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Cellulase Activity of *Trichoderma reesei* (RUT-C30) on Municipal Solid Waste

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ABSTRACT

This work presents a preliminary investigation of *Trichoderma reesei* (RUT-C30) grown on municipal solid waste (MSW). Such a process offers the potential for inexpensive production of cellulase enzymes while reducing the waste stream to landfills. Cellulase enzyme activity for batch-culture growth on MSW compared favorably with growth on refined cellulosic substrates. Cellulase productivity in an initial fed-batch culture reached a maximum of 22 IFPU/L-h with a maximum activity of 1.5 IFPU/mL.

Index Entries: Cellulose hydrolysis; bioconversion of municipal solid waste; cellulase production; enzymatic hydrolysis; *Trichoderma reesei* (RUT-C30).

INTRODUCTION

Enzymatic hydrolysis coupled with the fermentation of the resulting sugars presents a potentially strong alternative to landfilling and incineration for handling a sizeable portion of the municipal solid waste (MSW) stream with the added benefit of chemical production for feedstocks or fuels. The potential for MSW to serve as an inexpensive source for cellulase production for the hydrolysis step in the overall fermentation process further improves the attractiveness of integrating this technology into the broad scheme of solid-waste management.

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This article focuses on the cellulase activity of *Trichoderma reesei* (strain RUT-C30) when grown on the cellulosic fraction of MSW. This constitutes approx 45–60% of the MSW waste stream (*1*). Experimental work reported herein involved initially growing RUT-C30 on a glucose-based medium to promote high cell densities. Then seen inocula were transferred to flasks containing one of several cellulosic substrates, including MSW. The resulting cellulase activity and glucose concentration were then monitored throughout the batch process.

These preliminary results deal with the utilization of MSW that has not been pretreated. Prior research has shown that, with the considerable "pretreatment" (e.g., bleaching, pulping, and so on) of the paper component of MSW (approx 70% of the biodegradable fraction of MSW on a dry basis), its susceptibility to hydrolysis is only nominally enhanced by additional pretreatment in the laboratory (*2*).

The potential for using the cellulosic fraction of the municipal solid-waste stream as a substrate for producing cellulase enzymes promises a significant reduction in the mass loading entering the landfill. Simultaneously a useful intermediate (the cellulase enzyme complex) is obtained that may be deployed in a two-step or simultaneous saccharification/fermentation process to obtain fuel ethanol (*3,4*).

MATERIALS AND METHODS

Substrates

All individual MSW components were air-dried at ambient conditions, and then ground with a Wiley mill to pass through a 2-mm screen. Each component was then dried at 100°C to a constant weight and mixed according to the percentages reported in the literature (*5*). For comparison, cultures were grown on MSW, Avicel PH-101 (50 μm) (Fluka Chemika-BioChemika, Ronkonkoma, NY), Solka Floc (generously supplied by Ali Mohageghi of the National Renewable Energy Laboratory in Golden, CO), and cellulose powder (20 μm, Sigma Chemical Co., St. Louis, MO). Avicel is a form of microcrystalline cellulose that has been shown to be a somewhat recalcitrant substrate for microbial hydrolysis.

Culture Maintenance and Growth Conditions

Stock cultures of *T. reesei* RUT-C30 were maintained on potato dextrose agar (PDA) slants and plates (*6*), incubated at 28°C to establish growth, and stored at 5°C. For broth culture studies, RUT-C30 was inoculated into experimental media described below. The pH was initially set at 4.8 for all cultures inoculated onto plates or in broth. Two medium formulations shown in Table 1 (*7*) were employed to evaluate culture performance. Medium 1 was used as a growth medium to produce high cell densities in

Table 1
Composition of Media 1 and 2 Used for Experimentation

Component	Composition	
	Medium 1[a]	Medium 2[b]
$(NH_4)_2SO_4$	7.0 g/L	7.0 g/L
KH_2PO_4	10.0 g/L	10.0 g/L
K_2HPO_4		
KCl		
$MgSO_4 \cdot 7H_2O$	1.5 g/L	1.5 g/L
$MgSO_4$		
$CaCl_2 \cdot 2H_2O$	2.0 g/L	2.0 g/L
Urea	1.5 g/L	1.5 g/L
$FeSO_4 \cdot 7H_2O$	25.0 mg/L	25.0 mg/L
$FeSO_4$		
$MnSO_4 \cdot 4H_2O$	10.3 mg/L	10.3 mg/L
$ZnSO_4 \cdot 7H_2O$	7.0 mg/L	7.0 mg/L
$CoCl_2 \cdot 6H_2O$	18.3 mg/L	18.3 mg/L
$NaNO_3$		
Cellulose		variable
Glucose	25 g/L	1.0 g/L
pH	4.8	4.8

All percentages are given as weight per volume (w/v).
[a]"Growth Medium"—Turker and Mavituna (7).
[b]"Production Medium"—Turker and Mavituna (7).

seed inocula. Media containing soluble carbon substrates (e.g., glucose or xylose) have been utilized to promote an initial high cell mass and growth rate, resulting ultimately in a higher cellulase enzyme concentration (*8,9*). Medium 2 was used as a cellulase production medium. Seed cultures were prepared by washing PDA plates with 10-mL aliquots of sterile, distilled water. This spore suspension was then added to 125 mL of growth medium (Medium 2) and allowed to incubate for 48–72 h at 30°C and 150 rpm. Batch culture experiments were conducted with 200 mL of medium in 500-mL shake flasks at 30°C, with pH 4.8 and an agitation rate set at 150 rpm. Uninoculated control flasks were maintained under identical conditions and examined for background activity.

A preliminary fed-batch study is reported as well. It was conducted in a New Brunswick Bioflo III fermenter with a 1.5-L vessel (1-L working liquid volume). One liter of growth medium (Medium 1) was inoculated with RUT-C30 seed culture (prepared as described above) and allowed to grow until log-phase growth was well established (as determined by rapid depletion of glucose). At this point, MSW was fed to the reactor daily at a rate of 10 g/L/d. Reactor conditions were identical to those described previously for the batch experiments.

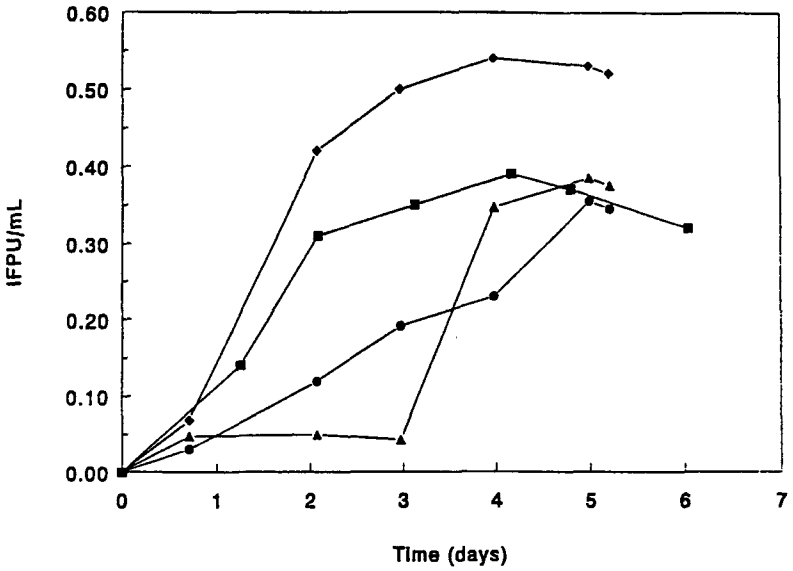

Fig. 1. RUT-C30 with various substrates comparison of MSW with refined cellulose. 2% Solids: —▲— cell. pwdr., —●— Avicel, —■— MSW, and —■— Solka Floc.

Analyses

Glucose concentrations and enzyme activities were determined by the methods described by Ghose (*10*). The method for cellulase enzyme activity is expressed as International Filter Paper Units/(IFPU)/mL of culture broth (IFPU/mL) as recommended by the International Union of Pure and Applied Chemistry (*10*). All experiments were run in duplicate with plots depicting averaged values for data obtained.

RESULTS

Batch Studies

A study of RUT-C30 was conducted in broth cultures using Medium 2 containing 2% solids (w/v) for four different cellulosic substrates, including MSW. Although data on the degree of crystallinity for each of the substrates were unavailable, other work (*11*) has shown that Avicel has a significantly higher degree of crystallinity in comparison to milled or powdered cellulose. Avicel was therefore chosen as a more recalcitrant substrate—thereby attempting to differentiate further cellulase activity among the various substrates examined. Indeed, the relative "completeness" of various cellulolytic enzyme systems (e.g., endo- and exo-glucanases and β-glucosidase) has been correlated to their ability to hydrolyze cellulosic materials of varying degrees of crystallinity (*12*). Figure 1 compares the cellulase activity of RUT-C30 for each of the substrates tested.

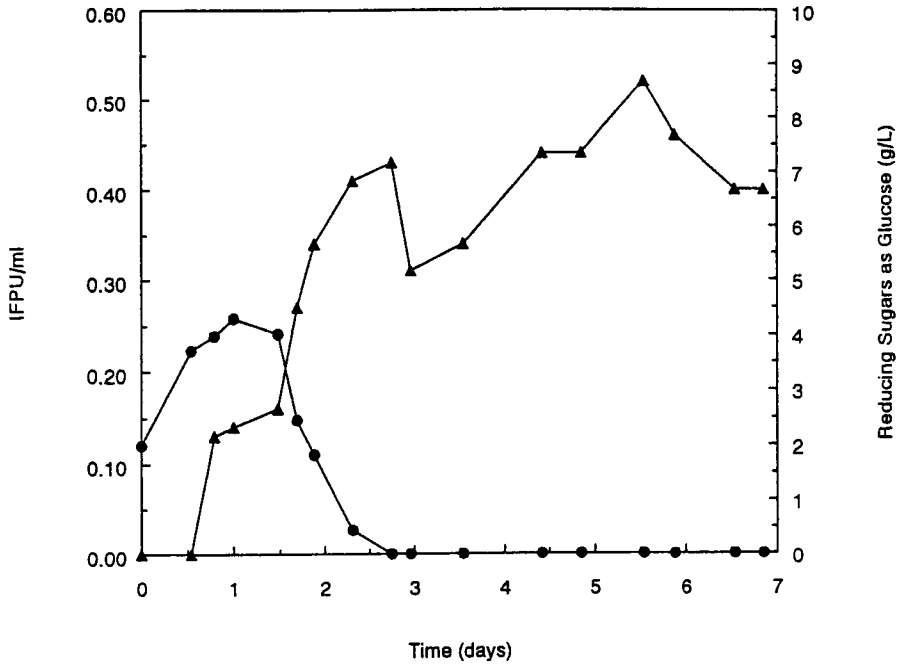

Fig. 2. RUT-C30 grown on MSW 2% solids concentration. —▲— Enzyme conc., —●— Red. sugars.

The flasks containing MSW attained an enzyme concentration of 0.5 IFPU/mL in 4 d, whereas the flasks containing cellulosic substrates reached a peak of approx 0.35 IFPU/mL in that same time period. It should be noted that the MSW prepared for this work contained approx 60% cellulose. Therefore, although fed in an equivalent solids concentration to other substrates (to maintain approximately equivalent mixing characteristics), significantly less substrate was available for conversion. The enzyme activity on MSW is somewhat higher than indicated when evaluated with this consideration. As expected, the cultures containing Avicel (and the cellulose powder) exhibited a delayed increase in enzyme activity in comparison to the flasks containing MSW and Solka Floc. However, the ultimate level of cellulase activity was comparable for the three pure or "refined" cellulose substrates. One condition noted in the preparation of Medium 2 is the release of additional sugars from the MSW during the autoclaving process. This may be seen in Figs. 2–4 with initial sugar concentrations of approx 2, 3.5, and 5 g/L, respectively, exceeding the 1 g/L of glucose contained in the medium preparation. The hydrolysis of hemicelluloses and possibly amorphous cellulose constituents makes these sugars available for faster cellular growth and ultimately higher enzyme activity.

Additional studies were run to examine the cellulase activity as a function of MSW solids concentration. Duplicate flasks were prepared with Medium 2 containing 2, 5, and 8% MSW, respectively. Representative data

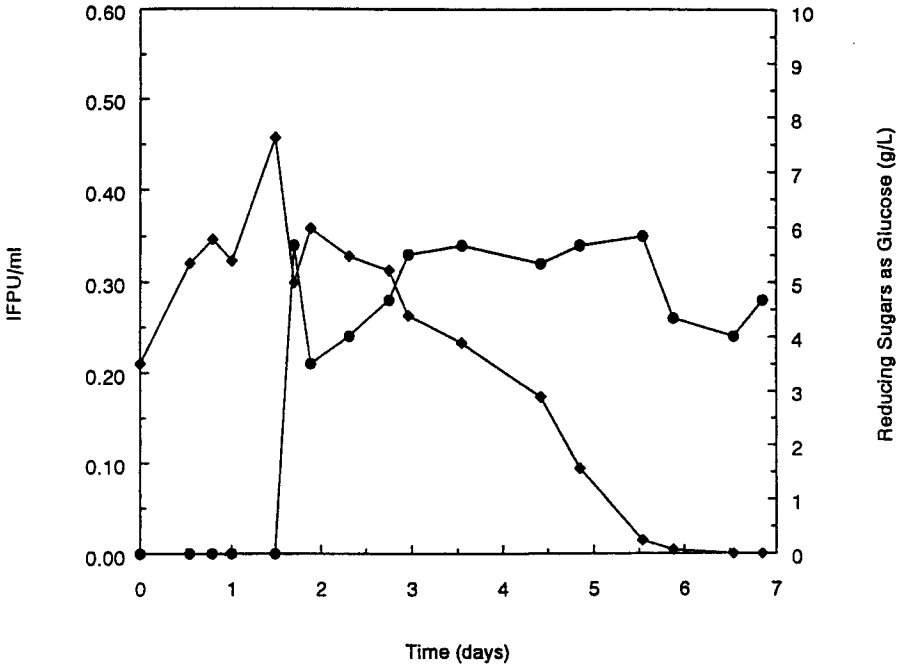

Fig. 3. RUT-C30 grown on MSW 5% solids concentration. —●— Enzyme conc., —◆— Red. sugars.

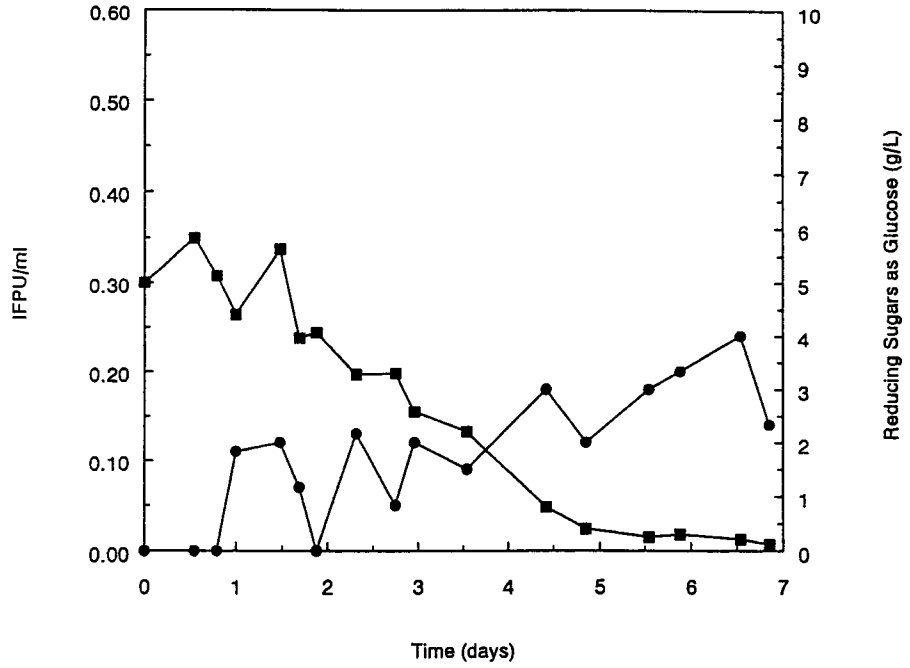

Fig. 4. RUT-C30 grown on MSW 8% solids concentration. —●— Enzyme conc., —■— Red. sugars.

are shown in Figs. 2-4. Although the enzyme levels did not reach those obtained for MSW in the previous batch experiment, the flasks containing 2% exhibited the highest activity (approx 0.5 IFPU/mL in 5 d). The culture growing on the higher solids concentrations utilized the available sugars more slowly and produced less enzyme. This may be owing, in part, to the heterogeneous nature of MSW (i.e., containing lignin)—likely to contain inhibitory or toxic constituents that manifest greater influence on culture behavior at higher concentrations. For each set of flasks, a decline in cellulase activity was observed after an initial increase. Such a phenomenon has been observed for other strains of *T. reesei* growing on cellulosic substrates. A possible explanation is a metabolic shift from the amorphous to more crystalline cellulose, resulting in a period of cessation in cellulase production and a decrease in cellulase enzyme from destructive shear (*13*). The absence of this observation in the first batch experiment is noted in that samples were only taken once daily, thereby allowing for the possibility that this shift in catabolic activity on the two forms of cellulose went undetected. Alternately, the complexation of the cellulase enzymes with lignin present in the batch culture may show an apparent decline in activity. Although offered only as conjecture, more work is needed to examine this phenomenon.

Fed-Batch Experiment

The results for the fed-batch experiment are shown in Fig. 5. The initial data represent the start of MSW addition to the reactor. The initial enzyme activity (without MSW addition) was approx 0.75 IFPU/mL. The cellulase activity gradually increased to 1.5 IFPU/mL in 13 d. Reducing sugars concentration closely tracked enzyme activity, reaching a peak of 7.75 g/L in the same time period with a significant decline 48 h following this peak. The tracking of reducing sugars with enzyme activity points to the possibility that cellular activity greatly decreased on addition of MSW. Additional work will focus on the initial growth period prior to MSW addition, replacement of air sparging with oxygen, and the use of soluble carbon substrates for initial growth, such as xylose (*8,9*).

DISCUSSION AND CONCLUSION

Batch cultures of RUT-C30 have shown a comparable cellulase enzyme activity when grown on MSW compared to refined cellulosic substrates. Preliminary results in batch cultures indicated an IFPU/mL of 0.5 for 5% (w/v) MSW compared to 0.35 for cultures grown on 5% (w/v) cellulose. The relative enzyme activity for RUT-C30 growing on MSW is even larger, noting that flasks were prepared on an equivalent solids concentration basis and the solids concentration of MSW is only 60% cellulose.

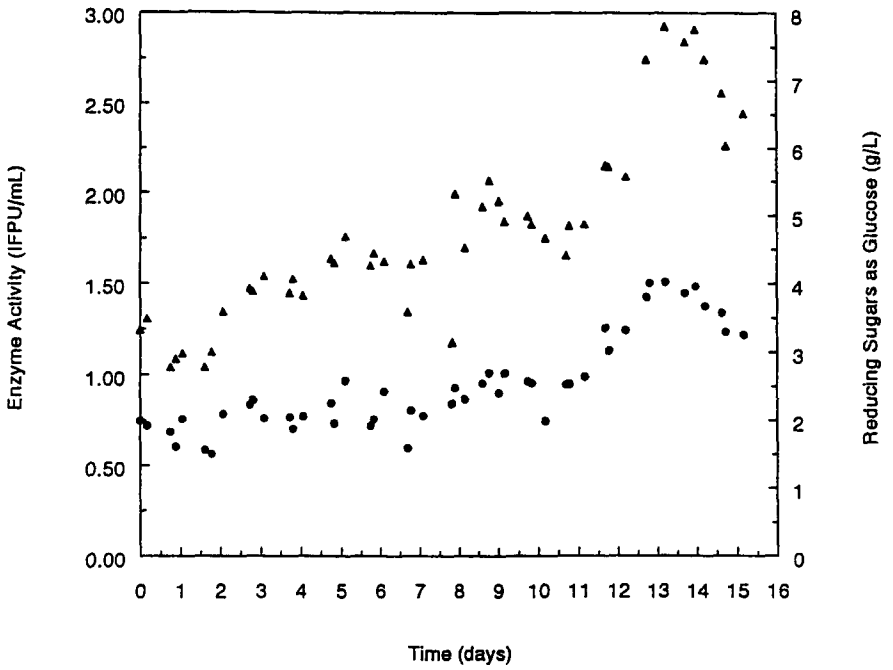

Fig. 5. Fed-batch culture RUT-C30 on MSW 10 g/L/d MSW feed rate. ▲ Red. sugars. ● Enzyme conc.

Table 2
Comparative Values of Cellulase Enzyme Activity
for *Trichoderma reesei* RUT-C30 Growing on Cellulosic Substrates

Reference	Substrate and system	Activity, IFPU/mL	Max. productivity, IFPU/L/h
Montenecourt et al. (*13*)	1% Cellulose/batch	1	
Montenecourt et al. (*14*)	5% Solka Floc/fed-batch	8	
Hendy et al. (*15*)	2% Cellulose/fed-batch (10-L fermenter)	4.2	46
Watson et al. (*16*)	1% Cellulose/fed-batch (20-L fermenter)	57	201

In fed-batch culture, RUT-C30 exhibited an enzyme activity of 1.5 IFPU/mL after 13 d with a maximum productivity of 22 IFPU/mL. Table 2 lists reported values for cellulase activity on cellulosic substrates for a variety of batch and fed-batch conditions. Although a direct comparison cannot be made with MSW as a substrate, these data serve to illustrate that considerable gains are needed in cellulase yield and productivity for MSW to compete as a viable substrate.

A significant reduction in the total solids of the biodegradable fraction of the MSW was observed. Summarizing data from a variety of cultures studied, batch cultures containing 2 and 10% MSW (w/v) experienced an

average reduction in solids concentration of 55 and 20%, respectively. Such information becomes important in the evaluation of process economics and gains made from reducing the landfill waste stream. Ongoing studies examining the improvement in the hydrolysis rates and yields through the employment of fed-batch culture and the identification of limiting factors (e.g., β-glucosidase activity) must be examined in the future to elucidate the quality of the cellulase enzyme system produced from MSW.

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REFERENCES

1. Leeper, S. A., Ward, T. E., and Andrews, G. F. (1991), *Production of Organic Chemicals via Bioconversion: A Review of the Potential*, Idaho National Engineering Lab, EGG-2645, pp. 1–79.
2. Holtzapple, M. T., Lundeen, J. E., Sturgis, R., Lewis, J. E., and Dale, B. E. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 5–21.
3. Galbe, M. and Zacchi, G. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 93–104.
4. Spindler, D., Wyman, C. E., and Grohmann, K. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 275–286.
5. Walsh, J. J. and Kinman, R. N. (1979), *Municipal Solid Waste: Land Disposal, Proceedings of the Fifth Annual Research Symposium*, pp. 41–57.
6. Jong, S. C. and Edwards, M. J. (1991), *Catalogue of Filamentous Fungi*, American Type Culture Collection, p. 442.
7. Turker and Mavituna (1987), *Enzyme and Microb. Technol.* **9,** 739–742.
8. Mohageghi, A., Grohmann, K., and Wyman, C. E. (1990), *Biotechnol. Bioeng.* **35,** 211–216.
9. Mohageghi, A., Grohmann, K., and Wyman, C. E. (1988), *Appl. Biochem. Biotechnol.* **22/23,** 263–277.
10. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
11. Liaw, E. T. and Penner, M. H. (1990), *Appl. Environ. Microbiol.* **56,** 2311–2318.
12. Mo, K. and Hayashida, S. (1988), *Agric. Biol. Chem.* **52,** 1675–1682.
13. Montenecourt, B. S. and Eveleigh, D. E. (1979), *Adv. Chem. Ser.* **181,** 289–301.
14. Montenecourt, B. S., Sheir-Neiss, G. I., Ghosh, A., and Ghosh, K. (1983), *Proc. Intl. Symp. Ethanol Biomass* **XX,** 397–414.
15. Hendy, N., Wilke, C. R., and Blanch, H. W. (1982), *Biotechnol. Lett.* **4,** 12, 785–788.
16. Watson, T. G., Nelligan, I., and Lessing, L. (1984), *Biotechnol. Lett.* **6,** 10, 667–672.