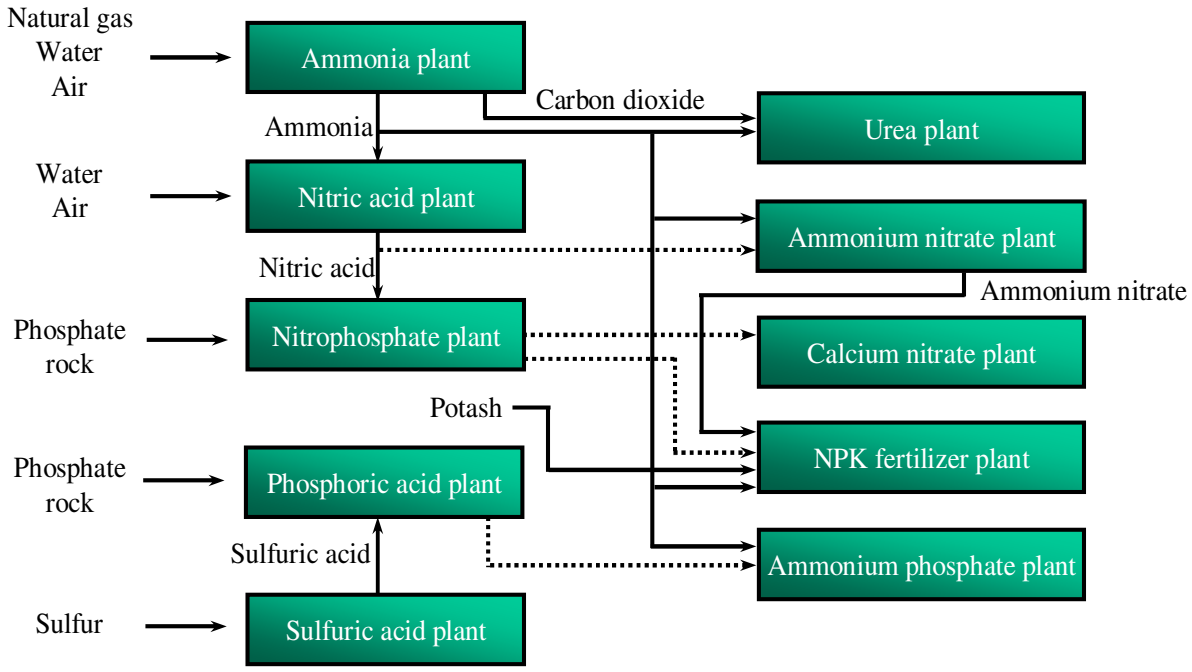


Figure 8. Nitrogen fertilizer production routes. (Food and Agriculture Organization of the United Nations and International Fertilizer Industry Association, 2000; Kramer, 2004)

Ammonia

More than 85 percent of the ammonia used in the United States is used for fertilizer applications. Ammonia can be directly applied to the field as a fertilizer, or more often, it is converted into another compound, such as ammonium nitrate, diammonium phosphate, UAN solution, or urea and then used as a fertilizer (figure 8). Figure 9 lists some of the uses for ammonia and the complex relationships among some ammonia-derived products.

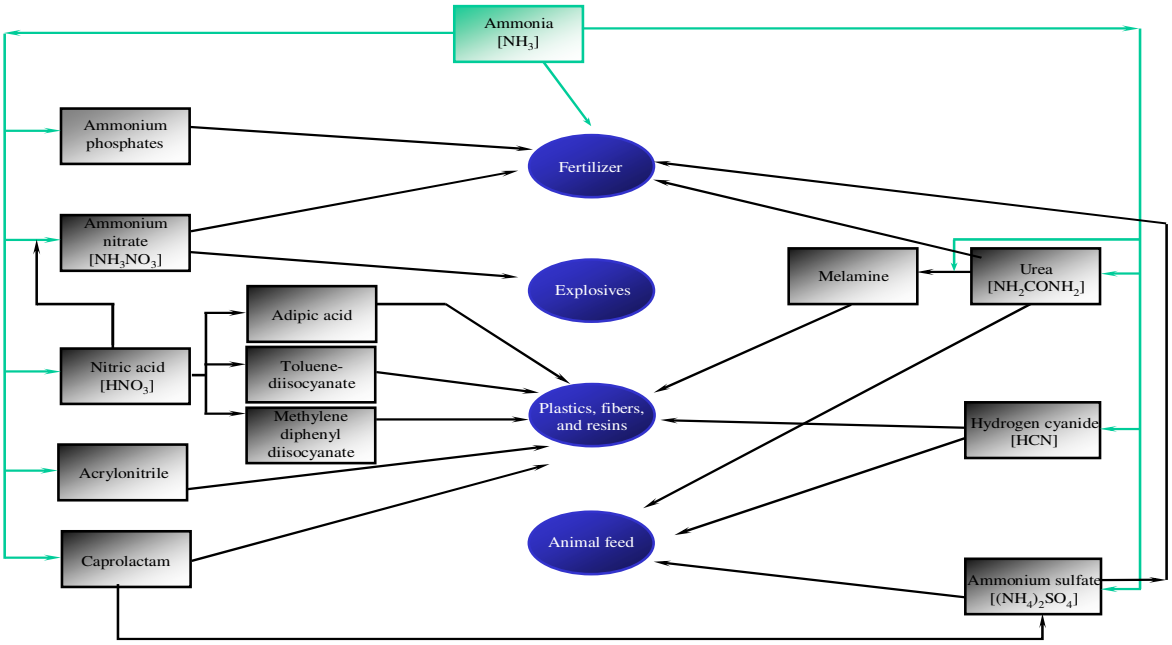


Figure 10. Principal downstream products of ammonia and their uses.

An average corn crop in North America will remove more than 2.7 billion kilograms (Gg) (6 billion pounds) of nitrogen from the soils every year. Each year, hay, which is grown to feed livestock, removes 3.4 Gg (7.4 billion pounds) of nitrogen from the fields, alfalfa hay, 2.2 Gg (4.9 billion pounds); and wheat, which is the most commonly used grain for human foods, 1.1 Gg (2.4 billion pounds). Fruits and vegetables are also big users of nitrogen. Bell peppers, grapes, snap beans, and sweet corn all take up about 112 kilograms per hectare (kg/ha) (100 pounds per acre) of nitrogen. Onions, peas, pineapple, and tomatoes take up from 168 to 224 kg/ha (150–200 pounds per acre) of nitrogen, and potatoes remove more than 280 kg/ha (250 pounds per acre) of nitrogen (Potash Corp. of Saskatchewan, 2001).

Fiber production is the principal nonagricultural use of ammonia. By means of the production of nitric acid, ammonia is used in the production of adipic acid, which is a key intermediate in nylon production. Ammonia also is used to produce caprolactam, which also is used for nylon production, by reaction with cyclohexanone. The caprolactam production process serves as the main source of the world's ammonium sulfate, which is a byproduct. Nitrogen & Methanol (2000) estimated that about 4.5 million metric tons per year (Mt/yr) of caprolactam is produced worldwide, which accounts for about 6.0 Mt/yr of ammonia consumption. Acrylonitrile is another ammonia-based product that is used in fiber production and is manufactured primarily through a catalytic reaction of ammonia with propylene. Global acrylonitrile production, which uses about 2.5 Mt/yr of ammonia, was estimated to be about 5.5 Mt/yr. Hydrogen cyanide, which is manufactured by catalytic synthesis from ammonia and hydrocarbons, is used in the manufacture of adiponitrile, which is used in the production of nylon.

Plastics production is another large nonagricultural use for ammonia. In addition to its use in fiber production, acrylonitrile also is used in the production of acrylonitrile-butadiene-styrene plastics and of synthetic rubber and other elastomers. Hexamethylenetetramine, which is produced from ammonia and formaldehyde, is used in the manufacture of phenolic thermosetting

resins. Through urea production, ammonia also is a component of melamine, which is used in adhesives, laminates, paper and textiles, and surface coatings. Global melamine production was estimated to be 450,000 t/yr. Ammonia can be converted, by means of nitric acid, to toluene diisocyanate, which is used in polyurethane production. Ammonia is also converted to nitrobenzene, which is used to make aniline dyes. In addition to its use as a dye, aniline is an intermediate in the formation of methylene diisocyanate, which, in turn, is a component of urethane foams. Acetone cyanohydrin, which is used in acrylic plastics, is manufactured from hydrogen cyanide.

Nitrogen & Methanol (2000) estimated that about 10 percent of nonagricultural ammonia, or about 2 Mt/yr, is used as a refrigerant gas, mainly in large commercial or industrial refrigeration systems. As a refrigerant gas, ammonia is highly energy efficient, relatively inexpensive, noncorrosive, and tolerant of impurities. Also, because of its distinctive odor, small leaks can be identified and repaired before they become serious. Although a significant portion of ammonia has been replaced by halogenated hydrocarbons in this use, the ozone-damaging potential of the hydrocarbons has resulted, in some cases, in a switch back to ammonia. Because ammonia refrigeration systems operate at elevated pressures, these systems must be maintained and operated to prevent releases; ammonia is considered to be a significant health hazard because it is corrosive to the eyes, lungs, and skin.

Ammonia can be a component in the synthesis of methamphetamine, which is of particular concern to drug and law enforcement agencies. Methamphetamines are synthetic amphetamines, or stimulants, that are produced and sold illegally in capsule, chunk, pill, and powder forms. Methamphetamines stimulate the central nervous system, and the effects may last anywhere from 8 to 24 hours depending on the dosage and concentration of the drug. Methamphetamines can be manufactured in small laboratories by using common ingredients. In one common manufacturing technique referred to as the "Nazi method," lithium that has been extracted from batteries and anhydrous ammonia are used to convert ephedrine from over-the-counter cold remedies to make methamphetamine. As a result, theft of anhydrous ammonia fertilizer from farms, retail outlets, and even ammonia pipelines for production of methamphetamines has escalated.

Urea

Solid urea, which contains from 0.8 to 2.0 weight percent biuret ($NH_2CONHCONH_2$), is primarily used for direct application to the soil as a nitrogen-release fertilizer; biuret is an undesirable component produced by heating urea at a high temperature, which causes the condensation of two urea molecules. Weak aqueous solutions of low biuret urea (0.3 weight percent maximum) are used as plant food applied to foliage spray. Mixed with additives, urea is used in solid fertilizers of various formulations, which include urea-ammonium phosphate, urea-ammonium sulfate, and urea-phosphate (urea plus phosphoric acid). Concentrated solutions of UAN (80–85 weight percent) have a high nitrogen content but a low crystallization point, and are suitable for easy transportation, pipeline distribution, and direct spray application.

Urea is used as a feed supplement for ruminants because it assists in the digestion of protein. Urea also is one of the raw materials used to manufacture urea-formaldehyde resins. At high temperature and pressure, urea (with ammonia) pyrolyzes to form melamine plastics. Urea is used in the preparation of lysine, which is an amino acid widely used in poultry feed. It also is used in some pesticides. Partially polymerized resins of urea are used by the textile industry to impart permanent-press properties to fabrics.

Ammonium Nitrate

Before World War II, most ammonium nitrate was used as an ingredient in high explosives. After World War II, its use as a fertilizer grew rapidly to reach about 90% of production in 1975. Most ammonium nitrate manufactured for the explosives market is used in blasting agents prepared by adding a fuel component, such as diesel oil, to the prilled product. This mixture is commonly referred to as "ANFO" (ammonium nitrate-fuel oil). More than 65 percent of the ammonium nitrate-based explosives is used in coal mining; the remainder is used in, in declining order, metal mining, nonmetal mining and quarrying, and highway construction. When used in blasting, ammonium nitrate is mixed with fuel oil and sometimes sensitizers such as powdered aluminum. Lower density ammonium nitrate is preferred for explosive formulation because it absorbs the oil more effectively.

A small but important use of ammonium nitrate is in the production of nitrous oxide gas; during the 1980s, consumption for this purpose averaged about 30,000 t/yr. The gas is generated by controlled heating ammonium nitrate to above 200 °C. Nitrous oxide is used primarily as an anesthetic and an aerosol propellant for food products.

Ammonium Sulfate

Ammonium sulfate is used mainly as a nitrogenous fertilizer and accounts for about 4 percent of the world's nitrogen fertilizer market (Nitrogen & Methanol, 2002). Ammonium sulfate has been replaced in some fertilizer applications because of its lower nitrogen content (about 21 percent compared with 34 percent for ammonium nitrate and 46 percent for urea). Ammonium sulfate, however, has about 45 percent sulfur by weight; this is a desirable attribute in areas where soils are deficient in sulfur. Nonfertilizer uses for ammonium sulfate, which account for about 5 percent of world ammonium sulfate consumption, include cattle feed, fire control, food processing, and tanning.

Nitric Acid

The largest use of nitric acid, which accounts for about 75 percent of total U.S. production, is for the manufacture of ammonium nitrate. The next three largest uses for nitric acid are in the manufacture of cyclohexanone (about 8–9 percent), dinitrotoluene (about 4 percent), and nitrobenzene (about 3–4 percent). Cyclohexanone is a raw material that is used to manufacture adipic acid, which reacts with hexamethylenediamine to make nylon-6,6. Dinitrotoluene is hydrogenated to toluenediamine, which is used to make toluene diisocyanate. Nitrobenzene is hydrogenated to make aniline, which is a raw material that is used to manufacture methylene diphenyl diisocyanate. Toluene diisocyanate is used to make coatings, elastomers, and flexible polyurethane foams, and methylene diphenyl diisocyanate is used for rigid foams. Other uses of nitric acid are in the production of explosives; metal nitrates; metal treatments, such as the pickling of stainless steels and metal etching; nitrocellulose; nitrochlorobenzene; nuclear fuel processing; and rocket propellants (Innovation Group, The, 2002).

Environmental Impact of Ammonia Production

The production of ammonia generates substantial quantities of CO_2, which contributes to global warming. If natural gas is used as the feedstock in a modern steam reforming plant, then about 2.7 metric tonnes (t) of CO_2 per metric tonne of nitrogen is produced. If coal or fuel oil is used, then this figure is about 25 percent higher. The production of urea, however, requires an input of about 1.6 t of CO_2 per ton of nitrogen. The fertilizer industry's share of the annual net addition of CO_2 to the atmosphere that results from human activities is estimated to be 2 percent; and human activities account for only 7 percent of the quantity released annually by biological processes. Consequently, the share of fertilizer production in the total annual release of CO_2 to the

atmosphere is very small (approximately 0.1–0.2 percent). Nevertheless, projected growth of fertilizer use makes it important that the industry keep CO_2 emissions as low as possible. Although ammonia plants continue to try to reduce CO_2 emissions through process improvements, future reductions of CO_2 emissions most likely will be from the replacement of old inefficient plants.

The production of nitric acid used for ammonium nitrate and nitrophosphate fertilizers leads to the emission of nitrous oxide (N_2O), which is a much more potent global warming agent than carbon dioxide. The U.S. Environmental Protection Agency (EPA) (2003, p. ES-10) estimated that N_2O is 310 times more effective at trapping heat in the atmosphere than carbon dioxide during a 100-year time period. It also is considered to be detrimental to the ozone layer. The rate of N_2O emission varies widely from 1 to more than 10 kilograms per metric ton (kg/t) of 100 percent nitric acid. Abatement techniques can reduce N_2O emissions significantly but are costly. The International Fertilizer Industry Association (1998, p. 43–44) estimated that fertilizer production accounts for about 6 percent of human-generated N_2O emissions compared with nearly 50 percent from motor vehicles. Most N_2O recycles to land and water, and as with CO_2, larger quantities are emitted through natural biological processes. N_2O is estimated to be responsible for 7.5 percent of the calculated global warming effect of human activities. Fertilizer production is estimated to be responsible for less than 0.5 percent of this effect.

Nitrogen oxides (NOx) also are emitted from ammonia and nitric acid plants. Nitric oxide (NO) is oxidized over a few days to nitrogen dioxide (NO_2), which has an atmospheric residence time of about a week and is deposited in air, rain, or as nitrate particulates. This contributes to acid rain and smog. In the case of ammonia, NOx emissions are about 1 to 2 kg/t of converted nitrogen. For nitric acid, however, NOx emissions amount to 6 to 9 kg/t of converted nitrogen. Selective catalytic reduction, which uses ammonia to convert NOx to nitrogen, can be an effective means of abatement, and more than 0.5 Mt of ammonia is used annually for this purpose (International Fertilizer Industry Association, 1998, p. 43–44).” (Kramer, 2004)

Biogas Refining & Renewable Ammonia Production

A typical modern ammonia-producing plant first converts natural gas (i.e., methane) or LPG (liquified petroleum gases such as propane and butane) or petroleum naphtha into gaseous hydrogen. The method for producing hydrogen from hydrocarbons is referred to as "Steam Reforming." Methane-rich biogas can be utilized in place of natural gas. Additionally, methane feeds for high temperature fuel cells allow for cogeneration of 1200 lbs of H_2 per day per MW of DG-CHP produced. The hydrogen is then combined with nitrogen to produce ammonia. In the latter case, the production of hydrogen from biomethane can be skipped, but hydrogen sulfide still needs to be removed from biogas.

Starting with a natural gas or biogas feedstock, the processes used in removing sulfur and producing the hydrogen are:

The first step in the process is to remove sulfur compounds from the feedstock because sulfur deactivates the catalysts used in subsequent steps and also contaminates fuel cell stacks when biomethane is utilized as a feed for high temperature fuel cells. Sulfur removal requires catalytic hydrogenation to convert sulfur compounds in the feedstocks to gaseous hydrogen sulfide. In organic chemistry, R-SH is a thiol compound (R) that contains a functional group composed of a sulfur atom and a hydrogen atom (-SH). For refining methane-rich biogas, hydrogen sulfide is already in gaseous form, hence this step can be skipped:

$$H_2 + RSH \rightarrow RH + H_2S(gas)$$

The gaseous hydrogen sulfide is then absorbed and removed by passing it through beds of zinc oxide where it is converted to solid zinc sulfide.

$$H_2S + ZnO \rightarrow ZnS + H_2O$$

Catalytic steam reforming of the sulfur-free feedstock is then used to form hydrogen plus carbon monoxide. For H_2 cogeneration via high temperature fuel cells using methane-rich biogas feeds, the following hydrogen production and carbon dioxide removal steps are skipped:

$$CH_4 + H_2O \rightarrow CO + 3H_2$$

The next step requires catalytic shift conversion or water-gas shift reaction to convert the carbon monoxide to carbon dioxide and more hydrogen:

$$CO + H_2O \rightarrow CO_2 + H_2$$

The carbon dioxide is then removed either by absorption in aqueous ethanolamine solutions or by adsorption in pressure swing adsorbers (PSA) using proprietary solid adsorption media. The final step in producing the hydrogen is to use catalytic methanation to remove residual amounts of carbon monoxide or carbon dioxide from the hydrogen gas:

$$CO + 3H_2 \rightarrow CH_4 + H_2O$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

To produce the desired end-product ammonia, hydrogen is catalytically reacted with inert nitrogen (N_2 derived from process air which is almost 80% nitrogen) to form anhydrous liquid ammonia. This step is known as the ammonia synthesis loop (also referred to as the Haber-Bosch process):

$$3H_2 + N_2 \rightarrow 2NH_3$$

The steam reforming, water-gas shift conversion, carbon dioxide removal and methanation steps each operate at absolute pressures of about 25 to 35 bar, and the ammonia synthesis loop operates at absolute pressures ranging from 60 to 180 bar depending upon which proprietary design is used. There are many engineering and construction companies that offer proprietary designs for ammonia synthesis plants. Haldor Topsoe of Denmark, Uhde GmbH of Germany, and Kellogg Brown & Root of the US are among the most experienced companies in this field.

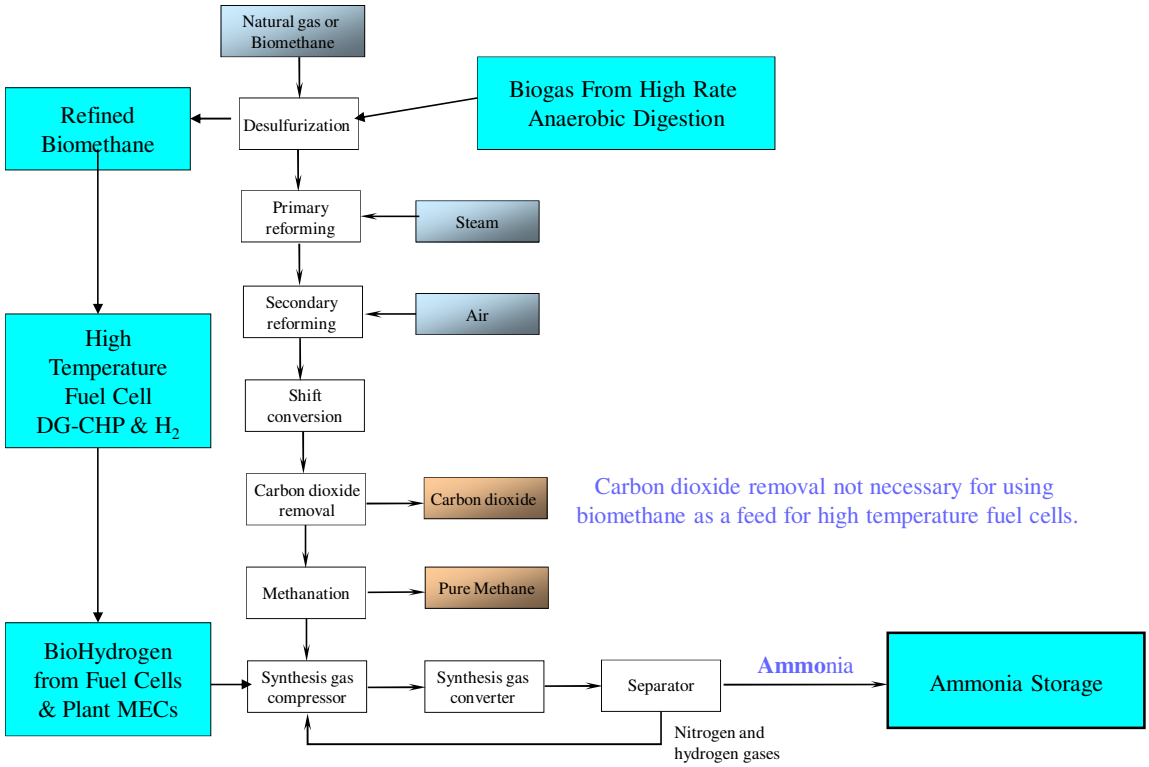


Figure 11. Basic Ammonia Production Process (steam reforming) & Integration of Anaerobic Digestion, Fuel Cells & Plant MECs which Provide Biohydrogen

Harvesting & Storage of High Moisture Corn Stover Biomass

Developing economical methods for harvesting, transporting and storing bulky lignocellulosic feedstocks is the first step for processing corn stover, cereal grain straw, and other crop residues at integrated biorefineries. Of the 500 million tons of crop residues produced annually in the US, 235 million tons of corn stover is left in crop fields according to USDA (Bernardo and Jung, 2008). At 90% conversion efficiency, 100 million tonnes of corn stover is enough to annually produce 11.3 billion gallons of ethanol.

In Nov 2008, 175 ethanol plants in the US had a total annual production capacity of 10.57 billion gallons according to the Ethanol Producer Magazine. However, many of these ethanol plants are currently idle or in bankruptcy due to relatively high corn and energy prices combined with low gasoline prices. In contrast to corn, corn stover and other crop residues do not compete with food production and are not subject to the volatility of commodity markets. In addition, integration of corn and stover processing allows for leveraging existing infrastructures and reducing biorefinery production costs. Processing 66% of corn stover at integrated biorefineries could more than double US ethanol production. Because of this, corn stover is one of the most obvious and practical alternative feedstocks for producing cellulosic ethanol.

Since 2005, USDA has been harvesting corn stover at four different heights to measure the amount and quality of stover that might be harvested using different removal strategies. The scientists found the lowest 30 inches of cornstalk are 64% water, which makes that portion of the stover more expensive to harvest, store, and transport – and also less efficient for conversion to ethanol due to more soil contamination and higher concentrations of silica and lignin, according

to Douglas Karlen, a research scientist at the USDA-ARS Soil Tilth Lab located in Ames, IA.

However, the corn plant material above the lowest 30 inches of cornstalk, the portion which includes the cobs and husks, produces significantly more ethanol and similar ethanol yields are possible using “normal-cut” stover (16-18 inches above the ground), which suggests that using a standard harvester is the fastest, most convenient, and least expensive method for harvesting stover (Biomass Magazine, Nov, 2008).

Corn stover can be harvested and chopped via Ceres Ag Residue Recovery System (Ceres Agricultural Consultants) which attaches to combines, and a CCX770 Cob Harvester pull-behind cob collection system developed by Vermeer Corp. The air separator developed by Vermeer could be eliminated since harvesting 75% of cobs, stalk, and leaves is proposed as an alternative feedstock for integrated biorefineries. 25% of corn stover would be spread on the top soil in order to maintain soil fertility and reduce erosion. In addition, about 16-18 inches of corn stubble and root balls remain after conventional harvests. The top portion of the corn plant, including the cob but minus the grain, is the best portion of the corn stover to use in the production of cellulosic ethanol according to new findings from the USDA Agricultural Research Service’s National Soil Tilth Laboratory in Ames, Iowa.

A typical modern corn head on a combine has a separate corn head assembly for each row of corn being harvested. (Heads come in four-row, six-row, eight-row, etc. widths.). The plants in each row enter gathering points leading to the corn head. Stalk rolls, which rotate in opposite directions to each other, grip the stalks and pull them through slots in a snapper plate, which pulls the ear (and assorted bits of leaves, husks and other "trash") from the stalk. Trash shields or ear savers are positioned on every gathering point to keep the ears of corn from falling off the head, and trash knives ensure that weeds or other trash doesn’t get wrapped around the stalk rolls.

Gathering chains move the ears of corn back to the cross auger, which conveys the crop to the center of the corn header and then feeds it into the threshing portion of the machine. Alternative corn heads exist that collect more of the stalk, and others that gather up the whole plant. In a study at the University of Wisconsin aimed at identifying the most efficient configuration of a single-pass, split stream corn harvesting method (one in which both grain and stover would be collected at the same time), the whole-plant header proved the most effective, capturing 90% of potential stover dry matter, compared to 67% when the stalk-gathering header was used and 30% when a conventional ear-snapper head was used (Shinners et. al., 2007).

Researchers are working to develop stover attachments that can be used on standard combines and allow single-pass harvesting. One such system, tested at Iowa State University, includes a modified row crop header and corn reel attached to the front of the combine, and a chopper and blower attached to the back.

The header and reel feed leaves and stalks into the combine along with the grain. This has the added benefit, from the point of view of using corn stover as a biomass feedstock, of never allowing the stover to touch the ground, where it can be contaminated with soil. In the Iowa State System, the chopper cuts the stover into two-inch pieces, which are then blown into a trailing wagon.

Research reveals that shredding stover lengthwise along the fibers requires 10 times less energy than chopping stover. For ethanol producers, less energy used at harvest time or during processing means less cost required to produce fuel from that feedstock. By shredding corn stover instead of chopping, as is commonly done, about 40% less energy is needed to gain access to more of the material stored in the plant. Using a technique employed in food processing and other industries to measure cell damage, shredded corn stalks in water produce 11% more leachates than chopped stover, and 5% more than stalks that had been chopped and put through a roller. A leachate is any plant substance that is dissolved out of a plant or soil when it is placed in water. These differences are all the more impressive when considering the energy savings tied to shredding, giving ethanol producers more cellulose for less cost. This is partially the result of increasing the surface area of the plant material by shredding which allows for better access to cellulose. This could reduce the need for more expensive pre-treatment processes (Buckmaster, 2008; Zhang et al., 2003).

A key to harvesting both grain and stover is accomplishing this task in as few steps as possible. Shinners et al., 2007 designed and tested a harvester capable of recovering both corn grain and stover in a single pass. Whole-plant harvesting was considerably slower than conventional grain harvesting, but good recoveries of both the grain and stover fractions were obtained. Up to 90% of the individual non-grain components (leaves, stalks, husks and cobs) of the corn plant were recovered, and the estimated ethanol yield of the recovered product ranged from 2,600 to 3,945 liters per hectare (278-422 gal/acre), depending on cutting height.

For spoonfeeding nutrients to twin row corn crops via sub-surface drip fertigation (SDF), the average yield could be increased from 150 bu/acre to 400 bu/acre. Harvesting 75% of corn stover would be equivalent to producing 10.16 tonnes/acre of lignocellulosic feedstock. At 90% conversion efficiency this would produce 1,148 gal/acre of ethanol. Including processing 90% of all carbohydrates contained in corn grain, e.g. 15.4% cellulose (pericarp, endosperm and germ fiber) and 84.6% starch (1,254 gal/acre), total ethanol production per acre would increase to 2,402 gal. This is a 14% increase resulting from processing both hexose and pentose sugars via integrated biorefineries. Hence, the potential for a two-fold increase in carbohydrate yields produced by processing both corn and stover (additional fiber) fractions provide an economic incentive for development of single-pass harvesting systems.

In a USDA-ARS project, “capture of potential stover dry matter (DM) varied from 48 to 89% for leaves, 49 to 92% for stalks, and greater than 90% for husks and cobs, depending upon corn head height. Stover aggregate moisture was 50.2, 43.1 and 36.4% (w.b.) when the corn head height was 10, 44 and 63% of ear height, respectively. Feed-rate of materials other than grain limited ground speed due to power availability, so area capacity was 2.3, 2.8 and 3.4 ha/h when corn head height was 10, 44 and 63% of ear height, respectively. Whole-plant harvesting reduced area capacity by nearly 61% compared to harvesting with a conventional snapping-roll head. Single-pass stover had an average particle size of 69 mm and bulk density of 51 and 110 kg DM/m^3 in the wagon and bag silo, respectively. Based on polymeric sugar content, estimated ethanol yield was 3,945, 3,230, and 2,600 L/ha when the corn head height was 10, 44 and 63% of ear height, respectively. Fermentation of single-pass stover in a bag silo was adequate with average losses of 6% of total DM (Shinners et al., 2007).”

If 50% of the stover is collected, the system operated at about the same speed as a normal corn harvester. If more than 50% of the stover is collected, the speed falls off. Shinners at al., 2009 have developed a stover-harvesting system which operates at least 80-90% as fast as a normal

harvester no matter how much stover is collected, leaving it up to the farmer to decide how much stover to collect without having to worry about its effect on his harvest speed. (Researchers Developing Machinery to Harvest Corn Stalks and Leaves.) Researchers will continue to try to improve one-pass harvest systems, as corn stover becomes more and more valuable as a biomass feedstock.

Replenishing Soil Organic Matter

For integrated biorefineries which reduce energy costs and diversify, feeding ethanol coproducts to livestock via CAFOs provides organic fertilizers which restores additional soil organic matter (SOM) via closed loop biomass production. Organic matter contains essential macro and micro-nutrients which are responsible for maintaining soil tilth and fertility. This is accomplished by recycling animal waste via anaerobic digestion and utilizing nutrient-rich effluents for subsurface drip fertigation (SDF). In addition, incorporation of nutrient rich ash minerals resulting from combustion of lignin in fluidized bed boilers also restores SOM for closed loop biomass production.

In addition to reducing SOM, harvesting corn stover may also increase soil erosion (Mann et al., 2002). Some authorities recommend that approximately 30% of the soil surface should be covered with crop residues after harvest to reduce soil erosion by wind (Padgitt et al., 2000). A 30% ground cover requires 1.07 tonne of corn stover per hectare (Renard et al., 1997). The quantity of corn stover for covering the ground depends on site-specific factors (e.g., slope, climate etc.), and the 30% ground cover specification is currently being debated (Mann et al. 2002). For this project, 25% of corn stover will remain in the cornfield in order to maintain soil erosion at a tolerable level. This will result in restoring about 3 ton of organic matter per hectare, which is almost twice the minimum recommended cover (Renard et al., 1997; Kim and Dale, 2005). The effects of harvesting corn stover on SOM and erosion will be taken into account in this study which incorporates nutrient-rich anaerobic digester effluents via SDF and simultaneous closed loop production of food and biomass. Utilizing this closed loop approach for integrated biorefineries, annual carbon sequestered by soil per hectare is predicted to be increased by about 50% in comparison to carbon sequestered by soil without recycling nutrients contained in organic matter. Thus, removing corn stover, processing animal waste resulting from feeding protein rich fermentation residues via CAFOs, and incorporating ash minerals from combustion of lignin, will actually increase accumulation rate of SOM, including carbon.

Ensiling Wet Biomass Feedstocks

In contrast to ensiling corn stover in plastic silo bags, wet concrete bunkers at the biorefinery offer an alternative processing and storage system. Silo bags are subject to being damaged by hail storms, livestock, rodents, all of which can result in catastrophic losses of carbohydrates due to microbial degradation. Storing silo bag systems on the farm also requires handling and transporting biomass twice. Concrete bunkers located at integrated biorefineries can eliminate handling feedstocks twice, are less susceptible to damage, and allow for intense management which can substantially reduce loss of carbohydrates. Utilizing ammonia treatment, enzyme inoculation and fine chopping of feedstocks during ensiling to eliminate air pockets, it may be possible to achieve less then 3% loss of carbohydrates. This would be considerably less than is possible to achieve with silo bags or bailing processes which require drying biomass first.

The chopped corn stover which is harvested can be ensiled and stored in wet concrete bunker systems. Ensilage is a truncated solid-state fermentation in which anaerobically produced organic acids accumulate to reduce pH and limit microbial activity. Ensilage can be used to both preserve

and pretreat biomass feedstock for further downstream conversion into chemicals, fuels, and/or fiber products (Ren et al., 2006). Experiments reveal that ensiling enhances sugar conversion and enzyme-added ensiling further increases the conversion rate. Sugar yield after enzymatic hydrolysis was 23 to 31% for hays, 31 to 45% for non-enzyme-added silages, and 49 to 59% for enzyme-added silage in this study. Ensiling with or without enzyme added has little effect on sugar conversion of straws (Chen et al., 2006). Ensiling did not significantly affect the lignin content of barley straw, cotton stalk, and triticale hay ensiled without enzyme, but slightly increased the lignin content in triticale straw, wheat straw, and triticale hay ensiled with enzymes. The holocellulose (cellulose plus hemicellulose) losses in the feedstocks, as a result of ensiling, ranged from 1.31 to 9.93%. The percent holocellulose loss in hays during ensiling was lower than in straws and stalks.

Ensiling of barley, triticale, wheat straws, and cotton stalk significantly increased the conversion of holocellulose to sugars during subsequent hydrolysis with two enzyme combinations. Enzymatic hydrolysis of ensiled and untreated feedstocks by Celluclast 1.5 L-Novozyme 188 enzyme combination resulted in equal or higher saccharification than with Spezyme® CP-xylanase combination. Enzyme loadings of 40 and 60 FPU/g reducing sugars provided similar sugar yields. The percent saccharification with Celluclast 1.5 L-Novozyme 188 at 40 FPU/g reducing sugars was 17.1 to 43.6%, 22.4 to 46.9%, and 23.2 to 32.2% for untreated feedstocks, feedstocks ensiled with, and without enzymes, respectively. Fermentation of the hydrolysates from ensiled feedstocks resulted in ethanol yields ranging from 0.21 to 0.28 g/g reducing sugars (Chen et al., 2007).

Processing and preserving energy crops such as sweet sorghum has been a challenge for the biofuels industry since drying and storing these feedstocks results in a decrease of conversion rates to sugars. Hence, these feedstocks must either be processed immediately after harvest or stored in an efficient and economical manner such as ensiling which preserves sugar concentrations (Phillip et al., 2007). Integration of ensiling biomass with efficient fractionation of lignocellulosic feedstocks could further increase sugar conversion rates for energy crops such as sweet sorghum and tropical corn in addition to crop residues such as corn stover and soy hulls.

Ammonia Treatment of High Moisture Corn/Stover & Ensiling Wet Biomass Feedstocks
Problems with preserving carbohydrate and protein content of lignocellulosic materials during ensiling are due primarily to fungal growth and heat. These issues can be largely resolved by using ammonia to simultaneously preserve and pretreat lignocellulosic biomass. An abundance of data exists for evaluating ammonia applications as utilized for preservation and enhancing digestion of livestock feeds. This data could be utilized for development of ammonia applications for use in the biorefining industry. Ammonia, which is a popular chemical used in production of fertilizers and industrial grade chemicals and products, is produced in large quantities in the US using hydrogen obtained from either methane or natural gas. For integrated CEA which incorporate plant microbial electrolysis cells (MECs) via controlled environment agricultural (CEA) applications, ammonia could be economically produced via closed loop production at integrated biorefineries.

Beginning in 1978, FDA approved use of anhydrous ammonia treatments for silage in the livestock industry. Treatment of corn silage with cold-flow anhydrous ammonia at the time of ensiling is a convenient means of adding non-protein nitrogen (NPN) to corn silage. A similar process could potentially be utilized as a pretreatment process for ensiling corn stover and other lignocellulosics as biomass feedstocks for integrated biorefineries. Such a process could provide

some of the same benefits obtained from ammonia steeping and ARP, but without the relatively high liquids to solids ratio and subsequent production costs resulting from energy intensive heating and water separation processes repeatedly utilized in pretreatment, fractionation, and biorefining. The fermentation process utilized by ethanologens in the biofuels industry is very similar to the fermentation processes utilized by microbial populations in ruminant livestock.

In terms of conserving carbohydrate content of corn silage utilized for livestock production, "net energy values for maintenance and gain were determined by the comparative slaughter technique for each of eight protein treatments. Among the protein treatments was corn silage treated with cold-flow anhydrous ammonia (ANAM) at the time of ensiling. Silages fed in three trials were analyzed for crude protein (N x 6.25), water soluble nitrogen (N), water insoluble N, lactate, pH and volatile fatty acids throughout the feeding period. Highest net energy values were obtained from the addition of a complete mineral mixture at time of ensiling to corn silage treated with 15.60 g ANAM/kg of corn silage dry matter (CSDM). Net energy values for maintenance (NE_m) and gain (NE_g) were 1.62 and 1.19 Mcal/kg diet dry matter. All protein treatments resulted in higher diet net energy values than the control treatment which contained no supplemental protein. Addition of ammonia to corn silage at the time of ensiling generally resulted in a higher pH and higher crude protein, soluble N, insoluble N and lactate (Lomas and Fox, 1982)."

Similar to ammonia pretreatment and fractionation processes utilized in the biorefining industry, ammoniation increases the digestibility of crop residues and grass hays by breaking lignin-cellulose bonds in plant fiber, thereby swelling the plant tissue to allow greater microbial activity, and improving dry matter digestion of ruminant livestock (TDN) 8 to 15 percentage units. Ammoniation boosts feed intake 15 to 20 percent or more because of improved forage digestibility and increased rate of passage through the digestive tract. Ammoniation usually doubles crude protein content by being a non-toxic source of non-protein nitrogen (NPN) and it is well utilized by microbial populations in ruminant livestock which are responsible for fermenting crop residues, e.g. lignocellulosic biomass. Ammoniation preserves forage that contains up to 25 to 30 percent moisture because it kills molds and fungi and prevents heating which reduces feed losses, e.g. loss of carbohydrates.

"Anhydrous ammonia or water ammonia mixes can be added to whole corn plants and corn stalks at the time of ensiling (Huber et al., 1979). Ammonia additions have resulted in the following benefits (Huber and Kung, 1983):

- addition of an economical source of crude protein
- prolonged bunk life during extraction (aerobic stability)
- less molding and heating during ensiling
- decreased protein degradation in the silo/bunk

Addition of anhydrous ammonia or water-ammonia mixes initially buffers the plant material. With anhydrous ammonia, corn forage will turn bright yellow immediately upon treatment. For example, corn forage may have a pH of 5.9 but treated corn forage will have a pH of about 8.5 to 9.0. We have observed (Kung et al., unpublished data) that ammonia treatment causes an initial delay, followed by stimulation, in growth of lactic acid bacteria. When fermentation in the silo is complete, corn silage treated with anhydrous ammonia usually is 0.1-0.2 units higher in pH, contains 0.5-1.5 % (DMB) more lactic acid, 0.5-1.5% more acetic acid, and less residual water soluble carbohydrates. Forages treated with ammonia have also been shown to be higher in insoluble N (Huber et al., 1979) and true protein (Buchanan-Smith, 1982) primarily because

ammonia reduces plant proteolysis. Ammonia and ammonium salts have been suggested to be anti-fungal in nature and result in improved aerobic stability (reduced molding and heating) during storage and feedout (Britt and Huber, 1975). However, recent data from our lab (Kung et al., unpublished) would suggest that improved aerobic stability is also due to an increase in acetic acid and decrease in residual water soluble carbohydrates after ensiling, in addition to a direct fungicidal effect from ammonia. In large bunks or pits where rapid feed out and bunk management are difficult, ammonia treatment can improve bunk life of silages.

Ammonia has also been added to high moisture ear corn (Britt and Huber, 1975) and high moisture snapped ear corn (Soderholm et al., 1988). In some instances (Alli et al, 1983 and Phillip et al., 1985) ammonia was added at about 1% of fresh weight. Addition of ammonia to high moisture corn has caused an increase in pH but lowered production of lactic acid. Decreases in plant proteolysis have also been observed. Improvements in aerobic stability have not been consistent. Mowat et al. (1981) reported high levels of free ammonia and reduced animal acceptance in ammonia-treated high moisture corn.

Table 7. Effect of Anhydrous Ammonia on True Protein Nitrogen Content of Corn Silage

Silage DM	28%		40%	
Treatment	Untreated	Ammonia	Untreated	Ammonia
CP, % DM	8.9	12.8	9.8	13.1
True protein N, % DM	0.72	0.94	0.88	0.99

(Table adapted from Buchanan-Smith, 1982)

Anhydrous Ammonia Addition to Other Crops. There has been recent interest in adding anhydrous ammonia to alfalfa silage, primarily to improve aerobic stability. Certain precautions must be considered for this application. First, alfalfa silage contains excess amounts of rumen degradable protein and added ammonia will compound this problem. Secondly, there is some research that shows when the moisture content of alfalfa is high (more than 70%) ammonia can cause an undesirable clostridial fermentation (Kung et al., 1989) leading to high levels of butyric acid and protein degradation in the silo. Glenn (1990) reported that adding ammonia to low DM alfalfa (20% DM) resulted in a decrease intake of digestible DM and energy whereas ammonia treatment of high DM silage (40%) increased intake of these nutrients. This author would not recommend use of anhydrous ammonia for legume silages that are going to be used as livestock feeds.

In grass-legume silages, Moore et al. (1986) reported that anhydrous ammonia applied at 3% of the DM improved digestibility and intake of poor quality grass-legume silage. Moderate levels of ammonia (1.5%) also resulted in high levels of butyric acid in treated silages (an indicator of clostridial fermentation). Such high levels of ammoniation are not recommended for normal corn silage or alfalfa silages. Whole-crop barley silage has also been successfully treated (1% DM basis) with anhydrous ammonia (Song and Kennelly, 1989).

Application. Ammonia can be added at the chopper, blower, bagger or bunk. Mixed ammonia solutions are bulkier than anhydrous ammonia but retention of ammonia is usually greater. In addition, molasses (to improve palatability and fermentation) and minerals can be added in these solutions. Some ammonia will be lost (between 10 and 30%) and losses will be greater if ammonia is not applied properly and if forage becomes too dry. Ammonia should be added at the end nearest the cutter in a chopper with an auger system. If no auger is used, ammonia can be added behind the cutter prior to entering the blower. For silage to be stored in a conventional up

right silo, ammonia should be applied to the forage before it contacts the blower to minimize losses. Ammonia can also be spiked into bunks between loads and it will disperse into the mass.

Application of anhydrous ammonia should be at approximately 6 to 7 lb of N per 700 lb of forage DM (Table 8). Excess ammonia (> 15 lb per ton) may result in poor silage fermentation (because of a prolonged buffering effect) and may add too much degradable intake protein to the forage. Adding 7 lbs of ammonia (5.7 lbs of N) per ton of 35% DM corn silage will increase the crude protein from about 8% to 12.5% (dry matter basis). Using the cold-flow method is the simplest way to add ammonia to silage. When gaseous ammonia is super cooled in a converter box, 80-85% of anhydrous ammonia gas is transformed to liquid ammonia.

Table 8. Addition of Ammonia & Urea to Corn Silage

	Anhydrous Ammonia	Ammonia-molasses mixes	Urea
Nitrogen, %	82	20-23[a]	46
CP equivalent, %	515	125[a]	282
Application, lb/ton of 35% DM forage[c]	7	± 25[a]	10-12[b]

[a] varies based on specific product.

[b] do not add urea to forage over 45% DM.

[c] application rate should vary depending on forage DM. Higher amounts should be applied to drier forage. In all cases, the desired application rate is 6-7 lb of N per 700 lb of forage DM.

Anhydrous ammonia should not be added to corn forage if the DM content is above 40-42% because fermentation is restricted in drier material and binding of ammonia will be less; thus normal fermentation may be disrupted. In instances where forage DM is above 40-42%, water-ammonia mixes or molasses-ammonia mixes should be used. Application for molasses-ammonia mixes should be as recommended by the manufacturer (Kung, 1985).

Due to the issues pertaining to length of pretreatment for ammonia steeping at ambient temperatures and the capital/energy intensive nature of ammonia steeping and ARP at elevated temperatures, integration of ammonia pretreatment with ensiling of lignocellulosic biomass provides an economically attractive alternative. This integrated process could allow for simultaneously enhancing digestion/pretreatment-fractionation via disruption of lignin bonds in addition to enhancing accessibility to cellulose similar to using ammonia steeping or ARP. However, the process would be conducted without regard for high liquid to solids rates and subsequent concerns for energy intensive processing and water separation, etc. The length of time exposed to ammonia during ensiling provides both a storage system for wet biomass and pretreatment of biomass via delignification and swelling of cellulose which increases surface area and access for enzymes during fractionation. Ammonia also provides an economical source of free amino nitrogen, an essential nutrient for microbes during fermentation.

This integrated approach would obviously have to be developed and fine tuned for the biofuels industry to limit acetic and lactic acid accumulation for most yeast fermentations. As discussed above, some researchers have evaluated enzyme inoculation for ensiling biorefinery feedstocks. If successful, ensiling crop residues and energy crops would serve as an economical storage and pretreatment process which would preserve both carbohydrates and protein contained in biomass feedstocks.

Once the biomass has been harvested, chopped, shredded and ensiled/pretreated, it is ready to be processed at the biorefinery. The ensiled biomass could then be mixed with water, processed via ultrasonics/microwave, and proceed directly with an accelerated ammonia steeping/SEAA process. This would be followed by alkaline oxidation using H_2O_2 to liquefy lignin and separate cellulose and hemicellulose. Oxidative cracking of precipitated hardwood lignin (PHL) by hydrogen peroxide (H_2O_2) in aqueous medium can proceed at relatively low temperatures (80-90 °C) in comparison with dilute acid cracking processes (130–160 °C) which produce low molecular weight lignin derivatives and organic acids. Subsequent to using SEAA to process ensiled biomass feedstocks and capture over 90% of xylan, dilute acid could then be utilized to further process hemicellulose into high value biochemicals. Separate processing of cellulose and hemicellulose, e.g. hexose vs. pentose sugars, allows for optimizing efficiency of hydrolysis and fermentation. Relatively pure sugar syrups which are produced from the hemicellulose fraction can be either refined or fermented.

Milling & Reducing Particle Size

Biomass particle size substantially affects pretreatment, enzymatic hydrolysis, and efficiency of fermentation. Particle size reduction increases the effective surface area to volume ratio; improving enzyme and microbial accessibility to active substrate sites (Mansfield et al., 1999). Uniform mechanical milling processes such as chopping and shredding followed by ultrasonics can substantially enhance efficiency of hydrolysis and fermentation by increasing physical access to polymers. As revealed above, when mixed with water during hydrolysis, shredded corn stalks produce 11% more leachates including polysaccharides than chopped stover, and 5% more than stalks that had been merely chopped and put through a roller. When the increased yields of polysaccharide sugars are considered along with the energy savings tied to shredding, more sugars and eventually more ethanol is produced for less cost. This is partially the result of increasing the surface area of the plant material by chopping and shredding which allows for better access to cellulose and hemicellulose. The result is a substantial reduction in the need for energy intensive pretreatment processes (Buckmaster, 2008; Zhang et al., 2003).

Though separating the more recalcitrant fractions (stalk- and cob-rich) of corn stover into stalk-, cob-, and leaf-rich fractions might be beneficial for improving hydrolysis and fermentation, adequate milling for reduction in particulate size followed by ultrasonification or exposure to specific microwave frequencies may provide the same benefits while reducing production costs. This is particularly true for fractionation platforms. Corn stover particle size reduction and washing can improve effectiveness of pretreatments and substantially improve sugar yields contained in hydrolyzates. For example, the time required for complete glucan hydrolysis of milled and washed AFEX corn stover (supplemented by commercially available xylanase) was reduced by 96 h, e.g. from 168 h to 72 h compared to the unwashed samples (Chundawat et al., 2006).

Ultrasonic cavitation further increases surface area of biomass (including organic waste feedstocks) in order to enhance efficiency of enzymatic and microbial processing. This process has been shown to be effective with corn starch and switchgrass (lignocellulosic) feedstocks, enhancing both liquefaction and saccharification. In experiments, corn slurry samples obtained before and after jet cooking were subjected to ultrasonic pretreatment for 20 and 40 seconds at amplitudes of vibration ranging from 180 to 299 microm (pp) (peak to peak amplitude in micrometers). The resulting samples were then exposed to enzymes (alpha-amylase and glucoamylase) to convert corn starch to glucose. A comparison of scanning electron micrographs

of raw and sonicated samples revealed the development of micropores and the disruption of cell walls in corn mash. The corn particle size declined nearly 20-fold following ultrasonic treatment at high power settings. The glucose release rate from sonicated samples increased as much as three fold compared to the control group. The efficiency of ultrasound exceeded 100% in terms of energy gain from the sugar released over the ultrasonic energy supplied.

Enzymatic activity of corn slurries is enhanced via sonication with simultaneous addition of enzymes as catalysts for hydrolysis (this is the process utilized in conjunction with either UHG SSF or UHG SSCF for both hexose and pentose sugars). This finding suggests that the ultrasonic energy did not degrade or denature the enzymes during the pretreatment. In addition, ultrasonic energy gelatinizes starch at relatively low temperatures (30-50 °C), much faster compared to heating. Hence, sonication appears to enhance raw starch hydrolysis via enzymes. Subject to verification, it is believed that sonication also promotes hydrolysis of the polysaccharides contained in lignocellulosic feedstocks (Grewell, 2008; Khanal et al., 2007). Various ultrasonic frequencies can possibly enhance both fractionation and hydrolysis processes. For switchgrass, sequential ammonia steeping-ultrasonic pretreatment released about 10% more fermentable sugars than did ammonia steeping alone. Similar results may be achieved for treating corn stover via fractionation and UHG SSF at integrated biorefineries (Montalbo-Lomboy, et al., 2007).

Optimizing Hydrolysis & Fermentation of Cellulose and Hemicellulose

Cohydrolysis of cellulose and hemicellulose via enzyme complexes results in conversion of 85.5% of glucan and 86.3% of xylan in corn stover to a liquid hydrolysate. Since lignin binds irreversibly to enzymes, this increases the enzyme loading rate required for efficient hydrolysis. Hence, removing lignin prior to enzymatic hydrolysis would reduce enzyme loading rate and increase efficiency of hydrolysis. In addition, contaminants released during hydrolysis of hemicellulose inhibits hydrolysis of cellulose (Novozymes' Cellic^{TM} CTec and HTec brochures, 2009). These factors appear to explain why 14-15% of glucan and xylan is not hydrolyzed in experiments in conjunction with sequential hydrolysis and cofermentation of hexose and pentose sugars (Lau and Dale, 2009). The hydrolysate used in cofermentations is comprised primarily of cellodextrin, xylose and other monomeric and oligomeric sugars. The 73 gal/tonne of ethanol produced represents 58.4% of theoretical yield (125 gal/tonne) for processing corn stover. Inefficient conversion of polysaccharides to monomeric sugars during hydrolysis is one of the primary limiting processes which decrease ethanol yields. Many yeasts and other microbes are incapable of fermenting oligosaccharides. Utilizing improved enzymes which are capable of converting close to 100% of olygomeric sugars to monomeric sugars via separate hydrolysis of relatively pure hexose and pentose sugars and/or utilizing ethanologens which care capable of fermenting both monomeric and oligomeric sugars would substantially improve ethanol yields.

The ethanologen utilized above is a genetically modified (recombinant DNA) *Saccharomyces cerevisiae* 424A(LNH-ST), an industrial yeast strain developed at Purdue University which is capable of cofermenting hexose and pentose sugars. Recombinant DNA is a form of synthetic DNA thereby combining DNA sequences that would not normally occur together. In terms of genetic modification, recombinant DNA is produced through the addition of relevant DNA into an existing organismal genome, such as the plasmid of bacteria, to code for or alter different traits for a specific purpose, such as immunity to sodium or tolerance to acetic acid. It differs from genetic recombination, in that it does not occur through processes within the cell or ribosome, but is exclusively engineered. The *S. cerevisiae* 424A(LNH-ST) strain is capable of fermenting both glucose and xylose to ethanol with higher tolerance for acetic acid than

conventional strains of distillers' yeast. Acetic acid is produced during hydrolysis of xylan and also by bacterial contaminants during fermentation.

In addition to enhancing tolerance of acetic in yeast strains, acetic acid can be prevented from inhibiting hydrolysis and fermentation of cellulose via separate hydrolysis and fermentation processes. This allows for separation of acetic acid as a high value coproduct prior to fermentation. Preliminary studies reveal that xylose fermentation is more sensitive to acetic acid than glucose fermentation when using *S. cerevisiae* 424A(LNH-ST) (Mosier et al., 2008). For integrated biorefineries, the ability to extract acetic acid and other organic acids including formic and levulinic acids provide opportunities for diversification and generation of additional revenue streams.

Efficiency of fermentation is based on the volume/concentration of ethanol produced during a certain time period. This is controlled by the loading rate of monomeric (fermentable) sugars, the fermentation rate, and yield for a particular ethanologen. The ability to incorporate UHG (increased titer/concentration of sugars) via SHF & SSF of cellodextrin and dextrin from starch for integrated biorefineries can dramatically decrease fermentation times to less than 27 hours for production of 18 v/v% ethanol for integrated biorefineries. This is in contrast to xylose fermentations which can require up to 90 hours for completion at higher pH and temperatures than those which are optimal for fermentation of cellodextrin and dextrin (Lau and Dale, 2009). Hence, separate hydrolysis and fermentation of xylan (pentose sugars) and glucan (hexose sugars) can increase fermentation efficiency and yields of xylose by over 3 fold. In addition to separate hydrolysis and fermentation of hexose and pentose sugars, a small volume of dextrin or glucose can be fed to pentose fermentors to further enhance fermentation efficiency for SSF and SSCF processes. Eventually, consolidated bioprocessing (CBP) holds much promise for using synthetic biocatalysts for simultaneous hydrolysis and fermentation of cellulosic hydrolyzates.

In contrast to cohydrolysis and cofermentation, over 84 gal of ethanol per tonne of corn stover can be produced via separation of cellulose and hemicellulose fractions prior to hydrolysis and fermentation. This represents a 14% increase in conversion efficiency. Fractionation provides over a 67% conversion efficiency of total carbohydrates in corn stover. Fractionation and separate hydrolysis provides the potential to eventually achieve 80-95% conversion efficiencies via ongoing development of SSF and CBP processes.

With current technology, ethanol yields can be substantially increased for processing the majority of corn biomass via separate hydrolysis and fermentation of xylose and glucose sugars. For 200 bu/acre corn crop yields, this would be equivalent to producing 550 gal/acre from corn, and 323 gal/acre from corn stover. Hence, ethanol yields produced per acre with current technology would be 873 gal/acre. This is based on processing corn biomass consisting of 50% corn and 50% stover with 75% of the corn stover harvested. 25% of stover would be spread in the field (in addition to stubble and root ball) in order to maintain SOM.

For incorporation of SDF and high density cropping systems via precision agriculture, 400 bu/acre crop yields could be consistently achieved. Processing the majority of the plant would allow for production of 20.32 tonnes of corn biomass per acre, 17.78 tonnes of which can eventually be fermented via mature processing technologies. For processing 80-95% of all carbohydrates in corn biomass, this would result in producing yields of up to 2,045 gal of anhydrous ethanol (99.5% ethanol), and 104.73 gal of corn oil produced for each acre of corn planted. These figures are based on production of 3.3 gal of ethanol per bushel of corn which is

achieved by processing cellulosic portions of corn grain, e.g. pericarp and endosperm fiber fractions along with corn stover via lignocellulosic biomass pretreatment and fractionation processes.

Ammonia & Development of Universal Pretreatment/Fractionation Processes

"Lignin in lignocellulosic feedstocks causes inhibition of enzymatic hydrolysis and microbial activity in ethanol fermentations. Besides the goal of reducing compounds that may inhibit fermentation of sugars to ethanol, pretreatment is also required to either partially remove or break up the lignin structure, so that enzymes can diffuse into the cellulose polymer and degrade it to monomeric fermentable sugars (cellodextrin). While a variety of pretreatment methods have been developed and tested at lab-scale (Wyman et al., 2005), pretreatment of biomass remains one of the most costly steps in lignocellulosic biofuels production and affects subsequent operations (Lynd et al., 1996).

For example, improvements in pretreatment can reduce the amount of enzymes (e.g., cellulases) used (Lynd et al., 1996). Teymouri et al. (2005) indicated effective enzymatic hydrolysis of ammonia fiber explosion (AFEX)-treated biomass at enzyme loadings as low as 7 FPU/g of glucan could be achieved by adjusting the pretreatment parameters. Kim et al. (2002) reported the enzymatic digestibility of corn stover treated by the ammonia recycled percolation to be 90% with an enzyme loading of 10 FPU/g–glucan. Although many biological, chemical, and physical methods have been attempted over the years, further development of pretreatment methods is needed to reduce overall costs of lignocellulosic bioconversion (Wyman, 1999).

Dilute acid treatment, water pretreatment with pH control, AFEX, ammonia recycle percolation (ARP), and lime pretreatment are among the most promising and most studied technologies (Wyman et al., 2005). Many of these pretreatment methods, however, require high temperature and/or high pressure. The extreme conditions used to increase the digestibility of the biomass decrease the reaction time required for the pretreatment, but they increase capital and operating costs. Extreme conditions may also cause the formation of compounds that are inhibitory to the fermentative organisms, and they may cause degradation of some fraction of the fermentation substrate. For these reasons, ambient temperature and pressure pretreatments are of interest.

Removing lignin with alkaline chemicals to improve cellulose digestibility and ammonia steeping/soaking at room temperature has been previously studied on several types of biomass (Kim et al., 2005; Dominguez et al., 1997; Cao et al., 1996). The steeping method is a simple method that does not require high pressures and high temperatures. Ammonia soaking of corn stover at room temperature can remove as much as 74% of the lignin, but retain nearly 100% of the glucan and 85% of the xylan (Kim et al., 2005). Sequential akaline oxidation processes using hydrogen peroxide (H_2O_2) allow for further removal of lignin. After treatment, both the cellulose and hemicellulose in corn stover become highly susceptible to enzymatic digestion. However, ammonia steeping requires a relatively high liquid to solids ratio that subsequently requires energy intensive separation processes to remove water prior to fermentation, particularly for achieving ultra high gravity (UHG) simultaneous saccharification and fermentation (SSF).

Currently, SSF is one of the most commonly used processes for ethanol production (Teymouri et al. 2005; Kim et al., 2002; Wyman et al., 2005; Iyer et al., 1996; Chang et al., 2001; Alizadeh et al., 2005; Chang et al., 1997; Kim et al., 2005). This process combines two steps in the same vessel to generate ethanol: enzymatic break down of the complex sugars primarily to glucose and xylose and fermentation of these sugars to ethanol by yeast and other ethanologens. This high

gravity approach for SSF via cellodextrin and dextrin substrates has been widely adopted because of the reduction of glucose inhibition during enzymatic hydrolysis. In addition, the risk of bacterial contamination and capital investments are lower, as both the hydrolysis and the fermentation steps take place in the same reactor (Isci et al., 2008).

Eventually, pretreatment and fractionation processes integrated with consolidated bioprocessing (CBP) may allow for surpassing the efficiency of SSF. Unlike SSF which is dependent upon production of exogenous enzymes, CBP utilize recombinant thermophilic ethanologens which produce the majority of their own enzymes.

Ammonia Steeping & Ammonia Recycling Percolation (ARP)

"Pretreatment of biomass with liquid ammonia exerts a strong swelling action and brings about phase change in the cellulose crystal structure from I to III. The benefits of this method include breakage of glucoronic acid ester cross-links, solubilization of lignin, and disruption of crystalline structure, swelling and increase in the surface area of cellulose. Pretreatment with liquid ammonia has been utilized mostly to increase *in vitro* digestibility of animal feeds, a process which serves a similar purpose for enhancing enzymatic hydrolysis via SSF.

"Batch treatment of corn stover feedstocks with aqueous ammonia (15–30 wt%) at 40–90 °C for 6–24 h. The optimum treatment conditions were found to be 15 wt% of NH3, 60 °C, 1 : 6 of solid-to-liquid ratio, and 12 h of treatment time. The treated corn stover retained 100% glucan and 85% of xylan, but removed 62% of lignin. The enzymatic digestibility of the glucan content increased from 17 to 85% with 15 FPU/g-glucan enzyme loading, whereas the digestibility of the xylan content increased to 78%. The treated corn stover was also subjected to a SSCF test using Spezyme-CP and recombinant Escherichia coli (KO11). The SSCF of soaked aqueous ammonia treated corn stover resulted in an ethanol concentration of 19.2 g/L from 3% (w/v) glucan loading, which corresponds to 77% of the maximum theoretical yield based on glucan and xylan (Kim and Lee, 2007)." Wheat straw treated with 50% ammonia hydroxide provides 20% delignification with a three fold increase in hydrolysis.

Ammonia Steeping & Dilute Acid

An improved pretreatment method involving two steps has been developed (Cao et al., 1996; Dominguez et al., 1997; Gong et al., 1997; and Tsao et al., 1996):

- steeping lignocellulosic biomass in aqueous ammonia at ambient temperature removes lignin, acetate, and extractives, followed by
- dilute acid pretreatment that hydrolyzes the hemicellulose fraction which is collected and washed.

Up to 90% of lignin can be removed through the ammonia steeping step (Cheng, 2001)."

Integration of this sequential separation process with advanced dilute acid separation processes could possibly provide over 90% pure cellulose, hemicellulose, and lignin fractions in addition to production of sugar syrup, furfural and a variety of organic acids from the hemicellulose fraction.

Alkaline Oxidation

This pretreatment process combines alkaline and oxidative pretreatments. Pretreatment with H_2O_2 in an alkaline environment or combining it with a preceding alkali treatment-step (ammonia steeping) provides an effective pretreatment of lignocellulosic biomass feedstocks. In weak alkaline media, H_2O_2 only selectively acts on phenolic compounds originated from partial

scission of lignin, causing its degradation without affecting the cellulosic fraction. Only the lignin and hemicellulose are solubilized. This treatment removes approximately 50% of lignin in wheat straw and corn stover. Sugarcane bagasse treated with 2% H_2O_2 at 30 °C for 8 h solubilized about 50% of lignin and most of the hemicellulose. Subsequent saccharification by cellulose at 45 °C provides 95% conversion to glucose in 24 h (Sun and Cheng, 2002). H_2O_2 to substrate ratio of 0.25 g per gram of substrate at 25 °C, pH 11.5, were the optimum pretreatment conditions. The lignin degradation products were not found to be toxic either for saccharification or fermentation. Reuse of the solvent up to six times after the initial pretreatment is possible.

"Pretreatment of two different softwood-based lignocellulosic wastes (newsprint and Kraft pulp mill sludge) was investigated. Pretreatment was conducted with aqueous ammonia and hydrogen peroxide (H_2O_2), two delignifying reagents that are environmentally benign. Three different treatment schemes were employed: ARP, mixed stream of aqueous ammonia and H_2O_2, and successive treatment with H_2O_2 and aqueous ammonia. In all cases there was a substantial degree of delignification ranging from 30 to 50%. About half of the hemicellulose sugars were dissolved into the process effluent. Retention of cellulose after pretreatment varied from 85 to 100% for newspaper feedstock and from 77 to 85% for the pulp mill sludge. After treatment with aqueous ammonia alone (ARP), the digestibility of newspaper and the pulp mill sludge was improved only by 5% (from 40 to 45% for the former and from 68 to 73% for the latter), despite a substantial degree of delignification occurring after the ARP process. The lignin content thus did not correlate with the digestibility for these substrates. Simultaneous treatment with H_2O_2 and aqueous ammonia did not bring about any significant improvement in the digestibility over that of the ARP. A successive treatment by H_2O_2 and ARP showed the most promise because it improved the digestibility of the newspaper from 41 to 75%, a level comparable to that of alpha cellulose (Kim et al., 2000)."

Oxidative Cracking for Production of High Value Lignin Derivatives

"Precipitated hardwood lignin (PHL) is a major byproduct in the biomass to ethanol process. Oxidative cracking of PHL by hydrogen peroxide (H_2O_2) in aqueous medium was investigated as a means to produce potentially useful chemicals. The cracking reaction takes place at moderate temperatures (80–160°C), giving mono- and dicarboxylic acids as the main products. The yields of these products are in the range of 30–50% of initial lignin. The reaction mechanism and the product distribution are dependent upon the reaction conditions, especially the pH. The reaction under strong alkaline condition proceeds well even at low reaction temperatures (80–90°C). Under acidic conditions, higher temperatures (130–160°C) are required to attain the same degrees of cracking. The reaction patterns of the oxidative cracking reaction involve the cleavage of lignin ring, aryl ether bond, or other linkages within lignin. By using the findings of this investigation and those of previous work, we have illustrated the reaction pathways for degradation of PHL under alkaline and acidic conditions. Aldehydes and aromatic acids are intermediate products in the oxidative degradation of lignin. However, they were produced only in trace amounts owing to rapid degradation induced by hydrogen peroxide (Xiang and Lee, 2000).

Soaking in Ethanol & Aqueous Ammonia (SEAA)

"A new process for pretreatment of lignocellulosic biomass was developed to improve hemicellulose preservation in solid form. In the SEAA process, an aqueous ammonia solution containing ethanol is used. Corn stover was treated with 15 wt% ammonia at 1:9 solid–liquid ratio (by weight) at 60 °C for 24 h with ethanol added at 1, 5, 20, and 49 wt% (balance was water). The extents by which xylan was solubilized with no ethanol and with ethanol added at 1,

5, 20, and 49 wt% of the total liquid were 17.2%, 16.7%, 14.5%, 10.4%, and 6.3% of the original xylan, respectively. Thus, at the highest ethanol concentration used the loss of hemicellulose to the liquid phase was reduced by 63%. The digestibility of glucan and xylan in the pretreated corn stover samples by cellulase was not affected by ethanol addition of up to 20 wt%. The enzymatic digestibility of the corn stover treated with 49 wt% ethanol added was lower than the digestibility of the sample treated with no ethanol addition.

Universal SEAA Fractionation Platform

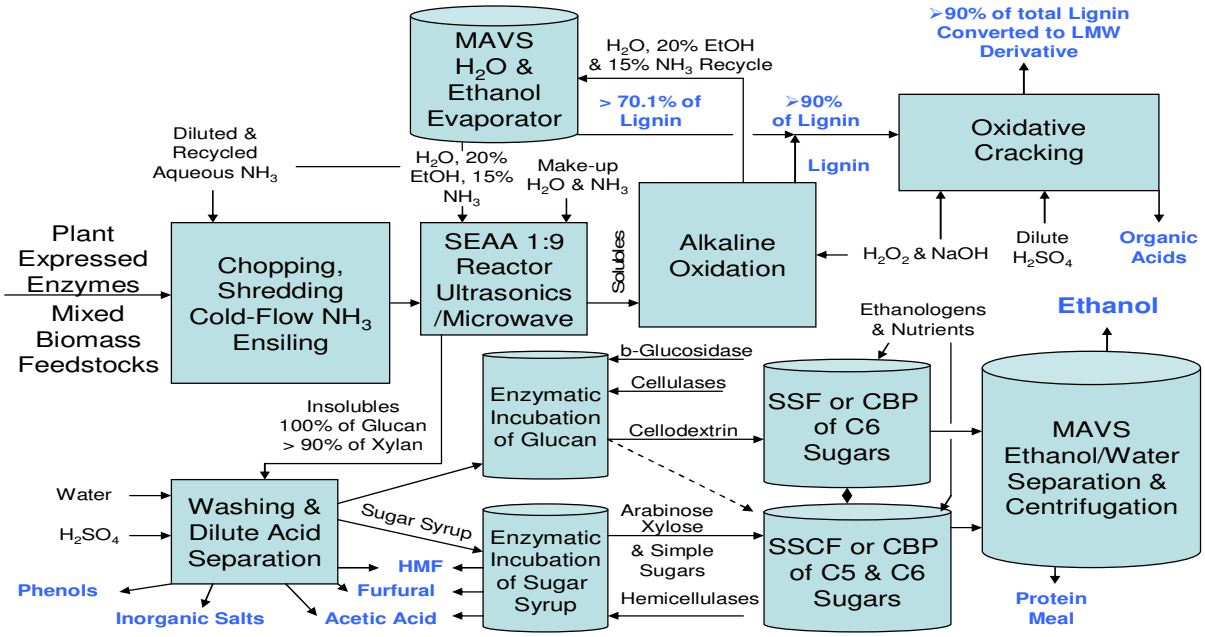


Figure 12. Integration of SEAA, Alkaline Oxidation & Oxidative Cracking of Lignin

Thus, based on these results, 20 wt% was found to be the optimum ethanol concentration for use in the SEAA process for pretreatment of corn stover. With optimum ethanol concentration at 20 wt%, the carbohydrate-rich solid sample obtained from the SEAA treatment contained 51.4% glucan, 26.7% xylan, and 6.2% lignin, which were almost 100% glucan retention, 89.6% xylan retention, and 70.1% lignin removal, respectively. The SEAA was proven to be an effective method to produce the carbohydrate-rich solid cake, which can be used for production of fuels and chemicals. It is anticipated that the ethanol used in the pretreatment process could be recovered and recycled about six times before the original ethanol stream is exhausted. Hence, an ethanol make-up stream is required to maintain the 20% ethanol in the aqueous ammonia solution (Kim et al., 2009)." The ethanol that is not recycled during the SEAA process would be distilled and sold as fuel so that this process would not significantly decrease ethanol yields.

As utilized in pretreatment and fractionation processes, dilute acid is ideal for liquefying and separating hemicellulose from cellulose, and preparing lignin for cracking. Dilute acid can also be integrated for extracting furfural, organic acids and sugar syrups (which contain primarily xylose, arabinose and mannose) from hemicellulose.

Ensiling feedstocks with ammonia and enzymes, reducing particle size via ultrasonics or microwave, and incorporation of the alkaline oxidation step could allow for increasing xylan

separation and total lignin extraction to over 90%. Pure low molecular weight lignin produced by oxidative cracking would allow for generating additional revenue streams via development of high value chemical products for industries such as the bioplastics markets.

Autohydrolysis & ARP

"A two-stage autohydrolysis treatment followed by treatment with aqueous ammonia, both applied in a flow-through (percolation) reactor. Autohydrolysis provides hemicellulose removal whereas the aqueous ammonia is intended for delignification. The pretreated material was nearly pure cellulose and both reagents are economical and environmentally benign. With proper operation of two-stage treatment, fractionation of biomass was achieved to the extent that the xylan fraction is hydrolyzed with 92–95% conversion, and recovered with 83–86% yields; and the lignin removal is 75–81%. The remaining solid after two-stage treatment contained 78–85% cellulose. The two-stage treatments enhanced the enzymatic digestibility to 90–96% with 60 FPU/g of glucan, and 87–89% with 15 FPU/g of glucan. In two-stage treatment, the composition and digestibility data indicate that the lignin content in the biomass is one of the major factors controlling the enzymatic digestibility (Kim and Lee, 2006)."

Universal ARP Fractionation Platform

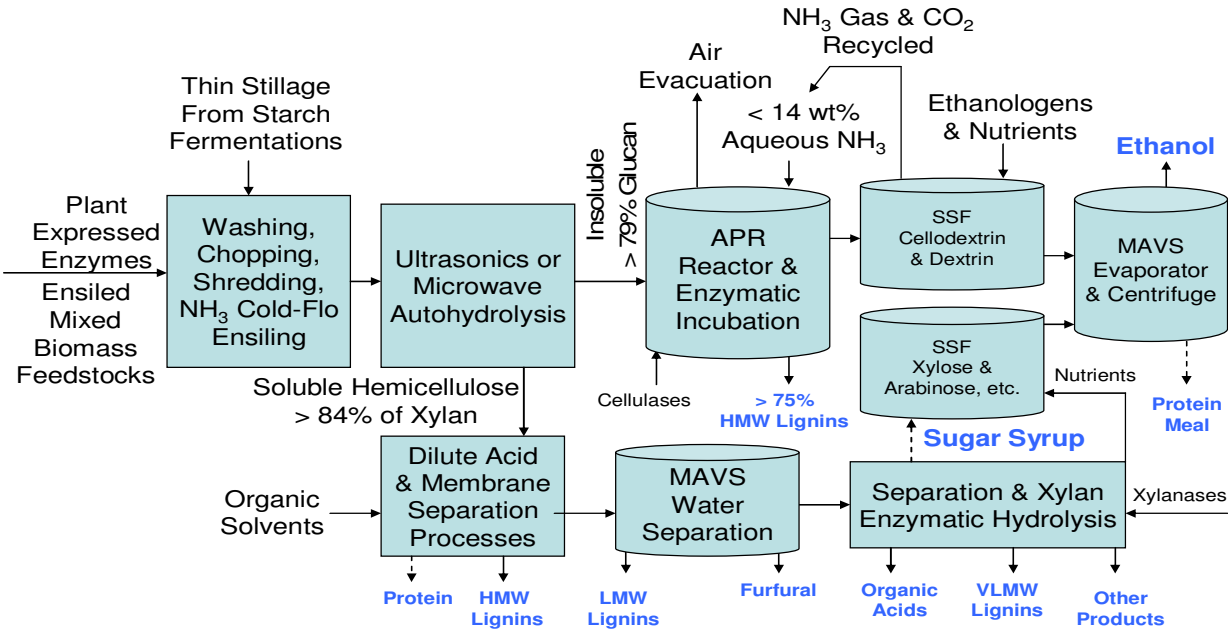


Figure 13. Integration of Autohydrolysis, ARP & Dilute Acid Pretreatment-Fractionation

Membrane Assisted Vapor Stripping (MAVS)

Depending on efficiencies of ethanol and water removal from various fermentation broths containing different ethanol concentrations (starch vs. lignocellulosic biomass processing), achieving optimal energy efficiency may require separate water-ethanol removal systems, particularly for production of hydrous vs. anhydrous ethanol.

In this hybrid system, the stripping column provides a high alcohol recovery and a low effluent alcohol concentration while the vapor compression membrane component enables the efficient recovery of latent and sensible heat from both the retentate and permeate streams from the membrane system. No rectification column is used. The actual costs associated with the

compressor and membrane components of the MAVS system in Figure 14 recovering 1 MGY of ethanol from a 5 wt% ethanol feed stream using a 35 °C stripper were determined to be as low as \$0.11 gal-EtOH^{-1} (\$0.036 kg-EtOH^{-1}) and the energy usage was about 3 MJ-fuel equiv kg-EtOH^{-1} (Vane and Alvarez, 2008).

This proposed MAVS process has been estimated to require as little as 8.9 MJ-fuel/kg-EtOH to produce 99.5 wt% ethanol from a 1 wt% ethanol feed stream, only 2.5 MJ-fuel/kg-EtOH for a 5 wt% ethanol feed stream (Vane and Alvarez, 2008; Vane et al., 2008). Comparable heat integrated distillation-molecular sieve systems would require at least 19.2 and 6.2 MJ-fuel/kg-EtOH for 1 and 5 wt% ethanol feed streams, respectively. To produce a 99.5 wt% ethanol product from a broth containing 11.5 wt% ethanol the MAVS process would require as little as 2.2 MJ-fuel/kg-EtOH (Vane et al., 2008). Thus, the MAVS hybrid ethanol separation process yields energy savings of at least 54% compared to the benchmark distillation-adsorption process. Côté *et al*. have proposed a hybrid distillation-vapor permeation process which replaces the rectification column and molecular sieve components of the conventional ethanol-water distillation-adsorption system with a vapor permeation dehydration system – such a process is referred to as the Siftek™ Dewatering System (Cote et al, 2007; Cote et al., 2008). Compression of the water rich permeate vapor from the first of two membrane stages allows for recovery of the latent heat from that vapor stream as heating steam in the reboiler of the beer column. (Cote et al., 2008; Vane, 2008)

Membrane Assisted Vapor Stripping (MAVS)

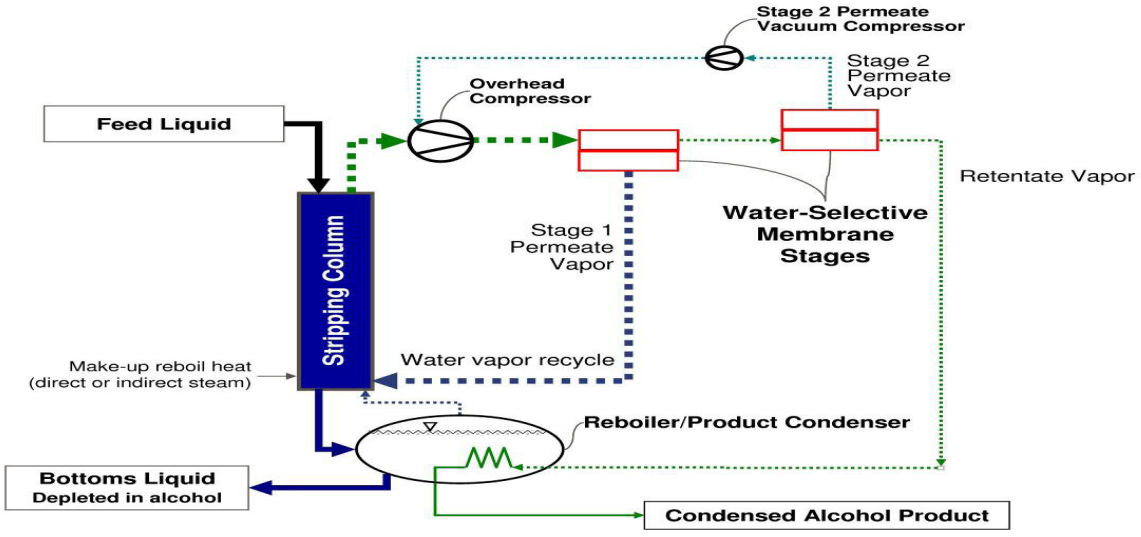


Figure 14. Schematic diagram of a hybrid ethanol-water separation process (Vane, 2008)

In summary, reductions in fuel-equivalents usage of 50% or more for MAVS vs. distillation + sieves - independent of feed concentration are commonly observed. Since this refers to fuel-equivalents, it should translate directly into greenhouse gas emissions reductions of 50% or more for that part of the separation process. This type of GHGE, associated with the production process is occasionally referred to as "upstream GHGEs".

MAVS data have been compiled for separating 5 wt% ethanol (circa 6 vol%). Distillation + sieves would require between 6.2 and 7.2 MJ-fuel/kg-EtOH to produce 99.5 wt% ethanol.

Estimates for the MAVS system would require 2.5 MJ-fuel/kg-EtOH for the same product purity. That assumes 90% fuel-to-heat boiler efficiency for distillation, 33% fuel-to-electricity efficiency, and 75% compressor efficiency. Varying some membrane parameters and process pressures/temperatures, the MAVS number can be as low as 2.2 and as high as 3.1 MJ-fuel/kg-EtOH. Though data has not been compiled for an 8 wt% ethanol scenario (circa 10 vol%), it is estimated that distillation + sieves would require 4.5 to 5.5 MJ-fuel/kg-EtOH and MAVS would require 2 to 2.5 MJ-fuel/kg-EtOH for producing a 99.5 wt% ethanol product.

"The hybrid stripping-vapor permeation MAVS approach is predicted to be more energy efficient and cost effective than distillation for the recovery of ethanol from ethanol–water mixtures and ultimate production of fuel-grade ethanol. The hybrid system takes advantage of the high ethanol recovery and low effluent concentrations offered by a stripping column with the high selectivity and ability to return the stripping phase made available by the vapor permeation system. Within the stripping options studied, steam stripping had the lowest potential energy usage and cost with estimated fuel-grade ethanol production costs of US$0.16 gal^{-1} from a feed containing 5 wt% ethanol and US$0.37 gal^{-1} from 1 wt% ethanol. These are both well below the current (December 2007) price of ethanol in the USA of ca US$2.00 gal^{-1}.43 Thus, even low concentration ethanol waste streams could be upgraded economically to fuel-grade ethanol using the hybrid MAVS technology. For example, if considered in the context of wastewater treatment, the MAVS technology would cost US$5 to treat 1000 gallons of 1 wt% ethanol wastewater. This same volume of wastewater would yield 12.6 gallons of fuel grade ethanol worth US$25, thereby more than paying for the treatment. Other separations which can be performed with the MAVS system include other alcohol–water systems and even mixtures of organic solvents in which efficient stripping column VLE and selective membranes can be combined advantageously (Vane and Alvarez, 2008)."

The Siftek™ Dewatering System is similar to the MAVS system, though the latter has some advantages according to patent applications which were recently published. One of biggest differences is the source of the driving force for membrane transport. As the patent application defines it, MAVS compresses the overhead vapor from the stripper to generate the feed vapor for the membrane process. The Siftek design does not compress the feed to the first membrane unit, it uses a vacuum pump on the permeate side of the membrane unit to generate the driving force. In most cases, the stripping column in MAVS will be under a vacuum and the temperature of the column can be controlled by changing the vacuum pressure. In Siftek, the stripping column has to be fairly hot and above ambient pressure to deliver a reasonable membrane feed pressure and, therefore, driving force. The MAVS technology decouples the feed pressure to the membrane unit from the pressure in the stripping column which can be advantageous, particularly if you don't want to kill organisms, deactivate enzymes, precipitate out proteins, etc. Vaperma has announced a partnership with UOP for marketing their Siftek technology (Ethanol Producer Magazine, Mar, 2009).

Protein Separation

An advantage of utilizing ammonia technologies for pretreatment and fractionation platforms is the ability to eliminate urea or ammonia supplements for enhancing efficiency of microbial fermentations. This allows for protein extraction processes and production of high value supplements and mixed feedstuffs for animals including integrated CAFOs.

Production of protein will not only bolster process economics but also increase land efficiencies by allowing the production of both fuel and animal feed on the same acre. The NHOC "Growing

Energy" report estimates the co-production of animal protein could lower the cost of cellulosic ethanol by $0.11-$0.13 per gallon, depending on the size of the production facility.

The leaves and stems of the plants are the source of protein found in cellulosic biomass feedstocks. The protein, referred to as leaf protein, is used in animal feed. Agricultural residues contain 4-6 six percent protein while crops like switchgrass and alfalfa contain 10% and 15-20% respectively. Leaf protein is extracted from the feedstock utilizing an alkaline water solution heated to 50-60 °C. Standard membrane filtration technology is employed to separate the protein from the other feedstock components. Up to 60% of the protein can be harvested with membrane technology and up 90% can be extracted with extensive washing. (Greer, 2005; Biomass Conversion Research Laboratory)

Extractives
In addition to polysaccharides and lignin, lignocellulosic biomass consists of extranesous substances often referred to as extractives (Fan et al., 1987, §2.1]. The extraneous substance refers to all the non-cell wall materials. Based on their solubility in water and neutral organic solvents, they can be classified as extractives, e.g., terpenes, resins and phenols, and non-extractives (mainly inorganics present in ash minerals), e.g., alkali, alkali earth carbonates and oxalates.

Integrated Biorefineries & Closed Loop Production
Closed loop production via integrated biorefineries reduces exposure to commodity markets and provides the best risk management strategy available as a hedge against market volatility. Purchasing feedstocks and energy currently represent the majority of inputs for biorefineries. Overhead costs are about $0.24/gal for conventional biorefineries. Hence, a $0.29 per gallon differential between the price of corn and the rack price of ethanol is required in order to make $0.05/gal net profit. Along with fertilizer and petroleum prices, commodity markets for corn, natural gas, and ethanol determine sustainability of the biofuels industry. Closed loop production and adoption of innovative technologies provides diversification and additional revenue streams resulting from marketing of high quality foods, bioproducts, provision of an abundant and free energy resource in the form of organic waste, and trading carbon credits. Unprecedented efficiencies of operation can be achieved while simultaneously reducing pollution and GHGEs via integrated biorefineries and closed loop production systems which leverage existing corn ethanol infrastructures for processing lignocellulosic feedstocks.

Fiber and germ meal can be utilized as livestock feeds, particularly for ruminants which utilize microbial populations and exogenous enzymes for efficient digestion of fibrous feedstuffs. Fractionation of corn stover biomass and subsequent fermentation of cellulose and hemicellulose fractions is predicted to produce over 84 gal/tonne via fractionation using current technology. This is a 68% conversion rate in comparison with a theoretical yield of 125 gal/tonne for corn stover. The current industry average is less than 60%. Enzymatic hydrolysis-SSF and CBP may eventually increase ethanol yields to 90%, e.g. over 112 gal/tonne of corn stover biomass.

Ag-Energy Parks & Municipal BioEnergy Complexes

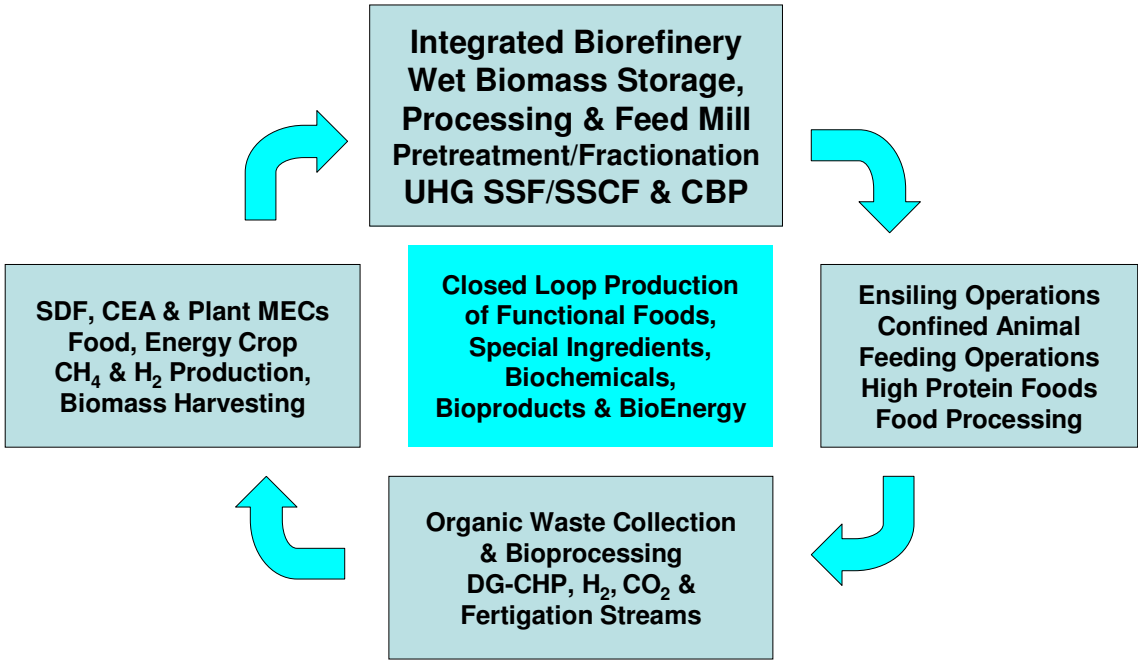


Figure 15. Comprehensive Nutrient-Energy Management: Sustainable Closed Loop Systems Architecture

Integration of lignocellulosic biorefineries with existing corn ethanol plants will substantially reduce energy requirements and capital investment. It also allows for recycling nutrient-rich thin stillage which contains undigested sugars for value added conversion to ethanol via industrial yeasts. The corn ethanol plant would provide power and additional heat via thermal integration and CHP technology. This is particularly advantageous for future use of lignin in higher value products rather tan using it as a combustible fuel to generate process steam.

Integration of processing cellulosic and starch feedstocks could benefit from fed batch systems which utilize sequential hydrolysis of cellulose to glucose. Glucose hydrolysates are inserted into fermentors utilizing SHF. This hybrid SHF/SSF process would feed glucose from hydrolysis of cellulose into dedicated cellulose and/or starch/dextrin fermentors at a rate that would not exceed the sugar concentration threshold for actively growing distillers' yeast or industrial yeast strains. This would allow for optimizing ethanol concentrations and fermentation efficiency and integration of processing cellulose and starch, thereby leveraging existing corn ethanol infrastructures.

Clean fractionation platforms allow for separation of lignin and optimizing efficiency of hydrolysis via separate processing of glucan and xylan fractions. Separation processes could include ammonia pretreatment/ensiling followed by SEAA, alkaline oxidation, and dilute acid in order to liquefy and separate lignin and hemicellulose. Lignin could be processed into high value low molecular weight (LMW) and very low molecular weight (VLMW) derivatives via oxidative cracking. Glycan and xylan would then be hydrolyzed via a combination of acid and enzymatic hydrolysis.

Contaminants such as acetic acid and furfural would be removed from hydrolysates as valued added products through dilute acid or membrane separation technologies prior to fermentation.

The xylose hydrolysate remaining after separation of acetic acid and furfural could be fermented separately from cellulose via pentose fermentors which utilize the thin stillage containing unfermented sugars from starch and cellulose fermentors as a value added backset. In order to optimize efficiency of xylose/sugar syrup fermentations, a certain amount of glucose could be fed to pentose fermentors via integrated biorefineries. Since fermentation of xylose can require upwards of 90 hours in comparison with less than 24 hours for glucose fermentations, separate hydrolysis and fermentation of xylose substrates would allow for optimizing fermentation efficiency and ethanol yields for lignocellulosic biomass.

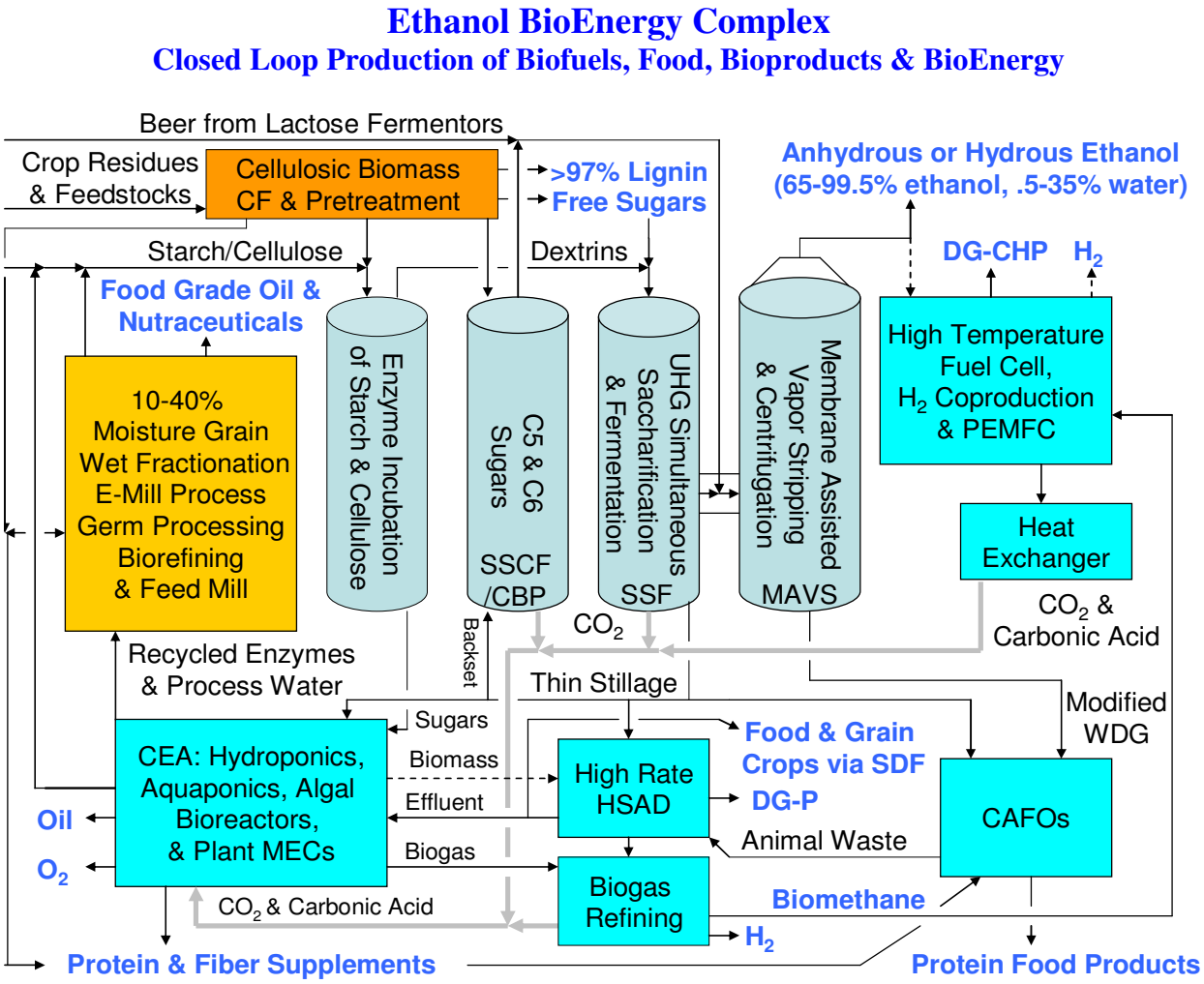


Figure 16. Ultra High Gravity Starch SSF, Split-Stream SSCF & CBP Integration of High Temperature Fuel Cells & MFC/MEC BioEnergy Technologies

Algal Starch & Lipid Feedstocks

"Algae are amenable to relatively simple genetic manipulations aimed at increasing photosynthetic efficiency, maximizing yields of desirable energy storage products, and optimizing conversion of photosynthetic products to fuels or chemical feedstocks." (US Department of Energy) There are over 100,000 different algae strains on earth which comprise the primary food source at the base of the food chain. It is the world's largest source of biomass.

Production of plant and algal oils rich in essential fatty acids (primarily omega-3s and GLA) offer much hope for the future of bioeconomies via integrated biorefineries and closed loop architecture which simultaneously produce food and bioenergy. Synthetic algae strains range from as high as 75% oil, to as high as 70% starch. Microalgae strains high in starch contain no lignin and limited amounts of cellulose in cell walls. Therefore algae comprised primarily of starch can be utilized to produce low cost dextrose and simple sugars for integrated biorefineries. In addition to use as starch feedstocks, algae strains high in oils can be utilized for production of high value nutraceuticals such as omega-3 oils DHA and EPA, phospholipids, and antioxidants.

The above example for fractionation of corn and producing corn oil at integrated biorefineries illustrates the economic advantages provided by integrated biorefineries and innovative technologies. The ability to produce either biodiesel or food grade corn oil (with a current retail value of $7/gal) provides flexibility, diversity, and additional revenue streams, thereby providing sustainability via simultaneous production of biofuels, bioproducts and high quality foods. However, algae oil is much higher in omega-3s (long chain polyunsaturated fatty acids) than corn oil and hence considerably more valuable as a high value nutraceutical coproduct. Algal oil is very appealing for production at integrated biorefineries which incorporate bioreactors in the form of fermentation vats (heterotrophic production from sugars and cellulosic hydrolyzates).

Fats (fatty acids) are chemicals made up of long chains of carbon atoms surrounded by hydrogen atoms. When a carbon chain is attached to the maximum number of hydrogen molecules that it can hold, it is called a saturated fat. When one or more carbon atoms are joined by double bonds, the fat is unsaturated. These double bonds allow the molecules to be easily broken down and used for a variety of essential body functions.

Omega-3 fatty acids have many different uses in the body. They are essential for the production of new cells and give flexibility to cell membranes, allowing for better absorption of minerals and nutrients. Doctors indicate that modern food processing, pollution, unhealthy lifestyles, and poor eating habits have resulted in 90% of Western populations requiring omega-3 fatty acid supplements.

With combined global retail sales over $300 billion, today's cosmetic, nutraceutical and functional food markets are exciting growth opportunities driven by increasing consumer demand for natural, science-based products that deliver real benefits. Martek, the world's largest algal DHA provider, currently generates revenues of over $400 million annually.

Essential Fatty Acids, Phospholipids & Antioxidants

Omega-3 fatty acids obtained from algal biomass retail for over $4,500/gal ($600/lb). This is in comparison with biodiesel which retails for about $3-$4/gal ($0.40-$0.53/lb). The nutraceutical astaxanthin, a super antioxidant also obtained from potentially processing algal biomass at integrated biorefineries retails for over $1,700/lb. Production of omega-3s and astaxanthin (nutraceutical and pharmaceutical grade products) represent an exponential increase (150-850 orders of magnitude) in value in comparison with liquid biofuels. Obviously, there are strong economic and environmental advantages for integrating production of high value foods and other coproducts with production of commodity biofuels, all of which displace petroleum products and reduce economic dependence on fossil fuels. This is accomplished via integrated biorefineries which simultaneously reduce pollution and GHGEs.

Ethanol biorefineries provide nutrient-rich biological effluents which can be utilized in conjunction with anaerobic digestion to provide value added fertigation streams for controlled environment applications (CEAs). This includes algal bioreactors and hydroponic greenhouses. In addition to value added fertigation streams, waste heat, CO_2 and low cost sugars produced from crop residues and organic waste provide opportunities to produce biomass with unprecedented efficiencies via closed loop architecture.

Food Ingredients: Emulsifiers such as those produced by ADM from vegetable oils (lecithin made from soy or sunflower oil, and possibly algal oil, etc.) for the food industry range in price from a low of $0.50/lb for a standard fluid lecithin to $10/lb for a highly purified sucrose ester. Most emulsifiers used as food ingredients would be priced in the $1-$5/lb range and would be used to solubilize hydrophobic oils/etc into water-based systems. The revenue generated by these types of high value food products provide diversification. In addition, commercial scale marketing does not usually saturate the market, so specialty food ingredients generally maintain value and provide relatively higher values than commodity based biofuels which currently retail for about $0.36/lb.

Vegetable and algal oil emulsifiers could also be utilized in the cosmetics and personal care industries in with a higher product price can be absorbed. A nutrient-rich product such as development of fruit drinks and other functional foods which incorporate omega-3s while providing emulsification could add substantial value. ADM has offered to provide testing to determine value for simultaneous emulsification and nutrient enrichment for projects of mutual interest/value which commercialize algal oils. Algal oil appears to be much more promising for processing as emulsifiers than either fish or krill oil. These types of functional ingredients and enriched functional foods high in DHA, DPA, EPA and phospholipids could potentially wholesale for much more than double the price that ($5-10/lb) current food ingredients/emulsifiers wholesale for. The same proprietary processing of soy and sunflower oils used by ADM could be utilized for processing algal oils. Total use of emulsifier for food/nutritional/cosmetics/personal care (all food-grade products) should be around 575,000 metric tons in 2010. About half of that total would be phospholipid-based emulsifiers.

Nutraceuticals: Omega-3s such as algal DHA, DPA & EPA currently retail for as much as $4,500 gal. Phospholipids can enhance bioavailability of omega-3s by 50-70%. Super antioxidants such as astaxanthin can also increase bioavailability and provide added value for algal nutraceutical and pharmaceutical grade products produced via heterotrophic production of algal biomass from lignocellulosic feedstocks.

Emulsification: During the development of Pharmax's WisdOM-3™ omega-3 product it was discovered in human clinical trials (Garaiova et al, 2007) that we normally absorb less than 50% of the omega-3 contained in standard cod liver oil or fish oil. However, if the fish oil is emulsified using WisdOM-3™, then the absorption level doubles to a full 100%. In effect WisdOM-3™ doubles the absorption of omega-3s DHA, DPA & EPA.

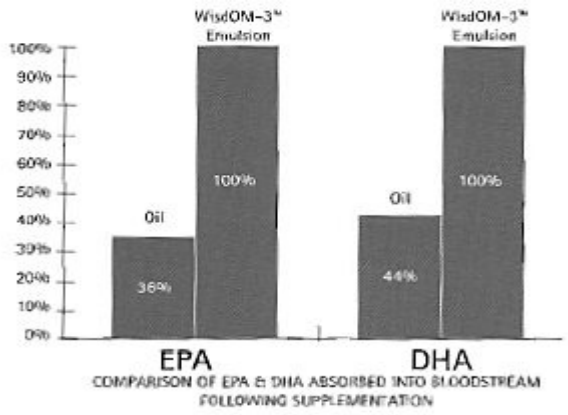


Figure 17. Comparison of EPA & DHA Absorption Rates in Bloodstream

When we digest most fats and oils in our diet, the emulsification that naturally occurs in the stomach and intestine is sufficient to ensure that complete absorption takes place. However, the problem with the most important omega-3's, EPA and DHA, is that they are very long molecules and our digestive tract finds them much more difficult to digest and absorb. The result is that over half are left behind and not absorbed, and is probably the reason why people often complain of 'repeating' after taking cod liver and other fish oils.

WisdOM-3™ emulsification also has the benefit of allowing other nutritional ingredients such as vitamins, plant sterols and glucosamine to be added, enabling development of a variety of nutraceutical products with multiple benefits.

Finally WisdOM-3™ causes oils to lose their 'oiliness' and also allows natural flavors and fruit concentrates to be added, making WisdOM-3™ products delicious to take either directly from the spoon or mixed into fruit juices or smoothies.

The following Pharmax products are made using the WisdOM-3™ process:

- Frutol
- Berry Frutol
- High DHA Berry Frutol
- Frutol with Full Spectrum Multivitamins
- Finest Pure Fish Oil - Emulsified
- Finest Pure Cod Liver Oil - Emulsified
- Cardol

The WisdOM-3™ emulsions offer a further alternative to the traditional capsule and oil products normally associated with omega-3 oils. Because of the enhanced absorption, you can effectively double the levels of EPA and DHA on the label when comparing with that stated for normal oils.

In addition, WisdOM-3™ emulsified oils have other unique advantages:

- They taste great—both for children and adults. So if you don't like taking lots of capsules and don't like the mouthfeel of oils, then products made using WisdOM-3™ offer a great alternative.

- Combination nutrients—products like the unique Cardol combine oils and other cardiovascular nutrients to produce probably the most complete cardiovascular supplement available anywhere. Cardol can only be made using the WisdOM-3™ process.

Pharmaceuticals: Enriching omega oils for the pharmaceutical and nutraceutical industries is possible via low temperature fractionation, a process known as crystallization. The low temperature enables saturates and mono-unsaturates to be transformed into crystals which facilitates the physical separation of the polyunsaturated stream. The removal of most saturates and monounsaturates leaves polyunsaturates in higher concentrations. The oils can then be deodorized for maximum palatability.

The key advantage in using crystallization is that the resulting enriched triglyceride or phospholipids have undergone no chemical manipulation, and so the natural state of the oil is maintained. It also allows the enriched oil to be marketed as natural, and is 'label friendly'. The technique can be applied to any marine or botanical oil, and will enrich EPA, DHA, GLA, & Free Fatty Acids.

Equateq, the developer of the crystallization technology, splits its business between pharmaceutical and nutraceutical clients. Concentrations for the pharmaceutical industry are about being “super pure”, whereas the nutraceutical industry is concerned with providing customized blends. All of Equateq’s ingredients are manufactured to pharmaceutical standards. Essentially, crystallization offers high-dose omega-3 potential (www.equateq.com).

Pharmax provides both enzymatic and cryogenic fractionation processes. Pharmax is the only company to use tWO different techniques to naturally concentrate both fish and cod liver oils. NEO-3™ uses an enzymatic process to concentrate oils while CRYO-3 uses a low temperature fractionation process similar to Equateq. The nature of the original fish oil and the required characteristics of the resultant concentrate dictate whether NEO-3™ or CRYO-3 is used in the concentration process.

Many premium fish oil products are processed to produce higher levels of EPA and DHA. However, almost all of these processes involve both chemicals and alcohol in the enrichment process and result in the fish oil being converted into a 'mixture of ethyl ester', where the individual fatty acids, including EPA and DHA are bound to alcohol molecules. The ethyl esters that contain the shorter chain fatty acids are lighter than those containing long chain fatty acids (such as EPA and DHA) and hence can be 'boiled away' in the process known as molecular distillation, leaving a higher concentration of the longer chain fatty acid (EPA and DHA) esters. This mixture either becomes the finished product (many softgel capsules contain ethyl esters), or further processing takes place to re-convert the ethyl esters back into triglycerides. These triglycerides however have a different configuration of fatty acids to that found in the original fish oil.

As such, virtually all concentrated fish oil products on the market are either ethyl esters or reconstituted triglycerides—both of which are unnatural. This is particularly true of ethyl esters, which are completely *de novo* molecules that are not naturally found in any whole food product.

The CRYO-3 Process
When subjected to low temperature, triglycerides that have a high proportion of saturated fatty acids start to solidify, due to the higher 'freezing point' of saturated fatty acids compared to unsaturated fatty acids such as EPA and DHA.

The CRYO-3 process has now been optimized whereby the fish oil is cryoprotected as it is gradually taken to very low temperatures, enabling accurate fractional crystallization or 'precipitation' of the triglycerides carrying high levels of saturated fatty acids. Once the solid fractions have been separated, the remaining liquid fraction has a higher ratio of EPA and DHA.

This process is used by Pharmax to produce its high EPA omega-3 oils and it may be possible to use it for processing algal oil.

Plant Oils
Much emphasis is placed on our requirement for fish, krill and algal oil derived EPA and DHA, and while these two fatty acids are of utmost importance, the precursor omega-3 fatty acid, alpha linolenic acid (ALA) is an equally important part of our overall omega-3 intake. Alongside the omega-6 fatty acid, linoleic acid, ALA is the only other universally recognized essential fatty acid. The richest sources of ALA are plant oils, particularly flaxseed oil, which is approximately 50%-60% ALA by weight, making it an important source of omega-3 fatty acids for vegetarians.

Pharmax research reveals that flaxseed oil is more resistant to oxidation than is generally believed, particularly if it contains a potent antioxidant system such as Multox. Pharmax research shows that high quality flaxseed oil can be kept at ambient temperature after opening and even used in short duration/low temperature cooking for up to 15 minutes without any significant increase in peroxidation value (Figure 18).

As such, flaxseed oil-containing products should be considered a cornerstone for any program aimed at boosting overall levels of omega-3 intake.

The Pharmax Omega 3:6:9 Range
According to Pharmax, fresh flaxseed oil is so flavorsome, the best way to increase intake is to use it as a culinary oil. With the Omega 3:6:9 range, flaxseed oil has been blended with a mixture of extra virgin olive, sesame and evening primrose oils to produce a delicious, nutritionally balanced culinary oil to be used as a salad dressing or in short duration cooking. Other oils such as CLA (conjugated linoleic acid) and phytosterols are added for those individuals interested in weight or cholesterol reduction respectively.

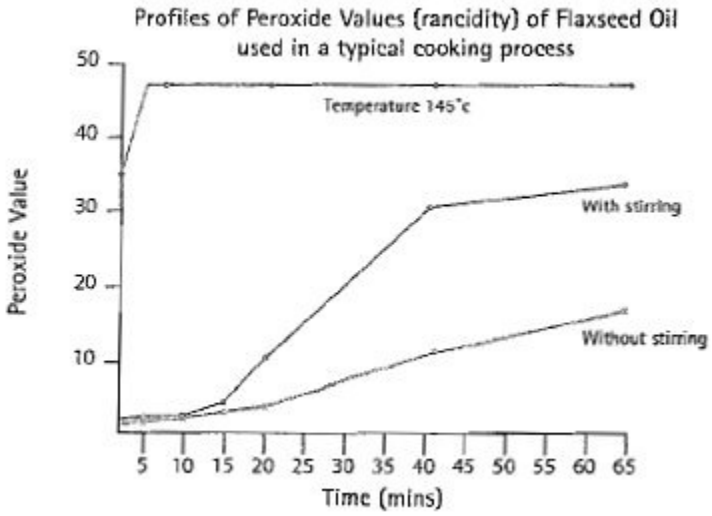


Figure 18. Profiles of Peroxide Values (rancidity) of Flaxseed Oil used in a Typical Cooking Process.

Ensiling & Integration of CAFOs

Though lignin is not chemically reactive during fermentation or anaerobic digestion processes, it is biodegradable via certain fungi in soils and aerobic ensiling processes when utilized with wet distillers grains in silage feeds for livestock (Garcia and Kalscheur, 2006; Kalscheur and Garcia, 2004; Trenkle, 2007; Barsaul and Talapatra, 1971), and subsequently as an organic fertilizer for closed loop production of biomass. Ensiling allows for simultaneous production of protein foods and organic waste biomass using an economical bioprocess which is much less energy intensive than gasification and other thermochemical conversion processes. Ensiling is similar to the carboxylate platform utilized for biorefining. Both biochemical processes utilize a combination of aerobic respiration and anaerobic fermentation via specific microbial populations.

Ensiling corn distillers grains (DG) with lignin and fermentation residues is an excellent feed for ruminants. DG can usually be purchased as either wet (40-70% moisture) or dry feedstuffs. Due to lipid and fiber/carbohydrate content, conventional DDGS supply approximately 22% more energy than corn grain. Though nutrient composition for DDGS can vary substantially depending on several factors, on average they are comprised of approximately 30% protein, 10% fat and 1% phosphorus. These are high priced nutrients and thus desirable in animal feeds. Fiber digestibility varies across different feedstuffs. Feeds high in fiber (lignocellusloic biomass) can supply variable energy depending on their digestibility. For some by-products (e.g. conventional DDGS), a significant portion of the energy supplied comes from fat/oil content.

Not all feeds that have higher fiber content are necessarily lower in energy. Corn silage, for example, has an energy content comparable to that of soy hulls even though soy hulls have 33% more non-detergent fiber (NDF). On the other hand, feeds (crop residues) like straws and corn stalks are high in NDF and, as expected, low in total digestible nutrients (TDN). The protein content of high fiber feeds is in general medium to low. One exception to this “rule” is conventional DDGS products which have a NDF content of close to 40% and a protein concentration of approximately 30%. This results from the fermentation of 93% of the starch in corn to ethanol and the subsequent three-fold concentration of all other nutrients (protein, fat, fiber and minerals) (Garcia and Kalscheur, 2006; Kalscheur and Garcia, 2004).

Modified distillers grains (MDG) resulting from fractionation contain 58.5% protein, 4.53% fat, 3.25% ash, and only 2.03% NDF. NDF is reduced by 81% in comparison with conventional DDGS. MDG allows for higher inclusion rates in rations and higher feed to conversion rates for livestock and poultry in comparison with conventional DDGS and whole corn feeds (Singh et al, 2005). Lignin and fiber which are separated from grains, crop residues and energy crops (lignocellulosic biomass) during fractionation processes can be ensiled with WDG to increase digestibility and then utilized as value added feedstuffs for ruminants.

In addition to wet distillers' grains (WDG) products, a small portion of undigested residues remaining after fermentation of lignocellulosic feedstocks such as corn stover, sweet sorghum/sugar cane bagasse, switchgrass/miscanthus, and water hyacinth can also be ensiled to provide balanced feedstuffs for animals. For example, water hyacinth which is a rapidly growing invasive species and is ideal for biomass production via CEA, has been successfully ensiled with molasses, hay and concentrates for use as a feedstuff for swine (Manh, et al.) and ruminants (Kumar).

Silage is the feedstuff resulting from the preservation of WDG and green forage crops by acidification. Acidification is the result of the fermentation of the forage in the absence of oxygen. There are two main phases in the ensiling process. The first is the aerobic phase which occurs in the presence of oxygen (air). Oxygen is present in the forage as it is placed in the silo or concrete bunker. This oxygen is consumed by the living plant material through the process of respiration. Under aerobic conditions plant enzymes and microorganisms consume oxygen and burn up the plant water-soluble carbohydrates (sugars) producing CO_2 and heat.

CO_2 can be captured and utilized to enrich plant growth for production of biomass feedstocks for integrated operations. The length of this phase is variable depending on ensiling conditions; it could last for a few hours or for as long as several days. It is good silage making practice to limit this phase as much as possible since water-soluble carbohydrates are being consumed and other nutrients are being destroyed. The heat generated by an extended aerobic phase can raise the temperature of the ensiling forage material sufficiently to cause heat damage. Good silage making practices reduce the amount of time that aerobic microorganisms and oxidizing plant enzymes are able to function. This is accomplished by chopping the silage to a short length, packing it thoroughly, and effectively sealing the silo or concrete bunker.

The second or anaerobic phase begins when the available oxygen is used up through plant respiration and aerobic bacteria cease to function. Anaerobic bacteria (bacteria that grow in the absence of oxygen) then begin to multiply rapidly and the fermentation process begins. Ideally the microorganisms which grow most rapidly will be predominately lacto~ bacilli species which produce lactic acid from the fermented plant material. The lactic acid which is produced will lower the pH of the silage. Fermentation completely ceases after 3 to 4 weeks when the pH becomes so low that all microbial growth is inhibited.

If ensiling procedures are such that lactic acid producing bacteria aren't favored, clostridial type microorganisms will grow. These organisms utilize plant water-soluble carbohydrates, lactic acid and protein for growth and produce butyric acid. The quality of silage is greatly reduced if a clostridial type of fermentation predominates.

In addition to *Lactobacilli* and *Clostridial* microorganisms, silage also contains yeasts, molds, coliforms, bacilli and propionic acid producing bacteria. In addition to utilization of plant sugars as energy sources, silage microorganisms degrade protein to amino acids, amines and ammonia during fermentation. Literally hundreds of fermentation products are formed in addition to lactic and butyric acids (Ensiling, Alberta Agriculture and Rural Development, 2008)." Once ensiled or pretreated, lignocellulosic biomass resources can be more efficiently digested via microbial populations in ruminant livestock. Animal waste can then be processed via anaerobic digestion to produce biogas and value added organic fertilizers, particularly for closed loop biomass production.

In addition to ensiling, enzymes are aiding in digestion of ethanol coproducts which are included in animal diets. Use of enzymes in fibrous diets such as DG can enhance feed efficiency and reduce nutritional concerns for livestock and poultry. Enzymes and the effects of feeding yeast in DG products can essentially eliminate the need for ionophores, particularly for natural production programs. "Evidence suggests that the benefits of exogenous enzymes is synergistic to ruminal endogenous enzymes. This synergy appears to explain why relatively small amounts of enzymes can have such large positive effects on animal productivity (Beauchemin et al., 2003). Enzymes help bridge the gap between actual digestibility of the feed that occurs *in vivo* and the potential digestibility of the feed that would be possible if the conditions are ideal (Beauchemin et al., 2008a; Beauchemin et al., 2008b)."

Storing Wet Biomass Feedstocks

The same ensiling process utilized for production of silage can be modified and manipulated to process and store wet biomass for use as high moisture feedstocks for integrated biorefineries. Ensilage is a truncated solid-state fermentation in which anaerobically produced organic acids accumulate to reduce pH and limit microbial activity. Ensilage can be used to both preserve and pretreat biomass feedstock for further downstream conversion into chemicals, fuels, and/or fiber products (Ren et al., 2006). Ensiling enhances sugar conversion and enzyme-added ensiling further increases the conversion rate. Sugar yield after enzymatic hydrolysis was 23 to 31% for hays, 31 to 45% for non-enzyme-added silages, and 49% to 59% for enzyme-added silage in this study. Ensiling with or without enzyme added has little effect on sugar conversion of straws (Chen et al., 2006). Ensiling has also been successfully utilized to store sweet sorghum for use as a biomass feedstock, thus eliminating some of the concerns for dry storage of this particular feedstock (Phillip et al., 2007).

Harvesting of high moisture feedstocks can reduce agricultural production, handling, and storage costs by over 10% for both food and energy crops. This is partially accomplished by eliminating energy intensive drying processes currently utilized for storing and transporting corn, cereal grains, and other crops. Conservation of water resulting from harvesting wet biomass feedstocks also reduces the volume of process water required for production of ethanol. Siouxland Energy & Livestock has been utilizing a concrete bunker system for storing 17% moisture corn for several years now. The moisture rate for ensiling biomass feedstocks could be potentially increased to over 35% for well managed operations.

The primary concern for ensiling biomass feedstocks is preventing organic acids (particularly acetic acid which is produced by bacteria) from reaching inhibitory levels during ensiling and particularly during yeast fermentations. For brewers yeast, acetic acid levels must be kept below .05 w/v% in broths/slurries in order to achieve optimal fermentation. Lactic acid, which is produced by yeast and other fungi, must be kept below .8% w/v%. Acetic acid is less of a

concern for bacterial ethanologens and industrial yeasts as long as the majority of carbohydrates are converted to ethanol in order to maintain ethanol yields.

With progressive management it is possible to consistently produce corn silage that consists of .2-.5 w/v% acetic acid and less than 1.0 w/v% lactic acid. When diluted by a ratio of one part feedstock to five parts process water, acetic acid concentrations in ensiled feedstocks would be reduced by 2.5 fold to .0375 to .0938 w/v% in fermentation slurries. Lactic acid would be reduced to well below the .8 w/v% threshold concentration. Though the higher range of acetic acid becomes inhibitory, through proper management of ensiled feedstocks, acetic acid concentrations could be kept below .2 w/v%. This is accomplished through adequate wilting to achieve dry matter (DM) content of at least 28% for ensiling livestock feeds which will inhibit effluent losses. However, for use as a storage system for wet biomass feedstocks for biorefining, moisture content must be less than 33%, e.g. 67% DM.

Fermentation DM losses are more difficult to convert to CO_2 losses because there are so many different pathways that can result from the silo fermentation. To minimize these losses inoculation with an inoculant containing strains of homofermentative lactic acid bacteria is required as these maximize the production of the 'good' silage acid - namely lactic acid- and reduce other 'bad' acids -namely acetic and butyric acids. Again we can assume that for every gram of acetic acid in the silage analysis 0.733 g of CO_2 is also produced. Data taken from a recent publication from the Institute of Grassland and Environmental Research (IGER) showed that the acetic acid concentration in inoculated silage (10 g/kg DM) was significantly lower than that of the concentration in untreated silage (27g/kg DM). If we convert this back to 1 tonne then the inoculated silage would have produced 7.33 kg of CO_2 compared to 19.79 kg CO_2 for the untreated silage. Oxygen ingress into the silage will also encourage acetic acid rather than lactic acid production and increase DM losses and CO_2 production. Similar to ensiling livestock feeds, it is possible that CO_2 emissions produced by feedstock silage could be utilized for enriching biomass production via CEA.

In regards to acetic acids levels in fermentation slurries, as long as fermentation environments are optimized for actively growing yeast, acetic acid produced by competing bacteria will be minimized. After fermentations are complete for corn feedstocks, ethanol concentrations usually range between 17-20 w/v%, e.g. about 5-6 parts water. For high moisture feedstocks such as potatoes and lignocellulosic biomass, ethanol concentrations can be as low as 6-8%, e.g. over 12 parts water. This is equivalent to a 4-12 fold dilution of organic acids which are contained in ensiled feedstocks. For integrated biorefineries, the thin stillage from UHG corn ethanol fermentations would be utilized as nutrient-rich backsets for SSCF and CBP fermentations. This will prevent acetic acid from reaching inhibitory levels. Most of these fermentation technologies utilize synthetic bacteria and thermophilic ethanologens rather than yeast. Hence, these microbes are not as susceptible to stress from acetic or lactic acid concentrations.

Harvesting crops at near physiological maturity allows for increasing agricultural production via continual cropping systems. For sub-tropical and tropical climates, up to four corn crops can be produced annually via high moisture harvesting. For temperate climates, relay cropping systems utilizing cool season crops can also increase agricultural production. For example, after high moisture crops such as corn are harvested, triticale, barley, oats or winter wheat is immediately planted. The cool season crops, often referred to as bumper crops, are then harvested and ensiled prior to planting corn in the spring. High moisture harvesting and relay cropping systems can increase total crop yields by over 50%. High moisture harvesting and ensiling can also be

utilized for conservation of carbohydrates/nutrients and storing biomass feedstocks such as crop residues and perennial energy crops such as switchgrass and miscanthus.

Potential for Ensiling Lignocellulosic Feedstocks

The potential of using ensiling, with and without supplemental enzymes, as a cost-effective pretreatment for bioethanol production from agricultural residues was investigated. Ensiling did not significantly affect the lignin content of barley straw, cotton stalk, and triticale hay ensiled without enzyme, but slightly increased the lignin content in triticale straw, wheat straw, and triticale hay ensiled with enzyme. The holocellulose (cellulose plus hemicellulose) losses in the feedstocks, as a result of ensiling, ranged from 1.31 to 9.93%. The percent holocellulose loss in hays during ensiling was lower than in straws and stalks.

Ensiling of barley, triticale, wheat straws, and cotton stalk significantly increased the conversion of holocellulose to sugars during subsequent hydrolysis with two enzyme combinations. Enzymatic hydrolysis of ensiled and untreated feedstocks by Celluclast 1.5 L-Novozyme 188 enzyme combination resulted in equal or higher saccharification than with Spezyme® CP-xylanase combination. Enzyme loadings of 40 and 60 FPU/g reducing sugars gave similar sugar yields. The percent saccharification with Celluclast 1.5 L-Novozyme 188 at 40 FPU/g reducing sugars was 17.1 to 43.6%, 22.4 to 46.9%, and 23.2 to 32.2% for untreated feedstocks, feedstocks ensiled with, and without enzymes, respectively. Fermentation of the hydrolysates from ensiled feedstocks resulted in ethanol yields ranging from 0.21 to 0.28 g/g reducing sugars (Ye Chen et al., 2007).

Microbial Engineering & Consolidated Bioprocessing (CBP)

Similar to utilizing microbial processes for ensiling, biochemists are currently analyzing microbes contained in the guts of termites in order to mimic the efficiency at which lignocellulosic biomass is enzymatically processed and subsequently digested/fermented. A recent patented process called Organic Hydrolysis™ is based on lignin-consuming organisms (*Xylophagous cellulosomes* found in the intestines of termites). These microbes allow for processing alternative feedstocks at biorefineries. Acid hydrolysis and expensive pretreatment processes for lignocellulosic biomass are eliminated. This patented system claims the ability to produce up to 325 gallons of biofuels per ton of biomass when combined with an algae photobioreactor for biodiesel production (PR.com Press Release; For Fuel Freedom). This approach is similar to that being conducted at the University of California's Berkeley lab which is mapping the genome of termite microbes.

British Petroleum (BP) has recently announced that it has selected UC Berkeley and other affiliates to contribute $500 million for development of liquid biofuels primarily for the transportation industry via the Energy Biosciences Institute. "UC Berkeley was a pioneer in the development of genetic engineering and today is the leader in synthetic biology, the genetic manipulation of bacteria or yeast to turn them into microbial factories. It also has been a leader in applying biotechnology to the development of new drugs and the engineering of plants. The campus's top-ranked departments of plant and microbial biology, molecular and cell biology, chemistry and chemical engineering, and its College of Engineering — complemented by LBNL's state-of-the-art research tools, such as the Molecular Foundry and the Advanced Light Source — provide a unique foundation for tackling the problem of converting biomass into fuel. The institute, with about 25 faculty-level principal investigators housed at UC Berkeley and the University of Illinois, will concentrate on three aspects of the biomass-to-biofuel equation: developing feedstocks; creating techniques for breaking down plant material to its sugar building

blocks; and finding ways of fermenting the sugars into ethanol. These 'cellulosic' techniques could lead to process improvements for existing ethanol plants." (UCBerkeley News, 2007).

Similar efforts are being utilized by other research groups to enhance microbial processing via genetic engineering. This is leading to improved SSCF, CBP, fractionation technologies (for removing unfermentable portions of biomass feedstocks prior to fermentation), and ultra high gravity (UHG) fermentations. These technologies are reducing fermentation and biorefinery production costs by over 70% for simultaneous processing of simple sugars, starch, cellulose and lignocellulosic feedstocks for integrated biorefineries.

"Despite its simple chemical composition, cellulose exists in a number of crystalline and amorphous topologies. Its insolubility and heterogeneity makes native cellulose a recalcitrant substrate for enzymatic hydrolysis (despite its homogeneous chemical composition). Microorganisms meet this challenge with the aid of a multi-enzyme system. Aerobic bacteria produce numerous individual, extra-cellular enzymes with binding modules for different cellulose conformations. Specific enzymes act in synergy to elicit effective hydrolysis. In contrast, anaerobic bacteria possess a unique extracellular multienzyme complex, called cellulosome. Binding to a non-catalytic structural protein (scaffoldin) stimulates activity of the single components towards the crystalline substrate (Department of Microbiology, Technical University of Munchen)."

Similar to the cellulosomes produced by bacteria in termite guts, cellulosmes are also produced by *Clostridiunm thermocellum*. The thermophilic *C. thermocellum* is considered by several research groups to be the most complex and best investigated cellulosome. A scheme for the cellulosomes is slowly emerging. Many crucial details of the cellulose hydrolysis are still to be uncovered - yet, a mechanistic model for the action of enzyme-complexes on the surface of insoluble substrates becomes apparent and application of enzymatic hydrolysis of cellulosic biomass is now being addressed by several universities (Department of Microbiology, Technical University of Munchen), government research groups (Bioconversion Science & Technology, BioSciences Division, Oak Ridge National Laboratory), and private corporations (Mascoma).

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