Comprehensive Description of Dr. Ho's contribution to Science and Technology

Dr. Nancy Ho, a problem solver and a person with vision.

Dr. Ho's childhood was marked by numerous illnesses during which she spent much of her time convalescing and unable to be as active as other kids of the same age. This made her feel inferior to other children. She felt herself to be "worthless" and a burden to her parents. This is probably what seeded her strong desire to accomplish something worthwhile in her life and contribute to society if given the chance.

After Dr. Ho completed her Ph.D. at Purdue University, she stayed at Purdue for family reasons. Although she did not vigorously pursue a high-ranking academic position, she always thrived on solving important problems in science and technology. Two years after she received her Ph.D. from Purdue, she began to intensely study the development of a new method for fragmenting the structure of DNA – facilitating the analysis of its sequence. Back then DNA was still a "black hole" in molecular biology, with no suitable means to delineate its intricate structure. Her proposal for that project was the first formal proposal she had ever submitted; it received great reviews from the three agencies the proposal was submitted to – NIH, NSF, and the National Cancer Institute. They all awarded her grants. From that time on, she fully supported herself as well as her coworkers by relying on federal grants to continue her research at Purdue.

As a scientist, Dr. Ho's greatest strength has been her ability to critique her own work and appreciate other scientists' talent and innovation. After several years of hard work, the method that she was developing for determining DNA structure was proceeding well. However, she foresaw that the newly discovered restriction enzymes (at the time) were far superior for tackling the DNA structure. As such, she gave up her own pursuit and began working with these novel restriction enzyme-based recombinant DNA techniques. Within a year or so, she became an expert in recombinant DNA technology at Purdue.

The First Worldwide Energy Crisis

In the 1970s, the world suffered its first energy crisis. As a result, governments worldwide, particularly the US Government, strongly supported the development of alternative fuels for transportation that could be produced from domestically available renewable resources. Ethanol was thought to be an ideal renewable fuel. It could be produced by not only fermenting sugars derived from food crops – such as cornstarch and cane sugar – but also from cellulosic biomass. Cellulosic biomass (corn stover, rice and wheat straw, wood, grasses, waste papers, etc.) is the largest renewable resource in the world, particularly in the US. Thus, cellulosic biomass is the most attractive feedstock for the production of ethanol fuel via microbial fermentation of its sugar molecules. It was known even then that more than 70% of this resource could be converted to sugars and fermented to ethanol by microorganisms, preferably the *Saccharomyces* yeast known as the baker's yeast that has been used by mankind for thousands years to make wine and bake bread. However, the conversion of cellulosic biomass to ethanol required the development of new technologies because all cellulosic biomass contain polymers comprised of two major sugars – glucose and xylose.

The Natural Saccharomyces Yeast is Unable to Ferment Xylose to Ethanol

It was known at that time that the natural *Saccharomyces* yeast was unable to ferment xylose to ethanol. Thus, in the 1970s, research efforts were carried out worldwide to search for new microorganisms that could efficiently ferment both glucose and xylose to ethanol. However, no such natural microorganisms were found. By the end of the 1970s, scientists realized they would have to use recombinant DNA techniques to engineer the natural *Saccharomyces* yeast to enable it to ferment xylose. As such, many research groups worldwide, particularly those in the United States and Europe, feverishly worked to achieve this goal.

By then, LORRE (the Laboratory of Renewable Resources Engineering) at Purdue University had already become an established research group for developing various technologies for producing renewable fuels from cellulosic biomass. LORRE naturally wanted to participate in such important research.

Invited to Join LORRE to Lead the Efforts in Engineering the Yeast to Ferment Xylose to Ethanol

Dr. Ho, who was known at Purdue at that time as the leading expert in recombinant DNA techniques, was given a wonderful opportunity. In 1980, Dr Ho was sought by the Director of LORRE at Purdue University to help them modify the *Saccharomyces* Yeast using recombinant DNA techniques to ferment xylose together with glucose to ethanol. Even though, Dr. Ho had never done any research on yeast and had no knowledge or experience with biofuel research, she found the project extremely intriguing and accepted the invitation. Dr. Ho joined LORRE in the fall of 1980 to lead the program at LORRE to engineer the *Saccharomyces* yeast to ferment xylose, which had become a highly pursued endeavor worldwide. In the beginning, there were nearly ten international groups focused on developing such recombinant yeast with at least half of the groups in the US. Dr. Nancy Ho's group was the smallest. Furthermore, she had to solely obtain her own funding to support this project.

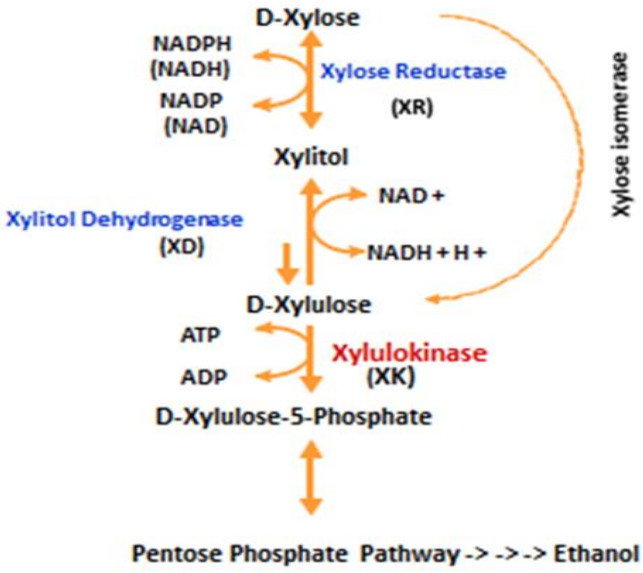
Successful Cloning of the First Xylose Isomerase Gene in Yeast

It was known at the time that yeast could use xylulose as a nutrient for growth, and could also ferment xylulose to ethanol. Thus, the *Saccharomyces* yeast was only **unable** to convert xylose to xylulose (see figure below). Theoretically, there were two approaches to engineering the yeast to metabolize and ferment xylose to ethanol:

One approach was to clone a xylose isomerase gene from bacteria to yeast since nearly all bacteria contain the functional enzyme, xylose isomerase, and can ferment xylose to ethanol. This approach is shown by the long curved arrow in the figure below.

The second approach was to clone the xylose reductase gene (XR) and xylitol dehydrogenase gene (XD) from other yeast (such as the *pichia* yeast). This would also theoretically enable the yeast to convert xylose to xylulose using xylitol as an intermediate. The cloned XR and XD genes would allow the yeast to produce the two enzymes, xylose reductase and xylitol dehydrogenase to catalyze the two reactions shown in the figure below.

Initially, all scientists involved in developing the engineered yeast used the easier approach by cloning a xylose isomerase gene. Dr. Ho's group did as well. She succeeded in cloning the first xylose isomerase gene from *E. coli* into yeast. Unfortunately, this approach could not produce a xylose-fermenting yeast. She showed that the enzyme produced by the cloned gene was not active in yeast. Dr. Ho knew this might happen because earlier scientists had shown that foreign genes cloned into a different organism were not guaranteed to function. In fact, since all bacteria have functional xylose isomerase, according to evolution, yeast species should also have functional xylose isomerase, assuming that yeast was evolved from bacteria. Thus, she did worry that the enzyme would not function in a yeast cell due to biochemical incompatibility even before she started the work. Subsequently, other scientists also cloned the xylose isomerase genes from a number of different bacteria, and none of the cloned genes could produce functional xylose isomerase in yeast. After these setbacks, the rest of the groups in the US that were working on the same project gave up on their pursuit. This was because most scientists worldwide believed that there would be insurmountable obstacles in trying to use the second approach by cloning the XR and XD genes from another yeast to make the *Saccharomyces* yeast ferment xylose to ethanol.



Thus, by 1985, there were only a few groups worldwide that persevered. Dr. Ho's group was the only US group that did not give up on this pursuit. That was also the time some of the scientists began to engineer bacteria to produce cellulosic ethanol (ethanol produced from cellulosic biomass) as a substitute to yeast. Dr. Ho was very much against engineering bacteria to produce cellulosic ethanol. This is because bacteria are not as stable as yeast, they can easily become mutated, and they are far less tolerant to ethanol and other inhibitors that are potentially present in the sugars extracted from cellulosic biomass. Besides, there might be other grievous consequences. This is because the conditions for using bacteria to produce ethanol is suitable for

most bacteria to grow including many bacteria that can make people sick or may even cause people to die. On the contrary, yeast ferments sugars to ethanol most effectively at low pH (pH around 5), which is not suitable for most bacteria to survive.

Dr. Ho is a scientist with strong convictions. It is her belief that with enough time and hard work, most scientific problems can be solved. She also had a specific plan that could allow the cloning of the XR and XD genes to make the yeast ferment xylose to ethanol. She was also passionate about enabling renewable cellulosic biomass to produce useful products, particularly renewable fuel. At the time, most of our cellulosic resources were considered as waste. She thought that it would be ideal if the safe effective *Saccharomyces* yeast could be made to harness these natural resources and produce renewable fuels for the world for generations to come. As such, she continued her work on engineering the yeast with great determination.

Successful Development of the Glucose/Xylose Co-fermenting Yeast

Her early realization that cloning the xylose isomerase gene in yeast might fail helped her prepare a strategy to use the second approach to make the yeast ferment xylose. The reasons most experts believed why the second approach of cloning the XR and XD genes in the yeast would fail because the enzyme xylitol dehydrogenase, produced by the XD gene, favors the catalysis of the conversion of xylulose to xylitol, not xylitol to xylulose. As illustrated in the figure above, the length of the arrow towards xylitol is longer than the length of arrow towards xylulose, which means the yeast is more efficient in producing xylitol than ethanol as the final product.

It was known at the time that xylulokinase was a very weak enzyme in the *Saccharomyces* yeast (only one tenth as strong as in other yeast such as the *Pichia* yeast). Dr. Ho knew that she had to improve the efficiency of this enzyme eventually since she was developing the yeast for the industrial production of fuel. The yeast should be made as efficient as possible for producing ethanol. With recombinant DNA technology, that is possible. However, she would only to do that when she was able to make the yeast ferment xylose first. Nevertheless, by early realization that the natural yeast produces very low xylulokinase activity that had helped Dr. Ho to form her idea on how to make the yeast to metabolize and ferment xylose by cloning the XR and XD genes from a xylose utilizing yeast such as the Pichia yeast. She realized that the xylolukinase produced in the natural yeast actually catalyze an irreversible reaction for conversion of xylulose to xylulose-5 phosphate, not the reverse reaction. Thus, she believed, if she included the cloning of a modified XK gene that allowed the yeast able to produce high xlulokinase activity together with the XR and XD genes from the Pichia yeast (or from other xylose utilizing yeast) in yeast, it would help "pull" the chain of reactions towards producing ethanol from xylose, not to produce xylitol. In 1993, Dr. Ho's Purdue group succeeded in developing the world's first genetically engineered yeast that could effectively co-ferment xylose with glucose to ethanol by cloning three highly modified genes, **XR (xylose reductase gene)**, **XD (xylitol dehydrogenase gene)**, and **XK** (**xylulokinase gene**) in the yeast.

Development of the "Stable Yeast" to Facilitate Cost Effective Industrial Production of Ethanol from Cellulosic Feedstocks

Dr. Ho's group at Purdue continued to improve the *Saccharomyces* yeast with the goal of making the recombinant yeast ideally suited for large-scale industrial production of cellulosic ethanol. In particular, they planned to make the cloned genes stably maintained in yeast without requiring the use of expensive chemicals, particularly antibiotics, which might have other undesirable consequences. Her solution was to develop a "stable" yeast by inserting all the necessary cloned genes into the yeast chromosome. In order to accomplish this, Dr. Ho invented a new technique that allows the insertion of multiple copies of multiple genes into the yeast chromosomes. This allowed her to insert all three genes, XR, XD, and XK together as a cassette into the chromosome in multiple copies - as many copies as needed to achieve the highest activity for yeast to ferment xylose to ethanol. In 1996, her group successfully developed the world's first "stable" yeast by inserting the XR-XD-XK genes in numerous copies into an industrial yeast, strain 1400, to make the recombinant 1400 yeast, designated as the 1400(LNH-ST) yeast, most efficient for co-fermenting glucose and xylose from cellulosic biomass to ethanol without requiring any chemicals to maintain the cloned genes in the yeast.

Chronological outline of the development of the ideal glucose/xylose co-fermenting Saccharomyces yeast by Dr. Nancy Ho and her co-workers at Purdue University:

1980-1985: The world's first scientist succeeded in cloning of a xylose isomerase gene in yeast.
1985-1988: Successful cloning and overproduction of xylulokinase in yeast.
1988-1993: Successfully developed the technology to genetically engineer any Saccharomyces (baker's) yeast to effectively co-ferment glucose and xylose to ethanol.
1993-1996: Successfully developed the stable engineered yeast with the cloned XR, XD and XK genes integrated (inserted) into the yeast chromosome.
1997-1999: Large-scale screening for better yeasts with no legal constraints for converting cellulosic sugars (mixed sugars recovered from cellulosic biomass) to ethanol.
2000-2006: Further genetic engineering of the best yeast, 424A (LNH-ST), to ferment other minor sugars and to test the production of high value co-products.

2006: The Establishment of Green Tech America, Inc. By 2006, Dr. Ho had no doubt that the Ho-Purdue Yeast strain 424A(LNH-ST) was the best microorganism in the world for the conversion of the sugars derived from cellulosic feedstock (glucose and xylose and other minor sugars) to fuels and chemicals. As such, Dr. Ho formally established Green Tech America, Inc. (GTA) in 2006 to facilitate the marketing of the Ho-Purdue yeast to benefit the whole world. Recently, the scientists at GTA have perfected the process to make the yeast produce high valued co-products during cellulosic ethanol production. These high-value co-products can be any important industrial enzymes or pharmaceutical proteins, which will make cellulosic ethanol production far more profitable. As such, ethanol produced by the yeast can be marketed at a much cheaper price to compete with gasoline.

2007-2012: Further improvement of the 424A(LNH-ST) to ferment cellulosic sugars (glucose, xylose, and other minor sugars) more efficiently and also more resistant to higher concentrations of ethanol and acetic acid. Two derivative strains – designated as 424A(LNH-ST)-ER and 424A(LNH-ST)-AR have been developed by the collaboration between her Purdue

Laboratory and GTA . These new derivatives can produce ethanol from the cellulosic feedstocks twice as fast. They are also much more tolerant to inhibitors present in the sugar mixtures. These latest improved derivatives of 424A(LNH-ST) can produce 10-12% ethanol in 24 to 36 hours with all sugars extracted from cellulosic biomass converted to ethanol.