N91-31785

USE OF INEDIBLE WHEAT RESIDUES FROM THE KSC-CELSS BREADBOARD FACILITY FOR PRODUCTION OF FUNGAL CELLULASE

R.F. Strayer, M.A. Brannon, and J.L. Garland. The Bionetics Corporation for NASA Biomedical Operations and Research Office, Kennedy Space Center, FL.

ABSTRACT

Cellulose and xylan (a hemicellulose) comprise 50% of inedible wheat residue (which is 60% of total wheat biomass) produced in the KSC-CELSS Breadboard Biomass Production Chamber (BPC). These polysaccharides can be converted by enzymatic hydrolysis into useful monosaccharides, thus maximizing the use of BPC volume and energy, and minimizing waste material to be treated. The evaluation of CELSS-derived wheat residues for production of cellulase enzyme complex by *Trichoderma reesei* and supplemental ß-glucosidase by *Aspergillus phoenicis* is in progress. A cellulase hyperproducing strain of *T. reesei* and *A. phoenicis* were grown in monoculture vs. coculture with different substrates--alpha cellulose and starch{controls}, wheat residue, and residue treated to remove lignin and hemicelluose. Enzyme activities were measured by standard filter paper and ß-glucosidase assays and by hydrolysis (1 to 4 days) of CELSS wheat straw. When compared to *T. reesei* monoculture, coculture of *A. phoenicis* with *T. reesei* resulted in increased levels of ß-glucosidase (0.27 vs 0.94 IU/mL) and filter paper activity (0.09 vs 0.13) but did not significantly alter the amount of glucose produced (21 g/L) during 48 hr wheat residue hydrolysis. Current monoculture studies suggest that ß-glucosidase production by *A. phoenicis* is higher on treated or untreated residue (~0.6 IU/mL) than on starch (0.13 IU/mL), but cellulase production by *T. reesei* is lower on residue (0.03 to 0.08 IU/mL) than on alpha cellulose (0.13 IU/mL). Separate preliminary bench scale studies have examined conditions for enzymatic hydrolysis of wheat residue cellulose and identified optimal enzyme loading rates (3 to 5 IU of *T. reesei* cellulase per g of CELSS wheat residue) and substrate concentration (5% w/v wheat residue) to yield high residue cellulose conversion efficiencies (up to 70%) and high product glucose concentrations (up to 23 g/L).

INTRODUCTION

The composition of wheat grown under the controlled conditions of the KSC-CELSS Breadboard facility Biomass Production Chamber (BPC) is shown in Figure 1. Edible biomass, e.g., harvest index, is typically 40%, with the remaining

C-3

60% of little nutritional value to humans. Nearly 35% of this inedible residue (or 21 % of total biomass) can be leached from the biomass by a simple, 4 hr water extraction (25°C), with soluble organics and inorganics both removed by this treatment. The characterization of these extracts and the potential to recycle the inorganics and further utilize the organics in a CELSS is another topic of current research at KSC. Preliminary results of this research were presented at the 1988 Annual Meeting of the American Society of Gravitational and Space Biology and, thus, will not be included in this paper.

The water-insoluble, particulate matter of the wheat residue is predominantly cellulose, a polysaccharide containing glucose subunits, and hemicellulose, which in wheat is mostly xylan containing xylose subunits and side chains of arabinose and glucuronic acid. Although these polysaccahrides are not of direct use by humans, they can be readily hydrolyzed into monosaccharides. The sugar produced may be consumed directly by the crew or be incorporated into edible biomass by yeasts or fungi (See introductory paper by Knott concerning the ongoing research at KSC, this publication).

The extremely low lignin content of wheat grown in a controlled environment noted in Figure 1 is of importance, because lignin complicates the conversion of cellulose and hemicellulose to monosaccharides, and because lignin is a true waste in CELSS. The same variety of wheat (cv. Yecora rojo) grown under field conditions will typically contain three to five times more lignin than noted in Figure 1 (Bugbee, personal communication).

The primary goal of biomass processing research at KSC is the minimization of the volume and energy required to (a) grow plants, (b) store the biomass produced and (c) recycle elements (C, N, etc.) through the waste processing systems while maximizing the utilization of volume, space, weight, light energy. To accomplish this goal, an efficient means of converting inedible crop residues into usable, preferably edible, products was researched.

Enzymatic hydrolysis of crop residue cellulose into glucose offered many advantages over other biomass conversion processes and, thus, was selected as the

first to be considered. The cellulase enzyme complex, especially of hyperproducing mutants of *Trichoderma reesei*, has an extensive literature database(1). In addition, the technology needed to convert cellulose to glucose enzymatically seemed to be readily available, without the need for extensive developmental research. Compared to acid hydrolysis of cellulose, enzymatic conversion avoids production of toxic byproducts, especially from the hemicellulose fraction of crop residue (2,3). Furthermore, the enzyme complex would be easier to produce and manage in the confines of a spacecraft than hot dilute acid, which would tend to corrode equipment and need neutralization and disposal after the conversion process was completed.

METHODS

Organisms

Aspergillus phoenicis QM329 and *Trichoderma reesei* QM9414 were obtained from the U.S. Army Natick Research Laboratory. Organisms were maintained on potato dextrose agar slants (PDA, Difco) at 4^{o}C, and transferred every 2 weeks.

Culture Conditions

The basal medium for production of enzyme and preparation of mycelial inocula contained the following compounds (g/L): 2.0 KH_2PO_4, 1.4 $(NH_4)_2SO_4$, 0.3 urea(Sigma), 1.0 proteose peptone (Sigma), 0.3 $MgSO_4.7H_2O$, 0.3 $CaCl_2$, 0.005 $FeSO_4.7H_2O$, 0.0016 $MnSO_4.H_2O$, 0.0014 $ZnSO_4$, 0.002 $CoCl_2$, 0.005 citric acid, 0.00025 $CuSO_4.5H_2O$, 0.00005 H_3BO_3, 0.00005 $Na_2MoO_4.2H_20$. Tween 80 was added at 1 mL/L. This medium is a modification of that of Ryu et al. (4), with the addition of selected micronutrients (3). Alpha-cellulose (Sigma) was added as carbon source for *T. reesei* QM9414 and potato starch (Sigma) for *A. phoenicis* QM329. Actively growing mycelial cultures were used to inoculate cultures for enzyme production. Erlenmeyer flasks of basal medium plus 1% (w/v)

alpha-cellulose (for *T. reesei*) or starch (for *A. phoenicis*) were inoculated with a 2% spore suspension (v/v) of the respective fungus. The suspension was prepared by washing two week old, sporulated fungal PDA plates with sterile deionized water. This suspension was then adjusted to a density of 0.4 absorbance units (660 nm wavelength). Mycelial cultures were incubated for 72 hrs at 25 degrees C while being shaken at 125 rpm on a rotary shaker. The resulting *T. reesei* mycelial suspensions could be used directly to inoculate enzyme production flasks, but the *A. phoenicis* mycelium grew in clumps and was gently dispersed with a glass tissue homogenizer to create a homogeneous inoculum.

Erlenmeyer flasks containing basal medium were also used for enzyme production. The medium was inoculated with the active, 72 hr mycelial suspensions, at selected densities, of *T. reesei* and *A. phoenicis*. Alpha-cellulose and starch concentrations varied with the experiment. Enzyme production flasks were incubated at 25 ^{o} C and 125 rpm. At the end of 7 days incubation, the fungal mycelia were removed from the medium by centrifugation at 3000 x g for 10 min, and the resulting supernatant was assayed for enzyme activities and for saccharification of wheat straw.

Assays

All enzyme assays were performed on the supernatant of seven-day old cultures (except for the timecourse of enzyme production depicted in Figure 2) after centrifugation at 3000 x g for 10 min to remove the fungal mycelia. Cellulase activity was estimated by the conventional filter paper assay (hydrolysis of purified cellulose contained in Whatman No. 1 filter paper) under standard assay conditions (4). Glucose (glucose oxidase/peroxidase, Sigma), instead of reducing sugar, was measured as the hydrolysis end product. ß-glucosidase activity was estimated with cellobiose as substrate according to Sternberg et al. (5) Results of all enzyme assays are reported as µmoles glucose produced per (minute x mL).

Saccharification

Saccharification of wheat straw was assayed by incubation of equivalent volumes of enzyme solution obtained from enzyme production supernatants and citrate buffer (pH=4.8) with 5% (w/v) ground wheat straw (50 mesh size) pretreated with alkaline peroxide to remove lignin and hemicellulose 6. The wheat straw was grown, hydroponically, to maturity (ca. 70 days) in the BPC ($23^{o}C$, 24 hr light at 300-500 $\mu moles \cdot m^{-2} \cdot s^{-1}$, 65% relative humidity) at KSC as part of a separate study. Sodium azide (0.02%) was added to the saccharification assay tubes to inhibit microbial growth. Saccharification assay tubes were incubated horizontally in a $50^{o}C$ water bath and shaken at 100 cycles/min. Tubes were removed at specified sampling times and centrifuged at 1000 x g for 2 min to remove particulates. Samples (0.1 mL) were removed from the supernatant, diluted 100 fold, and assayed for glucose.

RESULTS AND DISCUSSION

Studies of cellulase enzyme production.

When a monoculture of *T. reesei* QM 9414 was grown on alpha cellulose, a typical timecourse of cellulase enzyme production ocurred (Figure 2). ß-glucosidase activity reached a maximum at about 96 hrs and decreased slightly after 144 hrs. Cellulase activity reached a plateau at 96 hrs and then trended upwards at 192 to 240 hrs (240 hr data not shown). Relative to cellulase activity, ß-glucosidase activity was low, with a ratio of the two at about 1:1 near the typical enzyme harvest at 168 hrs. A number of published studies have recommended an optimal ratio of ß-glucosidase to cellulase for cellulose hydrolysis at 4:1 or greater (6,8).

This result was not unexpected, as hypercellulolytic mutant strains of *T. reesei*, such as QM9414, are known to produce an extracellular cellulase complex which is relatively deficient in ß-glucosidase (7,8). In practical saccharification reactions, this deficiency has caused a buildup of cellobiose, a competitive inhibi-

tor of the exoglucanase component of cellulase (9). A solution to this problem was suggested in the literature. The rate and extent of cellulose hydrolysis by the *T. reesei* cellulase complex can be increased by the addition of supplemental ß-glucosidase, produced by *Aspergillus phoenicis* in separate (8) or mixed (10,11,12) culture with *T. reesei*..

However, the benefits of increased glucose production must be weighed against the the added cost of *Aspergillus* production. Although mixed cultivation of *Trichoderma* and *Aspergillus* eliminates the expense of a separate fermentation step, starch is required as an additional carbon source for production of ß-glucosidase in *Aspergillus*. Furthermore, mixed culture may also involve problems resulting from interactions between the two fungi during growth. For instance, Duff et al.(11) noted a decrease in cellulase activity with increasing starch concentration in cocultures of these two fungi, and suggested competition for medium components, or production of inhibitory metabolites by *A. phoenicis* might be responsible.

An experiment was designed to evaluate the effects of relative inoculum density of the two fungi and starch concentration (substrate used for *A. phoenicis* growth and ß-glucosidase production) on the hydrolytic capacity of the cellulase enzyme complex produced in mixed cultures of *Trichoderma reesei* QM 9414 and *Aspergillus phoenicis* QM 329. A 4 x 4 factorial design was utilized, with starch concentration (0, 0.25, 0.5, and 1 % w/v) and *A. phoenicis* mycelial inoculum (0, 0.2, 1, and 5 % v/v) as factors. Alpha-cellulose concentration (1% w/v) and *T. reesei* mycelial inoculum (5% v/v) were held constant. The sixteen experimental treatments were repeated three times. All response variables were analyzed by three-way ANOVA with starch concentration and *A. phoenicis* inoculum as factors and replicates as a block. Bonferroni Multiple Comparisons were run when any significant differences occurred with either factor. Linear regressions were also performed on the data and correlation coefficients were obtained between all response variables.

The results of this experiment are shown in Figure 3 (for clarity, only the high and low starch concentrations are plotted). Both ß-glucosidase and cellulase

activities (Figure 3A and 3B, respectively) were affected significantly by the two factors: enzyme activities increased with increases in both starch and *A. phoenicis* inoculum density. Even at exceptionally low *A. phoenicis* inoculum (0.02%, v/v), significant increases in enzyme activities occurred at all starch concentrations tested.

The effects of starch concentration and *A. phoenicis* inoculum density on the extent of wheat residue (harvested from the KSC-CELSS BPC) hydrolysis after 48 hr (Figure 3C) were distinctive from those observed with the enzyme assays. A small, but significant, increase in cellulose hydrolysis with increasing *A. phoenicis* inoculum still occurred at 0% starch. Higher glucose yields from enzymes produced by cocultures containing *A. phoenicis* probably were a result of increased ß-glucosidase activity, as has been shown previously (12). When starch was included as an experimental factor, however, residue cellulose hydrolysis was unaffected. Correlations between glucose production from wheat residue and the enzyme assays were low (data not presented), and revealed the limitations of depending on enzyme assays alone to predict the hydrolytic potential of a cellulase complex acting on a natural cellulosic substrate.

An interspecies interaction, e.g., competition or antagonism, between the two fungi in cocultures containing starch and cellulose may be responsible for the results observed in Figure 3. Soluble protein levels (data not shown) indicate that *A. phoenicis* may have grown at the expense of *T. reesei*, whatever the reason. This interaction is apparently not significant in cocultures that contain only cellulose, with growth of *A. phoenicis* greatly reduced compared with cocultures containing starch. Although more precise definition of the nature of this interaction is beyond the scope of the present study, changes in media or other growth parameters could potentially reduce such competition.

To better understand the apparent need for increased ß-glucosidase for cellulose hydrolysis, enzyme production by monocultures of each fungus was examined. In addition to growing the fungi in monoculture for production of enzyme, the experiment also examined the substrate used for preparation of the inocula for enzyme production. Alpha cellulose and starch, purified substrates unavaila-

ble in a functioning CELSS, had been used in previous studies for fungal growth and production of the enzymes. Table 1 lists the factors examined in this experiment.

Table 1. Experimental design for monoculture production of ß-glucosidase by *A. phoenicis* and of cellulase by *T. reesei.* with different substrates used for generating inocula for enzyme production from wheat residue.

FACTOR	LEVEL
Inoculum substrate	
A. phoenicis	Starch, wheat residue, or base treated residue
T. reesei	Cellulose, wheat residue, or base treated residue
Enzyme ratios for wheat residue hydrolysis	
ß-glucosidase:cellulase	0:1, 1:4, 1:1, 4:1

Wheat residue was used for enzyme production.

To date, only one replicate of three has been completed, so conclusions drawn from the data are tentative. Figure 4 shows the enzyme activities for both fungi with inocula grown on different substrates and with enzyme production from wheat residue. ß-glucosidase production (Figure 4A) by *A. phoenicis* grown on wheat residue appeared to be considerably greater with inocula prepared from either wheat residue or base extracted wheat residue when compared with inocula grown on starch. ß-glucosidase production by monocultures of *T. reesei* appeared to be slightly greater for inocula prepared with cellulose, but enzyme activity from inocula prepared with treated wheat residue may not be substantially less.

Activities of cellulase produced by the fungal monocultures are presented in Figure 4B. Surprisingly, *A. phoenicis* produced measurable cellulase activity when cultured on wheat residue if the inocula were prepared with wheat residue

or treated wheat residue. To our knowledge, cellulase production by this fungus has not been demonstrated previously, but substrates such as starch and monosaccharides were used for growth of the inocula and enzyme production, instead of the complex lignocellulosic materials of our study (10). Cellulase activity of *T. reesei* monocultures grown on wheat residue (Figure 4B) were greatest when inocula were prepared with cellulose, but inocula prepared with treated wheat residue also caused significant production of cellulase.

To test the enzymes produced by the two fungal monocultures against natural substrates, culture filtrates were combined in different ratios of *A. phoenicis* ß-glucosidase to *T. reesei* cellulase and used to hydrolyze wheat residue for up to 168 hrs. Figure 5 shows the results of one set of these assays for *T. reesei* cellulase from wheat residue inoculated with mycelia grown on base-treated wheat residue. All three experimental sources of *A. phoenicis* ß-glucosidase are shown, i.e., monocultures of wheat residue inoculated with *A. phoenicis* mycelia grown on either starch (control), untreated wheat residue, or wheat residue treated with base to solublize the lignin and hemicellulose. Only the two extreme ß-glucosidase:cellulase ratios are plotted, i.e., 0:1 and 4:1. After 24 hrs of hydrolysis, glucose production from the wheat residue cellulose appears to be stimulated by the addition of *A. phoenicis* ß-glucosidase regardless of source, whereas at 168 hr, the addition of ß-glucosidase causes only a small increase in cellulose hydrolysis. These preliminary results (more replications to be run) indicate that supplementation of *T. reesei* cellulase complex by ß-glucosidase from *A. phoenicis* may not be needed. However, enzyme loading rates (ratio of cellulase to substrate cellulose) were kept low for this study as a result of poor enzyme yields from one of the *T. reesei* monocultures (inoculated with mycelia grown on wheat resiude). At these low loading rates, the need for additional ß-glucosidase at later times may have been diminished by the lack of cellobiose buildup. Additional studies at higher loading rates are needed before the role of supplemental ß-glucosidase can be determined.

In conclusion, *Trichoderma reesei* QM 9414 can be used to produce sufficient cellulase from treated wheat residue for the conversion of CELSS derived crop wastes. Contrary to current belief, the *T. reesei* cellulase complex may not need

supplemental ß-glucosidase, especially if long hydrolyses with high conversion efficiencies are desired. If supplemental ß-glucosidase is needed, then separate monocultures of *Aspergillus phoenicis* could be used. However, the effects observed may not apply to cocultures grown on other substrates.

Integration of biomass conversion into the KSC-CELSS Breadboard Facility:

Estimates of reactor sizes are needed for scale-up of enzyme production and of enzymatic hydrolysis of crop residues (saccharification) for preliminary design considerations in anticipation of integrating biomass conversion processes with the output/harvest from the BPC. To make these estimates, data were needed concerning substrate concentration, e.g.,wheat residue, and conversion efficiency at various enzyme (e.g., cellulase) loading rates (amount of enzyme per g of substrate). For these early studies, hydrolysis substrate was alkaline peroxide treated wheat residue, with the lignin and hemicellulose being chemically removed by the procedure of Gould and co-workers (7). The enzyme was prepared by cultivation of *T. reesei* on alpha cellulose. Results of 48 hr residue hydrolysis assays (Figure 6) indicate that the best conditions for optimal conversion efficiency were 5% residue concentration at an enzyme loading of 3 IU/g residue {IU = international units of enzyme = g glucose produced / (mL x min)}

These values were used to estimate reactor sizes. For convenience, continuous operation of the BPC was assumed, with one tray of mature wheat harvested per day. From the data shown in Figure 6, the anticipated hydrolysis (or saccharification) reactor size was estimated at 16 L (containing 5% residue for 48 hr, or the equivalent of 2 days harvested wheat residue). The amount of cellulase enzyme needed to support this saccharification would be 900 IU, with an assumed enzyme loading of 3 IU / g cellulose and residue with a cellulose content of 40% (Calculated from Figure 1, for cold water extracted residue). With an enzyme yield of 4 IU/ g of residue (calculated from results presented in Figure 5), then the enzyme production reactor would be 9 L and contain 225 g of residue. This quantity is nearly 29% of the residue available for both enzyme production and saccharification.

To better estimate reactor sizes and other parameters needed to scale-up enzyme production and residue saccharification, the data presented in Figure 6 should be repeated with cellulase enzyme produced from wheat residue instead of from alpha cellulose. Nevertheless, improvements can be suggested: (a) cellulase enzyme in the hydrolysis reactor should be recycled, thus reducing the requirement for use of residue to produce enzyme, (b) the enzyme production yield should be increased, and (c) the saccharification conversion efficiency should be increased through pretreatments to remove lignin and hemicellulose.

LITERATURE CITED

1) Mandels, M. 1982. Cellulases. Annual Reports on Fermentation Process 5:35-78

2) Lee, Y.Y., C.M. Lin, T. Johnson, and R.P. Chambers. 1979. Selective hydrolysis of hardwood hemicellulose by acids. Biotech. Bioeng. Symp. 8. 75-88.

3) Grohmann, K., M. Himmel, C. Rivard, M. Tucker, R. Torget, and M. Graboski. 1984. Chemical-mechanical methods for the enhanced utilization of straw. Biotech. Bioeng. Symp. 14. 137-157.

4) Ryu, D., R. Andreotti, M. Mandels, B. Gallo, and E.T. Reese. 1979. Studies on quantitative physiology of *Trichoderma reesei* with two stage continuous culture for cellulase production. Biotech. Bioeng. 21:1887-1903.

5) Sternberg, D., P. Vijayakumar, and E.T. Reese. 1977. ß-glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. Can. J. Microbiol. 23:139-147

6) Gould, J.M. 1984. Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Biotech. Bioeng. 26:46-52

7) Sternberg, D. 1976. ß-glucosidase: its biosynthesis and role in saccharification of cellulose. Appl. Environ. Microb. 31:648-654.

8) Howell, J.A. and J.D. Strick. 1975. Kinetics of solka floc cellulose hydrolysis by *Trichoderma viride* cellulase. Biotech. Bioeng. 17:873-893.

9) Allen, A., and D. Sternberg. 1980. ß-glucosidase production by *Aspergillus phoenicis* in stirred-tank fermentors. Biotech. Bioeng. Symp. 10. 189-197.

10) Duff, S.J.B., D.G. Cooper, and O.M. Fuller. 1985. Cellulase and beta-glucosidase production by mixed culture of *Trichoderma reesei* Rut C30, and *Aspergillus phoenicis*. Biotech. Lett. 7(3):185-190.

11) Duff, S.J.B., D.G. Cooper, and O.M. Fuller. 1987. Effect of media composition and growth conditions on production of cellulase and ß-glucosidase by a mixed fungal fermentation. Enzyme Microb. Technol., 9(1):47-52.

12) Duff, S.J.B., D.G. Cooper, and O.M. Fuller. 1986. Evaluation of the hydrolytic potential of crude cellulase from mixed cultivation of *Trichoderma reesei* and *Aspergillus phoenicis* Enzyme Microb. Technol. 8:305-308.

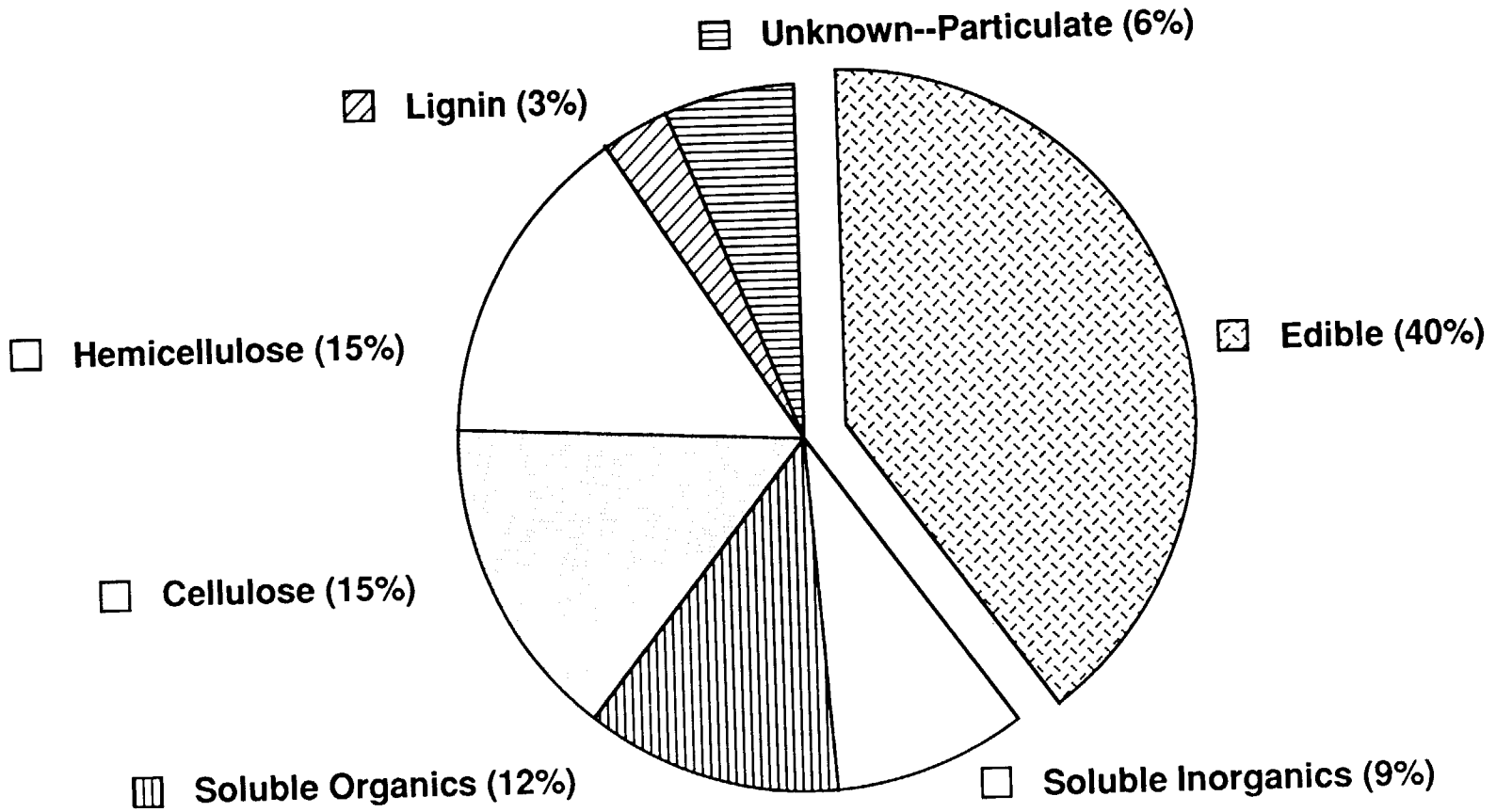

Figure 1. Composition of KSC-CELSS Breadboard wheat at harvest from the Biomass Production Chamber. Fiber analysis by Nutritional International Inc.

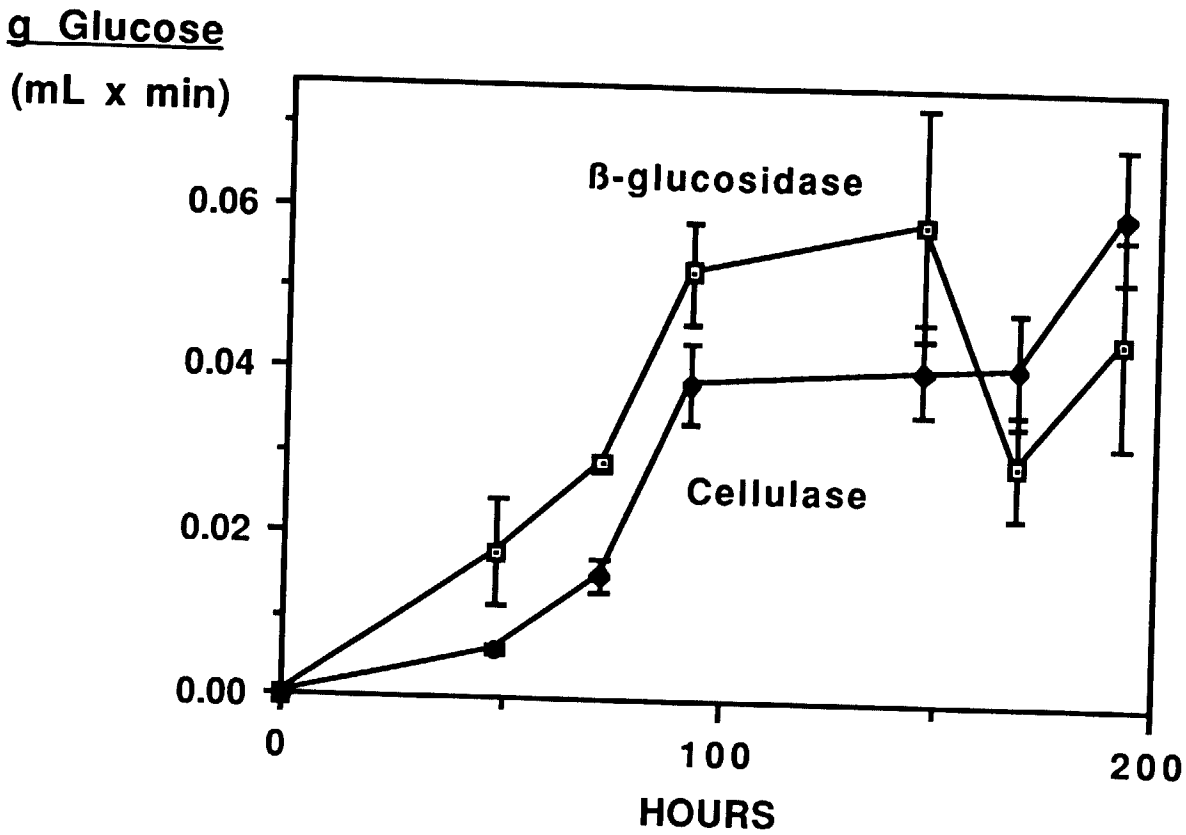

Figure 2. Time-course of soluble enzyme production by a monoculture of-*Trichoderma reesei* QM 9414 grown on alpha cellulose. Average of three replicates and standard deviations are plotted.

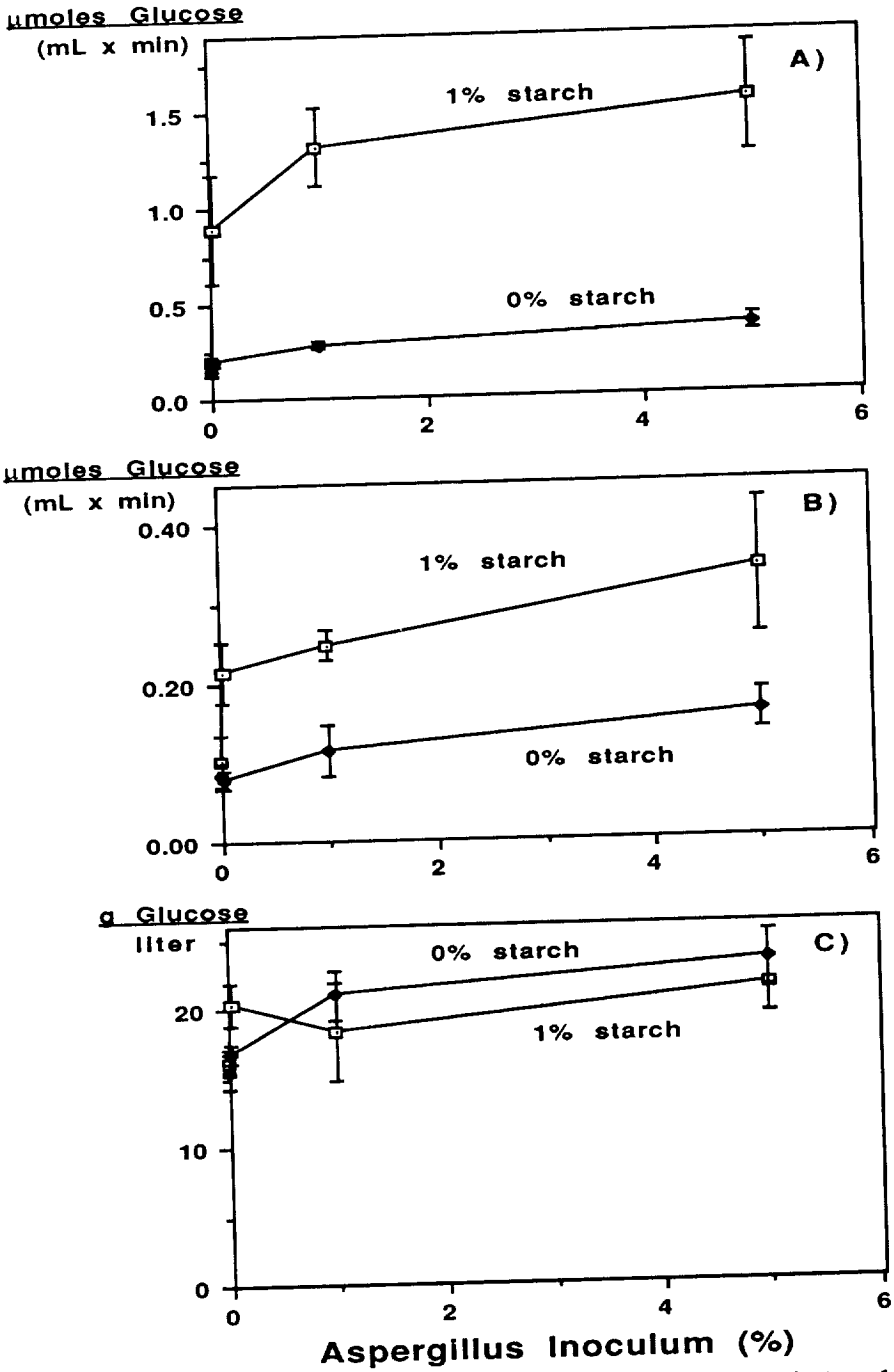

Figure 3. Effects of two starch concentrations and *A. phoenicis* relative inoculum concentration (v/v) on ß-glucosidase activity (A), cellulase activity (B) and 48 hr. wheat residue hydrolysis (C) for soluble enzymes produced in 7 days by cocultures of *T. reesei* and *A. phoenicis*. All cocultures were inoculated with the same inoculum concentration of *T. reesei* (1%) and contained 1% alpha cellulose.

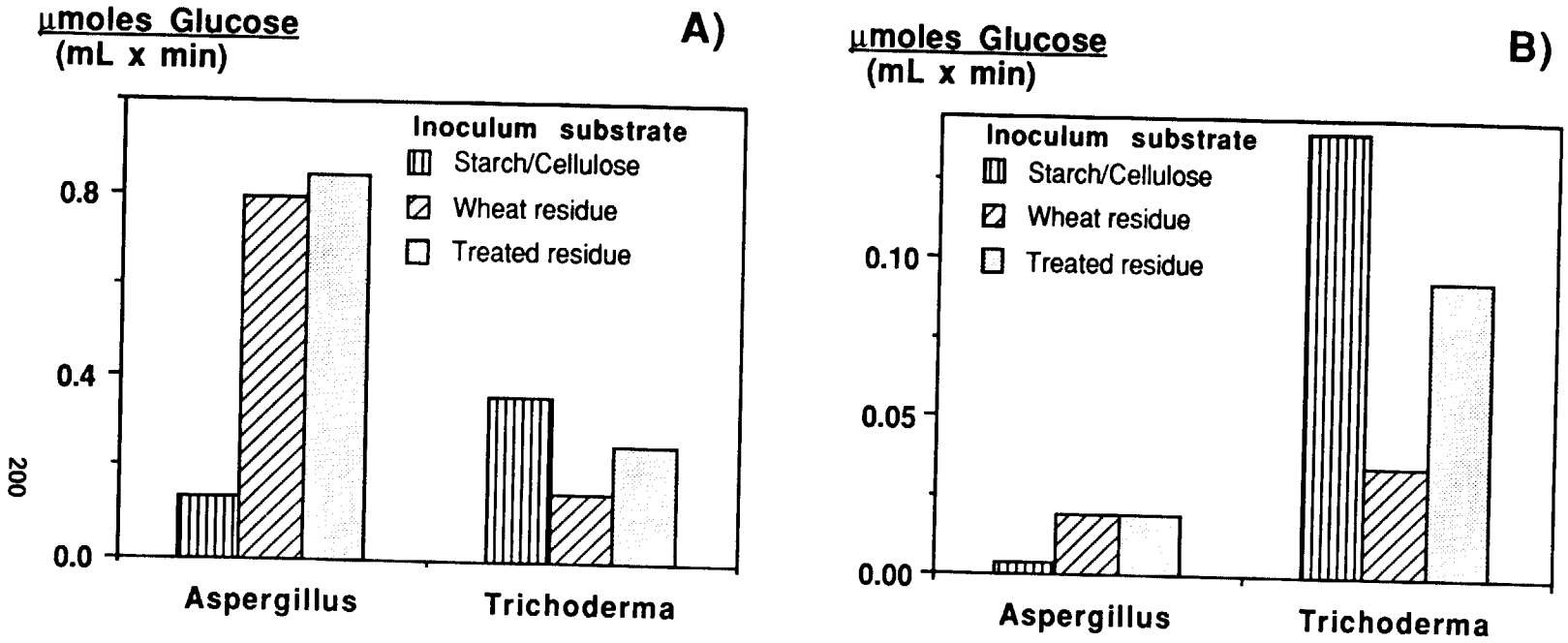

Figure 4. Activities of ß-glucosidase (A) and cellulase (B) produced in 7 days by monocultures of *T. reesei* and *A. phoenicis* grown on wheat residue. *A. phoenicis* monocultures were inoculated with mycelia grown for 3 days on either starch, untreated wheat residue or wheat residue treated with base to solublize lignin and hemicellulose. *T. reesei* monocultures were inoculated with 3 day old mycelia grown on either cellulose, untreated wheat residue or base treated wheat residue.

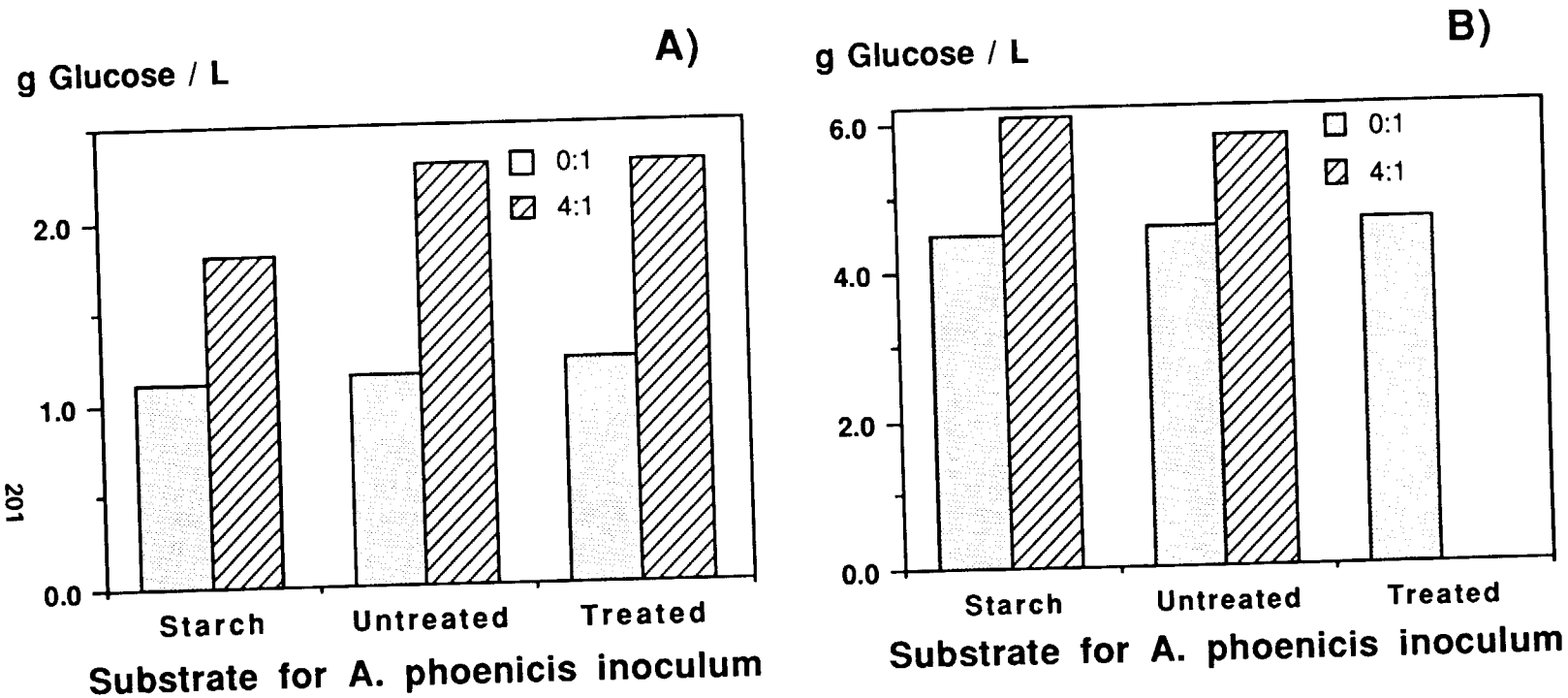

Figure 5. 24 hr (A) and 168 hr (B) wheat residue hydrolysis by soluble enzymes produced by monocultures of *A. phoenicis* (predominantly ß-glucosidase, Figure 4A) and *T. reesei* (predominantly cellulase, Figure 4B). Enzymes from the two monocultures were combined in different ratios (0:1 and 4:1 shown, *A. phoenicis* ß-glucosidase to *T. reesei* cellulase) according to the enzyme activities as determined for Figure 4. Monoculture conditions are described in the legend for Figure 4.

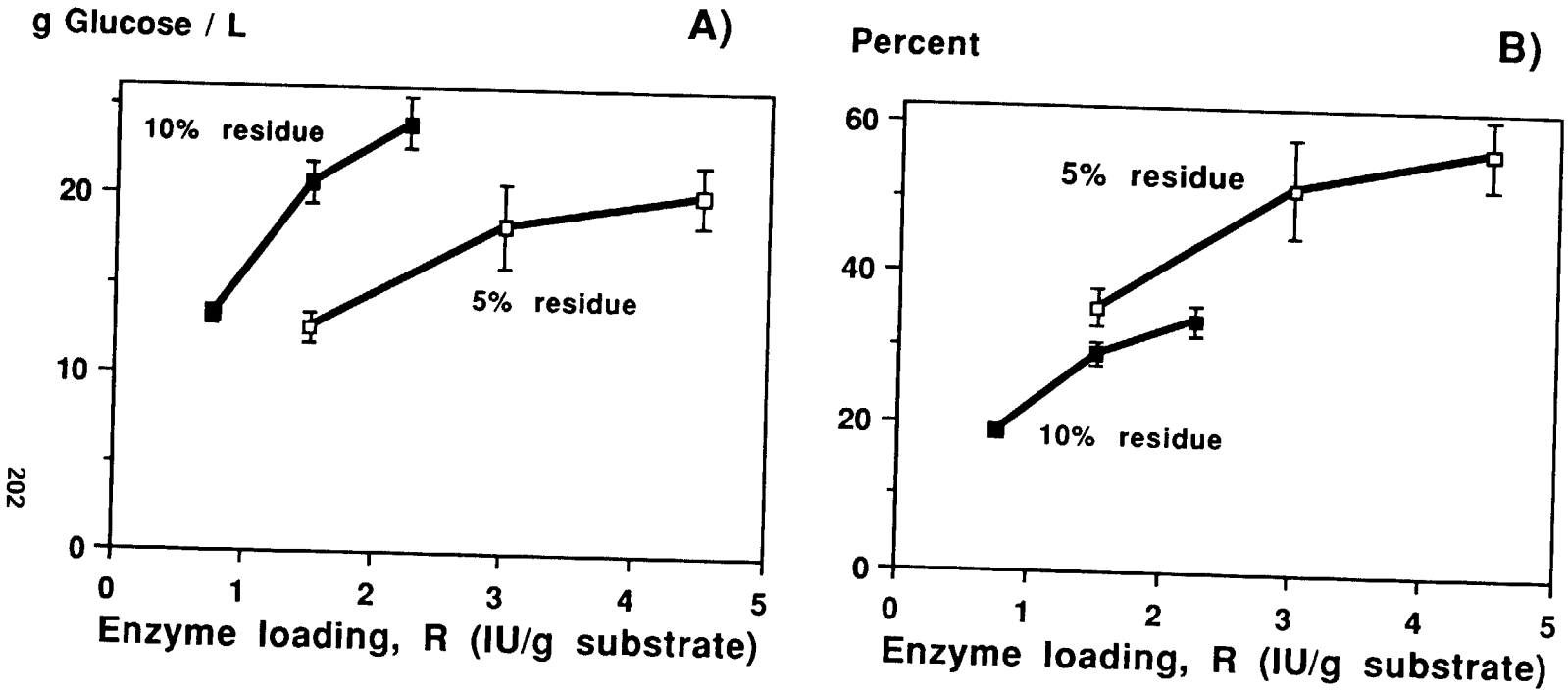

Figure 6. Effects of enzyme loading (IU/g substrate) on glucose production (A) and conversion efficiency (B) at the end of 48 hr saccharification/hydrolysis at two substrate concentrations. *T. reesei* cellulase was produced in monoculture from alpha cellulose. Saccharification/hydrolysis substrate was KSC-CELSS produced wheat residue that had been treated with alkaline peroxide to remove lignin and hemicellulose.