APPLICATION OF THE RATIONAL DESIGN APPROACH TO THE DEVELOPMENT OF ENVIRONMENTALLY BENIGN WOOD PRESERVATIVES.

Mark Namchuk[a], Dedreia Tull[a], Paul Morris[b] and Stephen G. Withers[a], University of British Columbia, Vancouver[a], B.C. and Forintek Canada Corp.,[b] Vancouver, B.C.

INTRODUCTION

The use of wood as a long term building material requires that it be protected from breakdown by wood-degrading organisms. Inexpensive, effective wood preservatives are currently available but, many are perceived as posing a short or long term threat to the environment. Traditionally, wood preservatives have been chemicals with a long history of use as general preservatives or have been broad spectrum biocides adopted from other applications such as agricultural disease control. The preservative formulations now being introduced are generally a single biocide or combinations of complementary biocides with narrower spectra of activity and thus a reduced potential for adverse environmental impact.

In recent years several research groups have independently begun to apply a rational design approach to the development of alternative wood preservatives of a more targeted variety. By examining the physiology and biochemistry of the wood-degrading organisms, systems unique to this class of organisms have been identified. Targeting wood preservatives to have activity against only these systems offers an approach for developing highly specific biocides which will still possess activity as wood preservatives but have little or no general toxicity. Some examples of systems targeted thus far for investigation are the hemicellulases (1) and ion metabolism (2). Our efforts in this area began in 1985 as the result of a co-operative program between Forintek Canada Corp. and the University of British Columbia designated to investigate the development of targeted wood preservatives for the control of wood-rotting fungi. Our research has focused on two approaches to the design of novel wood preservatives. One of the approaches discussed herein centers on the design, synthesis and testing of mechanism-based inactivators targeted at the enzymes which mediate the breakdown of cellulose (cellulases). By interrupting the breakdown of cellulose, the primary food source of the wood-rotting organism is made inaccessible, making further breakdown of wood difficult or impossible.

The degradation of cellulose in wood is generally believed to be carried out by a cellulase complex. This complex is composed of at least three enzymes, an endoglucanase, exoglucanase and β-glucosidase which function in concert to degrade crystalline cellulose to glucose (Figure 1). The activity of the β-glucosidase component is crucial since it has been demonstrated that exo and endoglucanase activity is inhibited by high concentrations of cellobiose which is the primary substrate of β-glucosidase and will accumulate if β-glucosidase activity is inhibited (3). It seemed reasonable therefore that inactivation of the β-

glucosidase component of the complex would be a suitable approach for design of a new fungicide since inactivation of that system would deprive the wood-rotting organism of its primary carbon and energy source, glucose.

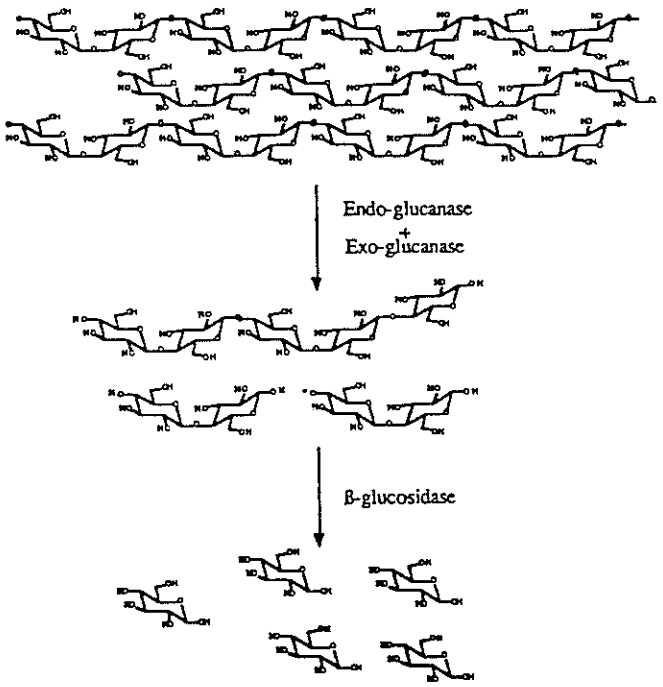

Figure 1. A representation of the roles of the component enzymes of the cellulase complex, in the degradation of cellulose to glucose.

The starting point for these studies is the proposed mechanism of β-glucosidase. Experiments performed in this laboratory (4,5,6) and others (7) have provided evidence to support the following mechanism (Figure 2). After an initial binding step, the substrate is attacked by an enzymic nucleophile (Nu^-) to form a glucosyl-enzyme intermediate. Departure of the aglycone (OR) is expedited by donation of a proton from an enzymic general acid moiety (AH). In the case where the substrate is cellobiose (the aglycone is glucose), this process results in the release of a molecule of glucose. The intermediate is then hydrolyzed by the general base (A^-) catalyzed attack of a molecule of water to release a second molecule of glucose with retention of configuration of the substrate at C1 and the free enzyme. Thus the enzyme is termed a *retaining* glycosidase. It is important to note that the formation and breakdown of the glucosyl-enzyme intermediate occurs *via* a transition state with substantial positive charge.

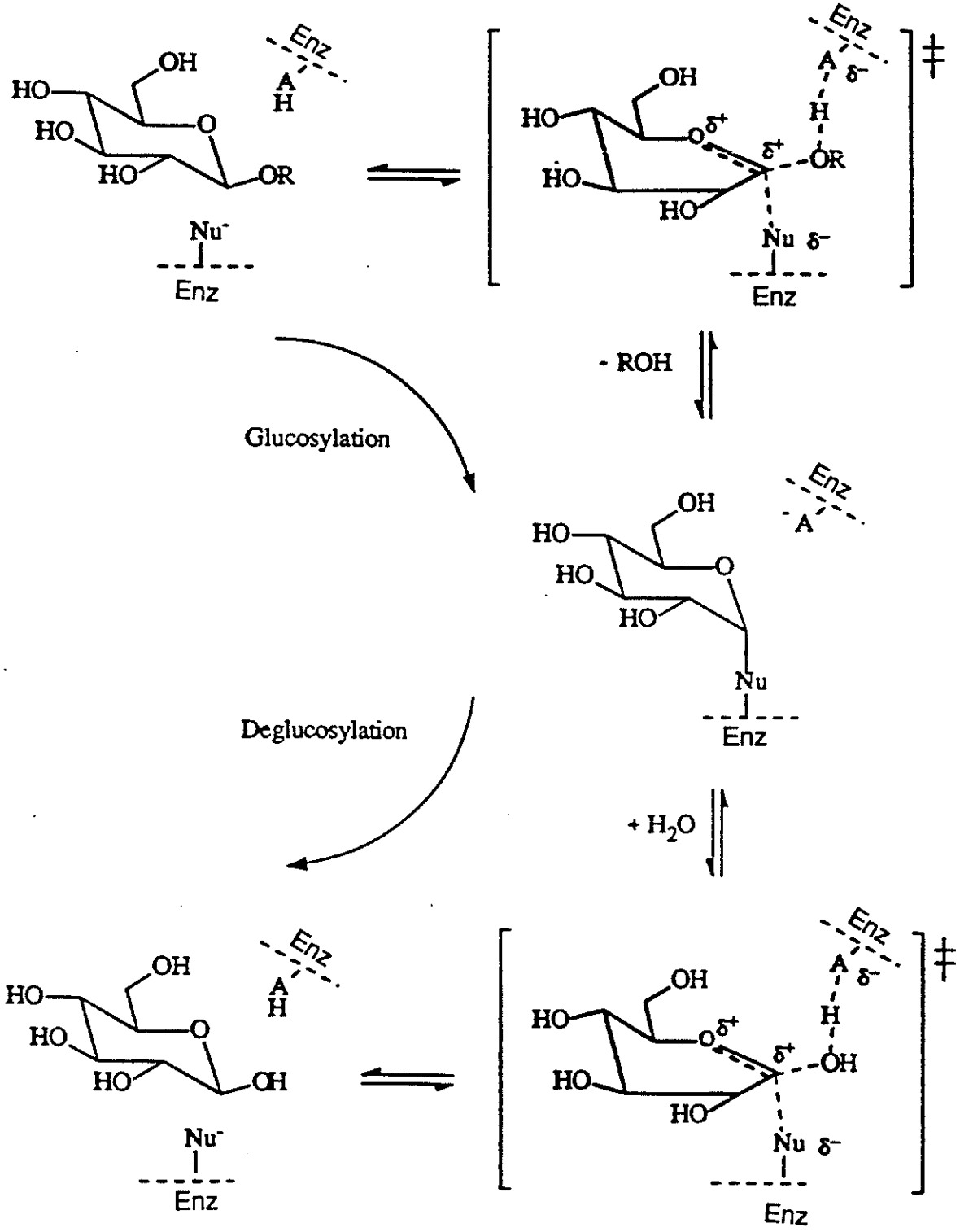

Figure 2. Mechanism of the hydrolysis of a glucoside by a retaining β-glucosidase. Taken from Street (8).

From this information, it was proposed that replacement of the C2 hydroxyl group by fluorine would result in a decrease in the rate of both formation and hydrolysis of the glucosyl enzyme intermediate due to destabilization of this positive charge. It was further proposed that use of an activated aglycone moiety such as 2,4-dinitrophenol (DNP) would increase the rate of formation of the intermediate but have no effect on its hydrolysis. Such a system should lead to the inactivation of the enzyme by accumulation of the glucosyl-enzyme intermediate (Figure 3).

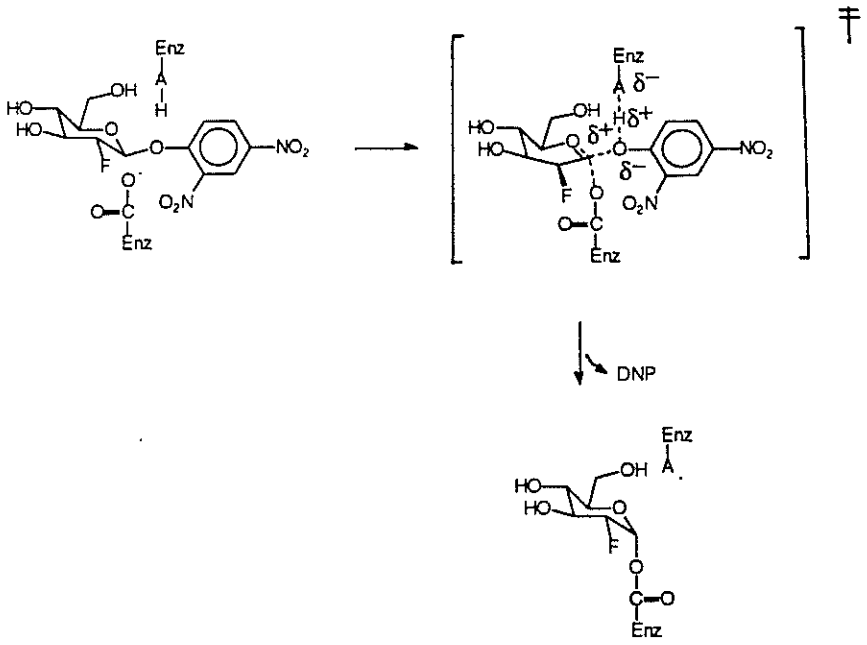

Figure 3. Inactivation of a retaining β-glucosidase by 2FDNPglucose.

MATERIALS

Synthesis of all of the compounds presented was carried out in the laboratory of Dr. Stephen Withers at the University of British Columbia. Details germane to the synthesis and characterization of these compounds have been published elsewhere (8). Isolation of the *Agrobacterium* β-glucosidase was carried out by the procedure of Day and Withers (9). Enzyme purity was assayed by Phast gel analysis using Silver stain to detect the proteins present. Preparations were generally found to be >95% pure. The *Cellulomonas fimi* exoglucanase was obtained from Dr. Neil Gilkes in the Department of Microbiology at the University of British Columbia. All of the other cellulase components tested were obtained through the same collaboration.

The test fungi *Coriolus versicolor* (W.F.P.L. no. 105e) and *Chaetomium globosum* (W.F.P.L. no. 47d) were obtained from the Forintek culture collection (Forintek Corp., Vancouver, B.C.). Culture tube cellulase activity assays on

these fungi were carried out by the method of Rautela and Cowling (10). All materials used in the preparation of media or in assays of enzyme activity were obtained from commercial sources and were analytical grade. Water used in the preparation of all media was Milli-Q quality.

RESULTS AND DISCUSSION

The class of compounds described above can be characterized as mechanism based inactivators or 'suicide substrates' for β-glucosidase. For reasons of availability, the initial testing of these compound was carried out on a β-glucosidase originally isolated from *Agrobacterium faecalis* (11) and subsequently cloned into *E. coli* (12). Treatment of this enzyme with 2',4'-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2FDNPglucose) lead to the rapid inactivation of the enzyme by accumulation of the 2-deoxy-2-fluoro-glucosyl enzyme intermediate. The identity of this intermediate was confirmed by ^{19}F NMR (5) and mass spectrometric analysis (John Gebler, Unpublished results from this laboratory) of the inactivated enzyme. The decrease in enzyme activity was extremely rapid and found to be strictly first order. Also, the incorporation of the inactivator was shown to be 1:1 with respect to the concentration of enzyme (4). Detailed studies of the mechanism by which 2FDNPglucose acts have largely confirmed the originally proposed mechanism for the mode of action of these inhibitors.

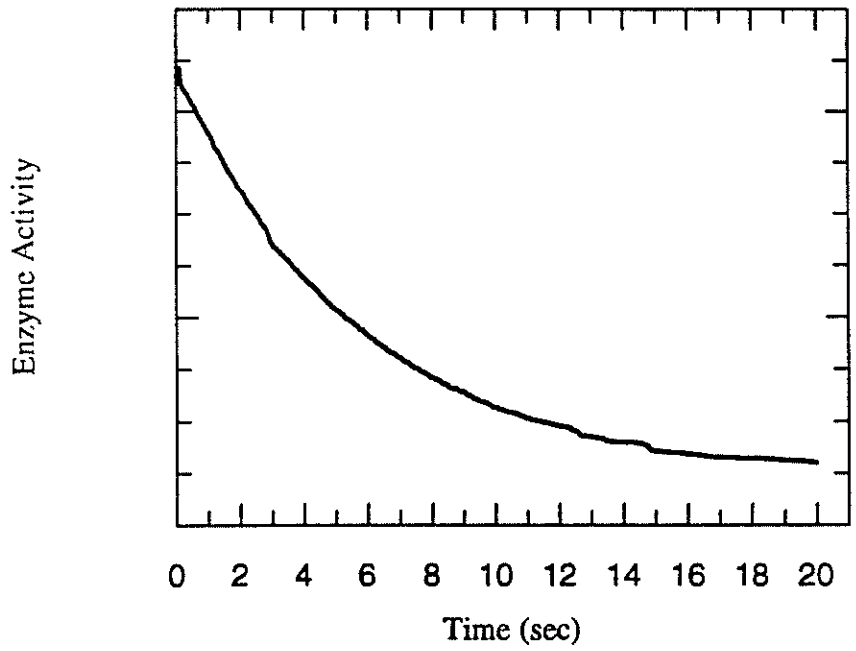

Figure 4. Loss of activity in the Agrobacterium β-glucosidase after treatment with 2FDNPglucose at 5°C.

In many cases, the mechanism of the other enzymes in the cellulase complex are believed to be similar to that of β-glucosidase. Tests were therefore carried out with 2FDNPglucose on the exoglucanase from another bacterium, *Cellulomonas fimi* (13). Although the rate of inactivation was found to be greatly reduced in this case, it was clear that 2FDNPglucose could still function as an inactivator in this system.

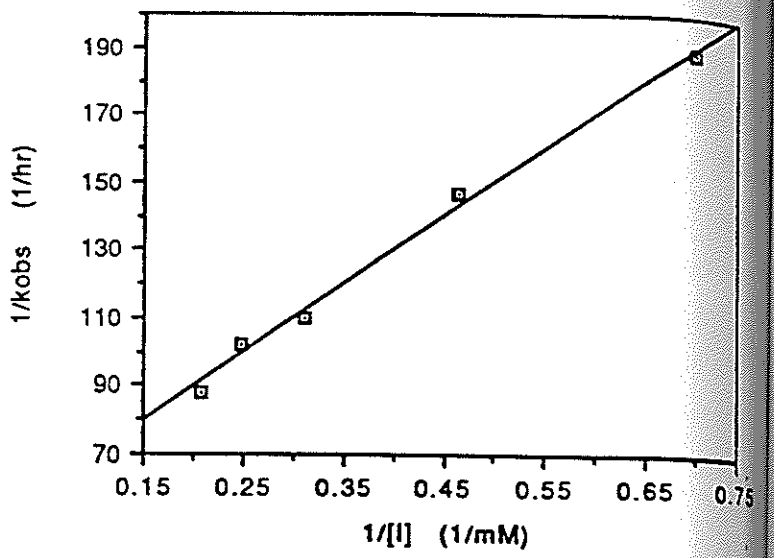

Figure 4. A Lineweaver-Burk representation of the inactivation of C. fimi exoglucanase by 2FDNPglucose. Taken from Tull (13).

Indeed, preliminary tests of 2FDNPglucose and derivatives thereof with the different enzymes within the cellulase complex isolated from several sources showed that these compounds displayed a broad spectrum of activity against the target enzyme systems (unpublished results). Tests of the specificity of this class of inactivators indicate the selectivity which would be expected from mechanism based inactivators. In all tests to date, this class of inhibitors has only been found to have activity against retaining β-glycosidases (unpublished results). This specificity is crucial since the goal of this research is to develop compounds which are only targeted at the cellulase system and have little or no general toxicity. Examining these data it would appear that 2FDNPglucose is capable of inactivating several of the components of the cellulase complex while still maintaining specificity for the target enzyme systems. Such broad spectrum activity is important for the application of these compounds as wood preservatives since it means that only one of the enzymes in the cellulase complex need be a retaining glycosidase (see Figure 1) for the compound to be effective.

Using the information obtained from the study of 2FDNPglucose, the putative wood preservative EDF and derivatives thereof were synthesized. These compounds were first characterized with regard to their ability to inactivate the *Agrobacterium* β-glucosidase and found to be efficient inactivators of that enzyme.

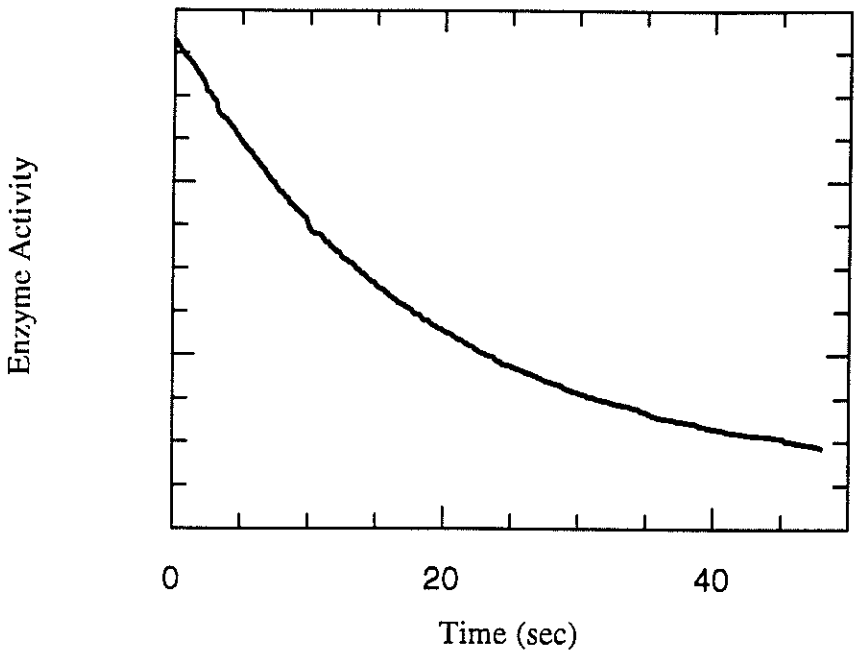

Figure 5. Loss of activity in the Agrobacterium β-glucosidase after treatment with EDD (a derivative of EDF) at 37°C.

Based on these results, tests with EDF were expanded to examine whether the compound could inhibit the degradation of cellulose in test cultures of two wood-rotting fungi *C. globosum* (a soft-rot fungus) and *C. versicolor* (a white-rot fungus). Cultures of these fungi were tested for their ability to digest acid swelled cellulose in the presence of EDF using an agar tube assay under the conditions described by Rautela and Cowling (10). Glass tube are inoculated with the test fungi on an agar medium containing the preservative and acid swollen cellulose as a food source. Cellulase activity is detected by clearing of the opaque test medium as the cellulose oligomers are broken down. An absence of clearing was therefore indicative of impairment of the digestion of cellulose in some manner. The advantage of this particular test method is that it allows the relatively small amounts of preservative which were synthesized (1-2g) to be tested in relatively short periods of time (4-6 weeks). This allows relatively rapid screening for compounds with biological activity which are then synthesized on a larger scale and further tested. Results of these tests showed EDF was capable of stopping the digestion of cellulose by the test fungi at a 5 mg/ml concentration of preservative.

Table 1 Results From Testing of EDF for Inhibition of the Breakdown of Cellulose by Wood-Rotting Fungi[a].

Concentration of EDF (mg/ml)	C. globosum	C. versicolor
0	-	-
2	-	-
5	+	+
10	+	+
15	+	+

a) + indicates no detectable breakdown of cellulose.

The data accumulated from these tests suggest that EDF may indeed be capable of functioning as a wood preservative. Tests of this compound in wood blocks will soon be initiated to derive further evidence for this claim. It should also be noted that direct evidence that the loss of enzyme activity is related to inactivation of the cellulase complex has not yet been obtained. For this reason, we are presently isolating small amounts of the cellulase complex from our test fungi to test for susceptibility to EDF.

CONCLUSIONS

The initial testing of this class of compounds has yielded some extremely promising results. Firstly, from *in vitro* enzyme testing we have some evidence that mechanism-based inactivators of this type are capable of inhibiting retaining β-glycosidases in bacteria which degrade cellulose. Further, from the *in vivo* studies carried out thus far we have preliminary evidence that these compounds are also effective against wood-rotting fungi. Tests are also presently underway to look for preservative activity for EDF against brown-rot fungi which is certainly relevant to the use of these compounds as wood preservatives. Nevertheless, these initial findings emphasize the power of the rational design approach and may represent the first stages in the development of a new class of environmentally 'friendly' wood preservatives.

REFERENCES

1) Highley, T.L. Hemicellulases of White- and Brown-Rot Fungi in Relation to Host Preferences, *Material and Organismen*, **1976**, 11Bd., Heft1.
2) Viikari, L. and Ritschoff , Prevention of Brown-rot Decay by Ion Chelators. International Research Group on Wood Preservation (1992), IRG/wp/1540-92.
3) Eriksson, K.E.; Pettersson, B., Extra Cellular Enzyme System Used by the Fungus *Sporotrichum pulverulentum (Chrysosporium lignorum)* for the Breakdown of Cellulose., *Eur. J. Biochem.*, **1975**, 51, 193.

4) Withers, S.G.; Street, I.P.; Dolphin, D.H., 2-Deoxy-2-fluoroglucosides: A Novel Class of Mechanism Based Glucosidase Inhibitors. *J. Am. Chem. Soc.*, **1987**, 109, 7530.
5) Withers, S.G.; Street, I.P. Identification of an α-D-Glucopyranosyl Intermediate Formed on a β-Glucosidase., *J. Am. Chem. Soc.*, **1988**, 110, 8551.
6) Kempton, J; Withers, S.G., The Mechanism of *Agrobacter* β-Glucosidase: Kinetic Studies., *Biochem.*, In Press.
7) Sinnott, M.L., Catalytic Mechanism of Enzymic Glycosyl Transfer., *Chem. Rev.*, **1990**, 90, 1171.
8) Street, I.P., Ph.D. Thesis, University of British Columbia (1988).
9) Day, A.G.; Withers, S.G., *Biochem. Cell. Biol.*, **1986**, 64, 914.
10) Rautela, G.S.; Cowling, E.B.; Simple Cultural Test for Relative Cellulolytic Activity in Fungi. *Appl. Microbiol.*, **1966**, 14, 892.
11) Han, Y.W.; Srinivasan, V.R., *J. Bacteriol.*, **1969**, 100, 1355.
12) Wakarchuk W.W., et. al., The Molecular Cloning and Expression of a Cellobiase Gene from an *Agrobacterium* in *Escherichia coli.* *Mol. Gen. Genet.*, **1986**, 205, 146.
13) Tull, D., M.Sc. Thesis, University of British Columbia (1991).

ACKNOWLEDGMENTS

The authors would like to thank Trevor Andrews for his expert technical assistance and the British Columbia science council for an STDF-AGAR grant and GREAT scholarship to M.N.