NSERC BIOCONVERSION NEWS

Bioconversion News

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FROM OLYMPIAN TO ENVIRONMENTAL LEADER: HON. DAVID ANDERSON (O.C.), BRINGS POLITICAL EXPERTISE TO NETWORK OBJECTIVES

Nicole Harner, University of Guelph

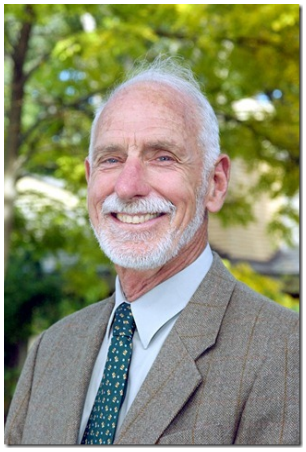
The NSERC Bioconversion Network is a Canadian research and development network aimed at streamlining the commercialization of biofuels and bio-refinery products. The network is guided by a diverse group of board members representing industry, academe, and government. Board members collectively bring knowledge and experience in forest and forest bio-products, and research and development. Board member Hon. David Anderson (O.C.), a distinguished former federal and provincial politician, brings decades of bureaucratic experience in governmental affairs, specifically the administration of environmental programs.

First elected Liberal MP for the constituency of Esquimalt-Saanich on Vancouver Island in the 1968 federal election, Anderson transitioned to British Columbia provincial politics as leader of the provincial Liberal Party in the 1970s. After this first stint in public life, he worked as an environmental consultant and adjunct professor teaching constitutional and administrative law and environmental policy at the University of Victoria's School of Administration. Anderson re-entered federal politics in 1993 as MP for Victoria. He won re-election in three subsequent elections and served for a decade in numerous cabinet positions, most notably as Minister of Fisheries and Oceans and Environment Minister.

Previous to his current work with the Bioconversion Network, Mr. Anderson's experience of the forestry industry was analyzing its impact on surface water run-off (temperature, volumes, timing), particularly on salmon and steelhead spawning streams. He had little direct involvement with by-products or biofuels but as Minister responsible for BC, he was involved in employment-related proposals for greater utilization and processing of wood fibre and wood waste.

As the network board member with the most experience in government, Anderson provides political context for research strategy. In other words, he suggests ways the issues can be presented, and suggests where in government they may find a receptive audience, in order to get the Bioconversion Network message heard.

During his time in public life, Mr. Anderson feels there was more emphasis on forestry and forest bio-products than there is today.



Hon. David Anderson

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"Today, the economy is more industrialized and diverse in comparison with 1968 when I was first elected an MP. Forestry is no longer the dominant industry that it was. True, the softwood lumber dispute took up a great deal of time in the nineties and early years of the last decade, so there was plenty of emphasis on the industry then, but that has declined, even though the Asian market has now soared. Part of neglect can be attributed to the rapid growth of Tar Sands, oil projects. These developments are extremely important and represent the bird in the hand. The assumption seems to be that biofuels will get their start elsewhere; meanwhile Canada is concentrating on the immediate opportunities that hydrocarbon development makes possible. There simply isn't the push that, in some countries, an impending energy shortage might provide."

Current state of environmental policy in federal politics

Anderson provides an interesting environmental perspective on the current state of federal politics. He admits the Liberal "Green Shift" of the 2008 election was unsuccessful, so there was no desire for the Liberals to make it an election issue in last year's federal election. The NDP, realizing the Green Party was going to attract its environmental vote, changed its position on carbon taxes and rarely mentioned climate change or any other environmental issue. The Conservatives continued their long held scepticism over climate change.

The presence of the Green Party and the lack of a serious effort by other parties to raise environmental issues during the election was contradictory, he mused. It seems that the environmental interest has been isolated with the Green Party; ironically the rise of the Greens has weakened environmental policies in the three main parties.

The Necessity of Government Support

Anderson is adamant that government support and investment in biofuels research is necessary. The enormous importance of the oil and gas industry in this country overshadows any other type of alternative energy work. "This is true for solar, wind and tidal power as well as biofuels. The exception, unfortunately, is ethanol biofuel from corn. The agricultural lobby is very strong, and much of the government support has been by way of subsidy for corn-based ethanol to satisfy that constituency. This is not the best basis for deciding research priorities."

On the forest biofuels and bioproducts front, David foresees Canada remaining behind other countries for the next ten to fifteen years. "There is not yet the economic or political drive that there should be, when you consider how much of the country is forest covered and how much raw material is generated by the forestry sector. In addition, long term or basic research is not a priority of the government. Short term commercial benefit has greater appeal to a business oriented government.

Even though we produce a substantial amount of grain-based ethanol, David still sees Canada lacking in biofuels funding and research. "With substantial hydrocarbon reserves in the Tar Sands, and substantial potential for both oil and gas in shale formations, we as a nation are following another path. However our potential is enormous, thanks to a very substantial forestry sector and a large source of waste wood from existing facilities. The raw material for forest-based biofuel should be plentiful and cheap. Further, as it would be a by-product of another manufacturing process, we may have substantial interest from an existing industry to take part."

Personal Life

Apart from his busy work schedule, Mr. Anderson maintains a repertoire of other hobbies and interests. An avid fly fisherman, he angles for trout on the Cowichan River in May/June and for salmon in Newfoundland in late June and early July. When in Victoria, he hikes the Sooke Hills on Tuesdays, sea-kayaks on Wednesdays, and gardens on Fridays. Every day, three times a day, he walks his two miniature schnauzers, who insist on an hour and twenty minutes of walking a day, except in wet weather when three minutes suffice.

Much of his remaining time has been devoted to park issues, particularly to campaigning for an expansion of the Juan de Fuca Provincial Park, which buffers a 47 kilometre hiking trail along the Vancouver Island west coast shoreline. It has the potential to rival the better known West Coast Trail, which is immediately to the north. Finally, for forty three years Anderson has been involved in trying to keep oil tanker traffic from the West Coast. The current threat is the Northern Gateway proposal from Enbridge, to establish a pipeline to a tanker port in Kitimat, to move substantial shipments of Tar Sands synthetic crude to Asia. David is taking part in the National Energy Board hearings and he predicts this will take up much of his time over the next two years.

If you would like to contact board member David Anderson, you can contact him at david@anderson-victoria.com

EDITOR'S NOTE

Rinee Mukherjee, University of Guelph

Welcome to the 3rd issue of NSERC Bioconversion Newsletter. In this issue we are highlighting Enzymatic Hydrolysis as our research theme. We take a closer look at the enzymatic hydrolysis of lignocellulosic biomass and the quest for enzymes with novel, superior properties. The projects in this theme are led by – Prof. Adrian Tsang, Prof. Reg Storms, Dr. Justin Powlowski at Concordia University and Prof. Jack Saddler at UBC. We hope that you find the articles enlightening.

In the upcoming, issues of the newsletter we hope to share other theme perspectives, such as those from enzymatic hydrolysis and fermentation. Please send me an email with your feedback and input on our newsletter, website, etc.

Thank You!

We welcome your feedback on the layout and scope of the Newsletter — Please ***Contact Us!***

ANNOUNCEMENTS:

PRETREATMENT WORKSHOP AND AGM; JUNE 4-8, 2012

NSERC Bioconversion Network's third Annual General Meeting (AGM) which will be held at the Forest Sciences Centre, University of British Columbia, Vancouver, BC on June 6-8, 2012. This year, in association with our network AGM we will be organizing a three day Pretreatment Workshop that will also be held at the Forest Sciences Centre, University of British Columbia, Vancouver, BC on June 4-6, 2012.

The Workshop will feature presentations/tutorials, and interactive discussions from world renowned international experts in biomass pretreatment technologies, pretreatment equipment manufacturers, pulp and paper companies and end users.

Registration is now open, Please Register. Please visit our website **www.nsercbioconversion.net/AGM2012** for more information.

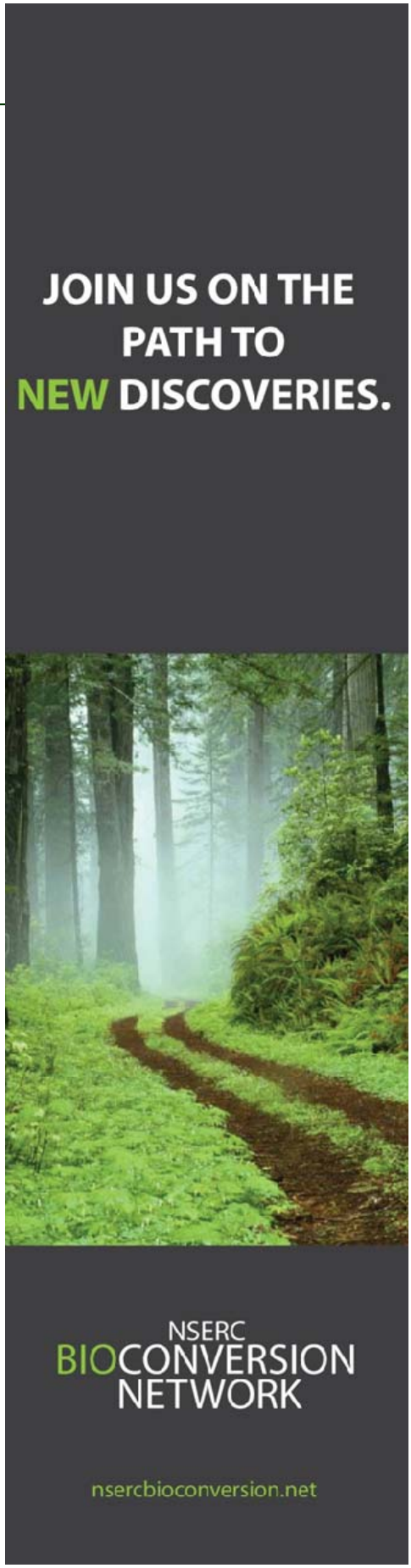
FIBRE

NSERC Bioconversion is pleased to announce that we are part of the FIBRE (Forest Innovation by Research & Education) Network. FIBRE network was launched in October, 2011 and is the organization that builds synergies among eight forest R&D networks in support of the priorities of Canada's vital forest sector innovation system.

Each FIBRE member network provides novel innovations to sustain and transform Canada's forest sector.

For more information please visit the FIBRE website

http://reseauxfibrenetworks.ca/



TAPPING INTO NATURES' JEWELLERY BOX - OR HOW TO TURN A FOE INTO A FRIEND

Theme 2 of the NSERC Bioconversion Network: enzymatic hydrolysis

Enongene Ekwe, Vanessa Blandford, Christina Sawchyn and Ingo Morgenstern, CSFG, Concordia University

The first newsletter in this series put a spotlight on the importance of various pre-treatment options of feedstock to increase bioconversion efficiency. In this edition we will take a closer look at the activities of theme 2 of the Bioconversion network, the enzymatic hydrolysis of lignocellulosic biomass and the quest for enzymes with novel, superior properties.

During the Second World War, when the mighty US Armed Forces moved slowly but steadily across the Pacific hopping from island to island, they encountered an unlikely foe. Military equipment such as tent canvas, tarpaulins, boots and uniforms were attacked by an unknown battlefield fungus and quickly rotted away. Army investigators were sent to collect and identify the foe. A collection taken in 1944 from tent canvas on Bougainville Island in the Solomons and labeled as strain QM6a was identified as the likely culprit due to its prolific cellulase secretion capability. First identified as *Trichoderma viride*, it was later recognized as a separate species and named *T. reesei* (Teleomorph *Hypocrea jecorina*).

Trichoderma reesei is a saprotrophic ascomycete with a pantropical distribution. In the post-war years, research interest shifted away from improving the mold-resistance of equipment to harnessing the extraordinary cellulases produced by *T. reesei* for industrial purposes, specifically the production of bioethanol. Improved strains of *T. reesei* quickly became the industrial workhorses for commercial cellulase production. The cellulases then known to be produced by this fungus included seven endoglucanases, two cellobiohydrolases, and several β-glucosidases. It came almost as a shock when in 2008 the fully sequenced genome of *T. reesei* QM6a was published and the total number of endo- and exo-cellulase genes detected increased only by one. The one newly discovered endoglucanase was identified as a member of GH family 61, which are no longer considered to possess endoglucanase activity. Furthermore the number of hemicellulases and pectinases in the *T. reesei* genome was also found to be much lower than in other filamentous ascomycetes sequenced so far. This discovery raised several questions such as 'what makes *T. reesei* such a prolific cellulose degrader, if not its gene complement? Are there still better fungal candidates for cellulase production in nature waiting to be discovered?'

The actual number of proteins in secreted saprophytic enzyme cocktails is unknown; estimates place it at more than 80. These proteins enable saprotrophs to feed on woody plant material. A brief look at the plant cell wall will enable us to better appreciate the relevance of these proteins. The intricate plant cell wall architecture composed mainly of cellulose, hemicelluloses, pectin and lignin (the so-called lignocellulose) is meant to stay tightly together to provide rigidity and stability in plants, which makes it extremely challenging to break apart. Cellulose from plant biomass is the most abundant biopolymer on earth and together with other polymers in lignocellulose is widely regarded as a sustainable biofuel resource. Although saprotrophs such as *T. reesei* and *A. niger* are designed by nature to thrive on plant cell wall material, their secreted multi-protein cocktails, which concertedly degrade lignocellulose into fermentable sugars, lack the efficiency required for direct applications in the lignocellulose-to-ethanol industry. Similarly, today's commercially available cellulase cocktails show this shortcoming. The reason for this challenge is that lignocellulosic substrates are degraded not by a single microbial species, but by the synergistic actions of enzymes from multiple species in the natural environment. The most efficient enzyme cocktails will therefore most likely contain a mixture of carboactive enzymes derived from several species. At CSFG we work toward this objective.

Diversity

Fungi are superior degraders of all kind of carbon containing materials. The strategies employed to break up plant cell walls and other carbon containing materials and hence the types of enzymes secreted, differ widely. Fungi belonging to the basidiomycetes cause either a white rot or a brown rot. In white rot all major wood components including lignin are broken down. Brown rot fungi leave lignin behind and degrade only the cellulose and hemicelluloses. Ascomycetes typically cause a soft rot, in which cellulose and hemicellulose are degraded and lig-

nin may be attacked to a certain degree. However, not much is known about lignin degradation by ascomycetes. Some ascomycetes reportedly cause a rot similar to the white rot afflicted by basidiomycetes. The approach at CSFG is to investigate a broad spectrum of potentially interesting fungi for lignocellulose degrading capacities (Fig. 1).

Figure 1. Plate cultures of thermophilic fungi *Thermomyces lanuginosus* (A), *Paecilomyces byssochlamydoides* (B) and *Thermoascus crustaceus* (C).

The main emphasis is on screening thermophilic fungi from different taxonomic groups because they may have evolved enzymes that are more valuable for industrial applications than those from closely related mesophilic species. After sequencing the first two genomes of thermophilic fungi (*Myceliophthora thermophila* a.k.a. *Sporotrichum thermophile* and *Thielavia terrestris*), two more thermophilic genomes have been sequenced last year by Genozymes (*Talaromyces thermophile* and *Thermomyces lanuginosus*) and six more are slated to be sequenced this year. Since thermophilic fungi have been detected only in a few orders of ascomycetes and the basal fungal group Mucoromycotina most genomes investigated fall into those groups as well. However, the genomes of the white-rot basidiomycete *Trametes versicolor, Amorphotheca resinae, Pseudocercosporella herpotrichoides* have been sequenced and annotated as well. Moreover, transcriptomic data has been generated for the brown rot fungus *Gloeophyllum trabeum*.

Utilizing over 30 different fungal species available to our team, we have been able to complete the genome and transcriptome sequencing of 22 species thus far. Within the context of the NSERC Bioconversion Network, RNA-Sequencing provides us with a large set of data suitable for mining fungal genomes for novel transcribed lignocellulolytic enzymes.

Transcriptomics and bioinformatics

RNA-Sequencing (RNA-Seq) allows researchers to identify genes transcribed within an organism at a given time point, temperature, and under other various growth conditions. Transcriptome data can be used to validate predicted gene models or used in comparative transcriptomic analysis. In the latter case, fungal cultures for transcriptome analysis are grown on a variety of simple or complex substrates (e.g. alfalfa, barley, avicel, xylan, olive oil, pectin and lignin) and the expression profiles of each substrate are studied in order to identify optimal expression conditions for potential genes of interest. The data from these samples are used to elucidate the pathways involved in lignocellulolytic degradation. Extracellular protein samples are also analyzed using mass-spectrometry in order to confirm that the target proteins are secreted.

RNA-Sequencing can also be coupled with genetic manipulation techniques to further understand the mechanisms which control gene expression. One example is the deletion of the xylanolytic transcription factor XlnR. Comparing transcriptomic and proteomic data generated from knockout versus wild-type strains will provide insight into the molecular switches controlling expression of biomass degrading enzymes. With each sequencing run yielding up to tens of millions of reads, there remains the challenge of positively identifying those candidate genes involved in lignocellulose degradation. What is the precise role of these genes and where do they reside in the genome? The analysis of this sequence information is understandably far from straightforward. Computational support for the abundant and complex sequencing is provided by CSFG's bioinformatics platform. In essence, the bioinformaticians provide the resources necessary to identify the differentially transcribed genes of interest, either already known or newly discovered, so that these fungal enzymes may be further cloned, expressed and characterized. Currently,

the group's main focus is the identification and annotation of cloning targets in fungal thermophiles.

Once the RNA-seq is assembled, the read data is sent back for extensive analysis to determine statistical and differential gene expression. Analysis of transcriptional patterns has proven to be far more complex than originally thought. Interestingly, the sequence data has provided various other kinds of valuable information. Most importantly, the transcribed sequences are indicative of their expression activity. When mapped back to the genome, the predicted gene models are validated, annotated (attaching biological information to a predicted gene, such as introns and exons) and curated (manual validation of annotated genes). The computational prediction of gene models and gene products is also being done in-house. In fact, the gene prediction pipeline, one of the group's major achievements, is able to efficiently and accurately predict gene models for fungal genomes. Currently, this can be achieved for a newly sequenced genome in one week or less.

The generated gene predictions and annotations are also integrated with a genome browser and a blast tool, and have recently published online into a single, public resource for fungal genomes and enzyme databases. This resource, which can be accessed through http://genome.fungalgenomics.ca, currently boasts 3 sequenced and annotated genomes with 8 more to be published later this year. As Dr Wendy Findlay, chiefly responsible for the development of the annotation pipeline explained that overall goal of a public resource for fungal genomes is "to sequence and annotate at least 20 genomes to have a community resource and to select out as many secreted, lignocelluloytic enzymes as possible."

With the implementation of genome sequencing, transcriptome analysis, and mass-spec analysis, over 2000 potential lignocellulolytic enzymes have been targeted, and are presently being cloned and expressed in *A. niger* by our collaborator at the DSM. In our group's recent publication "Comparative Genomic Analysis of the Thermophilic Biomass-Degrading Fungi *Myceliophthora thermophila* and *Thielavia terrestris*" (Nature Biotechnology 2011, 29(10): 922-927) one can see how this approach allows for the identification of novel enzymes and improved understanding of gene regulation and secretion in fungus.

Cloning

The list of target lignocellulases of interest generated during the bioinformatics annotation step is then passed on to the cloning group. Since 2009, this group has successfully cloned and expressed over 2000 genes in *A. niger*. Not only has the cloning group been able to express a considerable number of the target enzymes, but also they have improved their cloning strategy such that the current success rate hovers around 85%. In contrast to the widely used and effective Gateway cloning strategy, the group makes use of a far less costly ligation independent cloning (LIC) strategy. This approach is also less time consuming. From the first PCR amplification to obtaining positively expressing gene in *A. niger*, a single technician is now able to clone 100 genes in just one week. There are plans for even higher throughput of the cloning group's work and testing is currently underway with use of robotics.

Protein Production

At the CSFG, an easy-to-select *A. niger* PY11 (a derivative of *A. niger*N400) has been developed, which is used as a model industrial workhorse for recombinant protein production (Fig.2). Once genes are cloned and the production host transformed, *A. niger* PY11 conidia harboring the target gene are grown in liquid media at 30˚C for 5 days. During this period, the fungus expresses and secretes, among others, the target protein in the liquid media. At the end of the growth, the supernatant containing the secreted proteins is separated from the fungal material. The supernatant is then tested for activity of the target protein via an appropriate assay method and the active target later purified from the adjoining contaminants.

For the past few years, we have been faced with the challenge of losing the secreted recombinant protein due to protease activity (a class of predator proteins that chop up other proteins). To identify the best time to harvest the supernatant with intact target protein, traditional methods such as SDS-PAGE, which involve time-consuming procedures may not be the best to rely on. Our protein production team has developed a rapid, pH-dependent, on-line approach to overcome this challenge. David Taylor and his team in the protein production platform observed that acidity of the growth media increases from pH 5 to about pH 3 due to citric acid secretion in the growth phase of *A. niger*PY11. During protein secretion in expression phase, the acidity drops progressively to pH 5. Above pH 5 protease activity is observed, which damages the target protein. Consequently, by simply monitoring the acidity of the media and harvesting supernatants at pH5, we are able to avoid compromising the target proteins.

We produce proteins in small or large scale depending on the intended end-use. Small scale production which is done in 20ml petri dishes is meant for protein activity screening, biochemical characterization and other biophysical studies. Large scale production is done in 3 to 15 liters fermenters and is meant for applications requiring large amounts of proteins such as biomass hydrolysis and bleaching assays.

A protein would be of no use if it is not active. Synthetic or model substrates are generally used to assess the biocatalytic potential of expressed proteins. Enzymes that show activity are biochemically characterized with respect to their specific activity and temperature and pH activity profiles. Cognizance of the fact that the industrial lignocellulosic bioethanol process is designed for 65°C under slightly acid conditions, biochemical characterization is essential for the identification of proteins that will suit such physical conditions.

Biomass Hydrolysis

Enzymatic hydrolysis of plant biomass into fermentable sugars is an essential step in the production of second generation biofuels and high value chemicals. It therefore comes as no surprise that the search for efficient industrially applicable cellulase cocktails has intensified. Since enzyme activity tests on model substrates do not necessarily tell their hydrolytic capabilities on natural biomass, we directly screen the active enzymes on a variety of natural pre-treated lignocellulosic biomass, which are provided by our collaborators such as FP Innovation and Dr Jack Saddler's group at UBC. By adding small amounts of recombinant proteins (10%) and commercial cellulase cocktails (90%) to biomass and measuring the amount of sugars, especially glucose released, we have strategically been screening for recombinant proteins which can confer enhancement of cellulase-driven biomass hydrolysis. This approach, commonly referred to as spiking, has lead to the identification of a xylanase, a β-glucosidase and an α-L-arabinofuranosidase, which show significant cellulase enhancement potential. Screening, biomass hydrolysis and other microplate-based experiments at CSFG can now be designed to take advantage of a new high throughput workstation (Fig. 3).

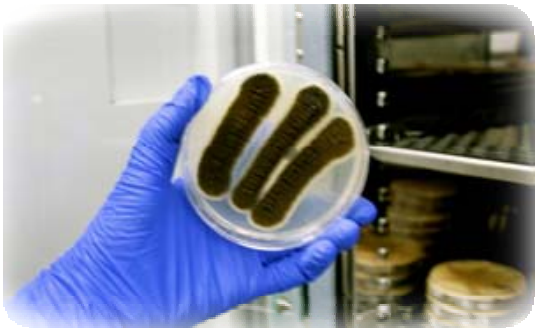


Figure 2. Transformed *A. niger* PY11 generating conidia.

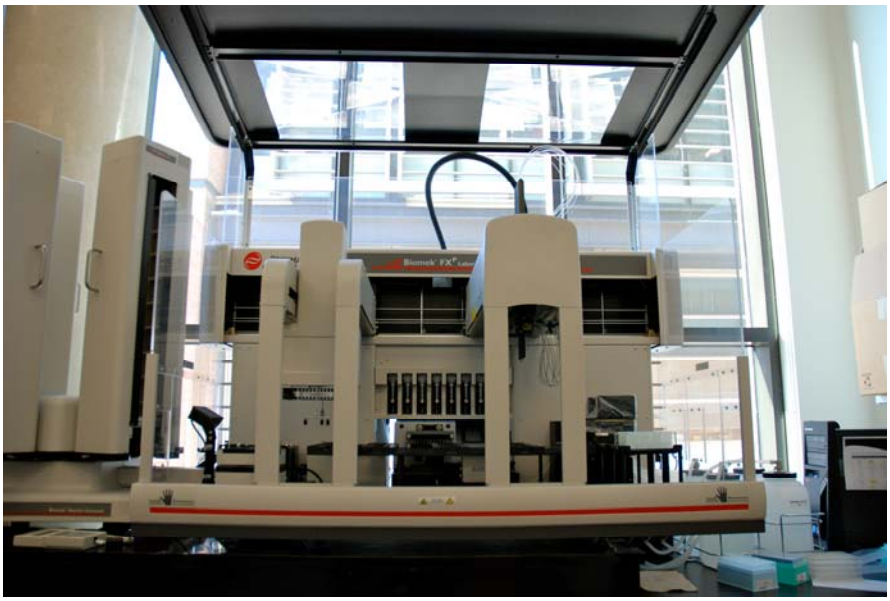


Figure 3. The brand new high throughput Biomek FXP Laboratory Workstation used for screening and protein characterization.

We also do biomass hydrolysability tests which involve the use of commercial cellulases to assess the maximum extent to which various pretreated biomasses can be degraded via established methods developed by the National Renewable Energy Laboratory (NREL) of the United States. Such data generated have been a valuable asset for our collaborators at UBC for identification of the best pre-treatment conditions of various biomasses.

Although high biomass consistency (≥15g biomass per 100 ml) is what an economically viable bioethanol process requires, most researchers perform these tests at low consistency (≤ 5g biomass per 100 ml). This is mainly because enzymatic hydrolysis at high consistency faces several constraints which severely impair performance of biocatalysts. For instance, the concentrations of biomass degradation products resulting from pretreatment, such as furfural and hydroxymethyl furfural (HMF) as well as residual lignin and related phenolic derivatives, increase with solids loading. These are strong inhibitors of cell wall degrading enzymes and some are toxic to fermentative microorganisms downstream. One way to overcome this challenge is to identify efficient enzymes which can tolerate high solids loading and such inhibitors. We are currently developing a microplate-based, high throughput-adaptable screening strategy that utilizes high consistency pre-treated biomass to identify those enzymes that are most tolerant to high solids loading and associated inhibitors.

For more information about the CSFG you may also want to visit our website http://genomics.concordia.ca/ and watch this short video http://www.youtube.com/watch?v=PL-SIQjK2KE.

Members of the Tsang lab from several platforms (Bioinformatics, Cloning and Expression, Enzyme Characterization, Fungal Culture, and Proteomics) in the foyer of their new building.

The new state-of-the-art facility housing the Centre for Structural and Functional Genomics.

A PLATFORM FOR LIGNOCELLULOSES ACTIVE ENZYME AND ACCESSORY PROTEIN PRODUCTION

Theme 2 of the NSERC Bioconversion Network: enzymatic hydrolysis

Rebecca Sydenham, Concordia University

When selecting an expression host for the production of secreted heterologous proteins, there are a number of things to consider. Will the host be equipped to handle all necessary post-translational modifications? Does the host produce proteins with similar activities to the proteins of interest? Are there secreted proteases that are likely to degrade those proteins? What kind of protein yield can be expected? How difficult will it be to scale up to large-scale production? How complicated and costly is it to grow?

As in many affiliated research groups, we use *Aspergillus niger* as our expression host. *A. niger* has been an industry standard for the production of secreted heterologous proteins for many years. It is capable of protein yields as high as 50 grams per liter and is relatively inexpensive to grow. There are also time-tested methods available for large-scale fermentations. The fact that it is a filamentous fungus is of further interest to us, given that all the enzymes we produce come from fungal species. The more closely related our host is to our proteins of interest, the more likely it is that it will be equipped to deal with things like gene splicing and post-translational modifications. We have also had much success in the past in producing functional secreted proteins using our old strain of *A. niger*.

There are, however, some notable disadvantages to using the old strain of *A. niger*.

- There are a number of secreted proteases that could degrade the heterologous proteins we are trying to produce.
- *A. niger* produces secreted homologs to most of the enzymes we study, some of which are constitutively expressed.
- In most cases, the expression of native proteins is better than that of heterologous ones.

We have come up with a number of complementary strategies to address these issues. These include using gene replacement to delete the various native genes that can compete with or interfere with production of the biomass active genes targeted by Theme 2b and 2c of this network; screening native promoters to find promoters that direct high levels of expression; using conditional gene expression and transcriptome analysis to study *A. niger*'s secretory pathway and a mutant selection strategy aimed at identifying mutations that enhance protein secretion.

Gene Replacement

We are using targeted gene replacement to knock out various genes. The gene replacement cassette is assembled using successive rounds of PCR (Fig. 1). It contains a selectable marker flanked by recombination sites. Promoter and terminator sequences of the target gene are PCR amplified and added outside the recombination sites. The resulting cassette can then recombine with the flanking regions of the gene of interest to generate the desired gene replacement. Successful gene replacements can then be selected for using the marker. If needed the marker can then be evicted using recombination making the new strain ready for another gene replacement.

Several genes have been successfully replaced using this method (Table 1).

Figure 1: Gene replacement cassette with target gene promoter and terminator sequences, selectable marker and recombination sites

Table 1:Deleting the kusA gene facilitates homologous integration

Targeted Integration Success Rate		
Genes	*kusA*$^+$	*kusA*$^-$
4 genes	16 out of 399 screened	ND
4 genes	ND	14 out of 15 screened

We have managed to reduce the amount of secreted proteases by 8 fold and total native secreted proteins by 10 fold. The absence of these proteins has significantly increased the purity of the heterologous proteins expressed by our mutant strains.

Screening for Strong Promoters

We have been using the glucoamylase (*glaA*) promoter to direct the expression of heterologous genes in *A. niger*, because *glaA* is one of the most highly expressed genes. There are, however, a number of issues associated with the continued use of this promoter:

- the glucoamylase promoter has been patented;
- *glaA* is induced by maltose and starch, which are carbon sources for *A. niger*;
- in order to achieve maximal expression with the *glaA* promoter, a minimum of five copies must be integrated into the genome;
- when expressing a heterologous protein using the *glaA* promoter, it is necessary to screen several independent transformants in order to identify one that directs maximum levels of expression.

We have characterized a number of novel promoters and found several that can support higher levels of expression than are obtained with the widely used *glaA* promoter.

Conditional Gene Expression

In order to assess the functional importance of various secretory pathway components, we have developed a promoter replacement cassette (Fig. 2). This cassette contains a selectable marker and a highly regulated promoter, Conpr. These are in turn flanked by the target promoter and ORF sequences. When inserted into the chromosome via recombination, the selectable marker and Conpr are inserted between the target ORF and its native promoter so that transcription of the targeted gene is now regulated by the Conpr promoter, and can therefore be induced and repressed by very simple means. We have successfully replaced several promoters using this technique and are currently attempting to use this approach to identify components of the secretory pathway that are rate limiting for protein secretion.

Transcription Profiling

Endoplasmic reticulum (ER) stress has been shown to induce the unfolded protein response (UPR), which can cause the transcriptional down-regulation and proteasomal degradation of foreign secreted proteins when using eukaryotic expression systems. We have completed a series of reconstruction experiments using semi-quantitative RT-PCR to identify treatment regimes that induce the expression of various secretory pathway components (Fig. 3). These treatment regimes will now be used to determine how secretory pathway stress induced by dithiothreitol, tunicamycin, heterologous protein secretion and various genetic modifications affects the transcription profile of *A. niger*.

Summary

We have developed:

- A set of complementary expression strains and vectors that lower levels of background protein expression and increase levels of target protein production;
- A conditional promoter replacement system that is being used to study secretory pathway function.

We are also developing a system of strains and complementary expression vectors for the selection of mutants capable of increased levels of heterologous protein expression.

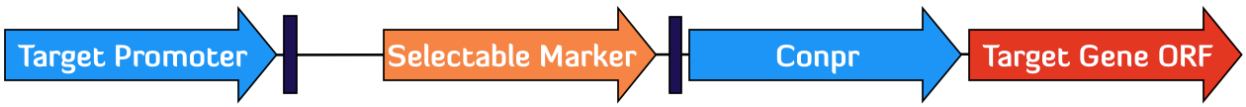


Figure 2: Promoter replacement cassette with a selectable marker and the tightly regulated Conpr promoter

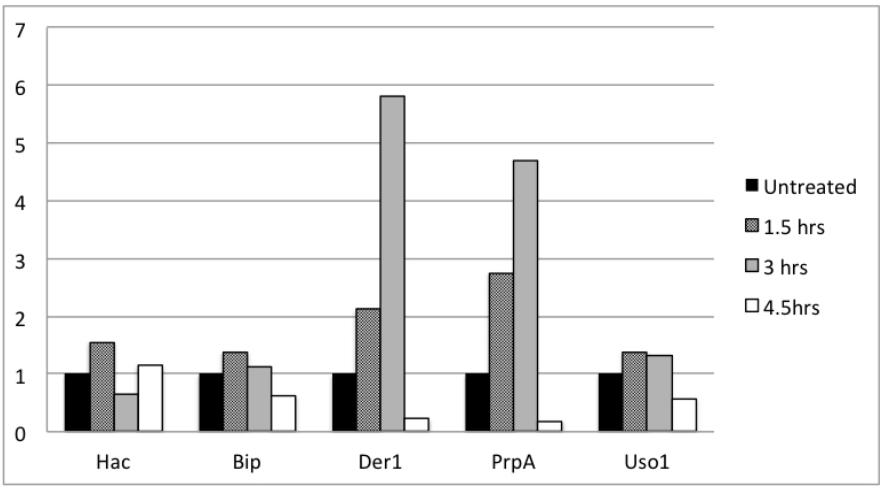


Figure 3: Reconstruction experiment. Transient changes in the transcript levels of 5 genes involved in the UPR following treatment with DTT.

Members of the Storm lab. From Left to Right: Yun Zheng, Edith Munro, Naïm Poonja-Tremblay, Gregorio Budkewitsch, Marco Martel, Gabrielle Tremblay, Simon Bourque, Rebecca Sydenham, Lina Mougharbel, Sonali Lagu and Reg Storms.

THE INDUSTRY INSIGHT: LALLEMAND ETHANOL TECHNOLOGY

Nicole Harner, University of Guelph

Lallemand Ethanol Technology (LET) is a business unit of Lallemand Inc., the Canadian-based yeast and bacteria producer. LET provides fermentation ingredients and value-creating services to the fuel ethanol, distilled beverage and industrial alcohol industries.

LET Research and Development Manager and avid Montreal Canadiens fan Dr. Frederic D'Aoust, is part of the senior management team overseeing R&D activities within the LET business unit of Lallemand Specialties Inc. Some of his responsibilities include: R&D leadership and direction, quality assurance of existing products, product performance & quality information, complaint investigation and response, technical reporting and communication, field sales support, regulatory affairs and management of strategic partnerships.

With the goal of providing fermentation ingredients for first generation and lignocellulosic fuel ethanol production, Dr. D'Aoust and his team undertake innovative projects with yeast strain, antimicrobial and nutrient package development. In addition to servicing the fuel ethanol industry, the researchers at LET are currently enhancing and developing new strains for the distilled beverage industry, including malt and grain whisky, rum, vodka, tequila and cachaça. Dr. D'Aoust also participates in the Alcohol School taught annually in Montreal and Toulouse, France, in addition to being an invited speaker at a number of international conferences and workshops.

Dr. D'Aoust describes the ethanol industry as 'dynamic and in constant flux', and is most interested in the innovation aspect of research and development. He most looks forward to seeing the recent years of work on second generation (cellulosic ethanol) come to fruition. Dr. D'Aoust admits that despite this interesting progress in cellulosic ethanol, the ongoing work on first generation ethanol cannot be dismissed. "We often see small incremental changes improving performance. What we intend to accomplish in the near future is actual step changes that will have a profound impact on the existing and future fuel ethanol industry." With first and second generation ethanol projects close to commercialization, Dr. D'Aoust is close to achieving what everyone strives for in R&D: to create successful commercial products from years of challenging laboratory research.

Upon joining LET as a research scientist, Dr. D'Aoust worked in the development of lignocellulosic biocatalysts. His responsibilities quickly grew, and he was appointed Senior Research Scientist supervising the day to day activities of the lab and acting as a liaison between laboratory staff and the R&D Manager. He jumped at the opportunity to work in conjunction with the R&D manager to develop plans and annual objectives for scientific staff. Part of this task required him to act on behalf of the R&D Manager at conferences, meetings, laboratory tours and speaking engagements. This unique situation allowed Dr. D'Aoust to learn and increase his skills under the guidance of an established and well-respected colleague. This experience helped transition him from pure research-oriented work to a more global position of understanding R&D in the corporate and business world, which he deems critical in fulfilling his current responsibilities as R&D Manager. Some key successes for Dr. D'Aoust and his team at LET include: the development and implementation of a genetic program in LET, developing a High Throughput Screening program, introducing the

Dr. Frederic D'Aoust

Ankom monitoring system that has been implemented in other Lallemand R&D laboratories, helped move several R&D projects closer to commercialization and facilitated strategic collaborations of genetic technology with industrial partners.

In collaboration with industrial and academic partners, LET has several ongoing lignocellulosic ethanol projects developing new yeast strains capable of producing ethanol from a wide variety of cellulosic substrates. As part of those projects, LET is busy screening their extensive collection of yeast cultures for industrial strains with properties conducive to cellulosic ethanol production. These strains will then serve as host strains for the integration of next generation genetic technology. In addition to the commercialization of a cellulosic biocatalyst, LET is also developing nutrient packages for the cellulosic ethanol industry. LET has partnerships with several research programs and networks including the Cellulosic Biofuels Network, the BBSRC Sustainable Bioenergy Centre: Lignocellulosic Conversion to Bioethanol (LACE) Program and the NSERC Bioconversion Network.

Dr. D'Aoust describes R&D at LET as much more development focused. "We often refer to it as little r and Big D. More projects are geared towards development and commercialization than pure applied research. We rely on collaboration and partnership with universities, small and large biotechnology companies and other R&D groups to conduct most of the pure scientific research." The NSERC Bioconversion Network provides LET with the opportunity to understand the current status of applied research while LET delivers industry perspective, insight into the fuel ethanol market, expertise in yeast production, management and application to the Bioconversion Network.

Dr. D'Aoust recognizes the diverse challenges facing cellulosic ethanol: "There is increased complexity associated with cellulosic ethanol not seen in the existing starch and sugar based (first generation) ethanol industry. For second generation ethanol to be economically feasible, all these difficulties must be addressed. These would include: feedstock availability and supply, hydrolysis, pretreatment, fermentation and regulatory issues." Dr. D'Aoust believes the NSERC Bioconversion Network assembles the wide range of experience and expertise required to overcome these challenges. Although he admits a slight bias, Dr. D'Aoust sees a bright future for the lignocellulosic ethanol industry, "There is more and more research dollars and funding entering the field making commercialization more attainable than before. Several hurdles have already been cleared and progress is continuing on other fronts. We are slowly seeing the progress from the lab bench to the market place with quick pit stops in the court rooms and parliament buildings."

With several petrochemical companies showing tremendous interest in making cellulosic ethanol a reality, they bring additional funding, resources and expertise to the table, "Who better to help get cellulosic ethanol over the hump. The concept of biorefineries has been perfected by the petroleum industry." Through production and supply of yeast and nutrients, LET is already a major player in the existing fuel ethanol market

and is positioning itself to take advantage of second generation production through their various collaborations.

As a resource rich country and a net exporter of energy, Dr. D'Aoust believes Canada is well represented in the international cellulosic ethanol industry, "Several NSERC Bioconversion Network members are highly respected scientific experts that play an important role within the global cellulosic R&D community. Several Canadian companies also share international recognition as industry leaders".

The vision, ambition and assets of Lallemand Inc. position them as world leaders in selected markets. Dr. D'Aoust believes the key to further success is "cooperation with customers, researchers, suppliers and other partners within the markets we serve." Lallemand Inc. strives to be more than a supplier, producer and employer, but to be a partner in success. As founder Fred A. Lallemand quoted, "Your success is our success."

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