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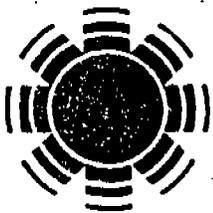
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Prepared for the
U.S. Department of Energy
Contract No. DE-AC02-83CH10093

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CELLULASE UTILIZATION RESEARCH

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PREAMBLE

Cellulase utilization research at SERI has taken a bidentate approach whereby multiple projects, some applied and some more background or basic in nature, are conducted concurrently. The applied projects are those which may directly lead to process methods, products or microorganisms capable of impacting immediately the economic picture of enzymatic conversion of biomass to liquid fuels. For FY1985-86 these applied projects include the evaluation of spray drying methods for cellulase preservation, optimization of the growth and cellulase enzyme production from a new SERI isolated aerobic thermophile, Acidothermus cellulolyticus, and the isolation of overproduced xylose isomerase from genetically modified E. coli (shown in "Overexpression of Xylose Isomerase" paper). Background studies conducted in FY1985-86 include the development of IMP/HPLC methods for the determination of the anomeric configuration of products from hydrolase action and the preparation of two textbook chapters on subjects in basic enzymology. The following paper consists of three sections describing these results.

I. EVALUATION OF SPRAY DRYING METHODS FOR CELLULASE PRESERVATION

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ABSTRACT

Spray drying processes are widely used for the large-scale preservation of biological goods. The application of this technology to the enhancement of the storability of cellulase and related enzymes would improve fermentation processes requiring input of specific quantities of enzyme catalyst of known activity. The present study found that very high (>90%) filter paper and carboxymethylcellulose hydrolyzing activities can be recovered from conventional spray dryers when operated at inlet air temperatures ranging from 90 to 180°C. The beta-

glucosidase and beta-xylosidase activity recoveries were somewhat lower and were optimal at temperatures ranging from 120 to 150°C. The individual performance of the 12 enzyme preparations studied was quite specific, however. An ultrasonic nozzle adaptation to the Yamato dryer demonstrated the overall feasibility of high energy atomization, inasmuch as the filter paper activity was retained after drying. The more labile beta-xylosidase activity was, however, entirely lost.

INTRODUCTION

Successful operation of industrial-scale fermentation plants based on enzymatic saccharification of biomass substrates demands the availability of appropriate quantities of saccharifying enzyme of known activity. Even with some redundancy in enzyme preparation facilities, losses of enzyme due to contamination or equipment failure can result in interruption of continuous operation. Access to stored and stabilized enzyme is clearly desirable.

Although some laboratory methods useful for the stabilization of enzyme preparations through dehydration (lyophilization, for instance [1]) have been successfully scaled up, spray drying is usually considered the most appropriate approach to large scale installations [2]. Unfortunately, the literature on conventional spray drying of biological materials has been largely concerned with the preservation and concentration of foodstuffs or food by-products. Some examples are cheese whey fractions [3,4], fish waste products [5], blood plasma proteins [6], bean protein [7], fungal mycelial fermentation broth [8], egg white [9], and many others. Studies describing the optimization of drying conditions for the successful spray drying of active enzymes are few, although those available include studies on fungal proteases [10], ribonuclease from Bacillus subtilis [11], milk additive enzymes (alkaline phosphatase) [12] and β -galactosidase from Aspergillus species [13]. Those specifically targeting cellulolytic and hemicellulolytic enzymes are not readily found in the literature.

In this study, preparations of commercially available enzymes and several cellulolytic enzyme-containing fermentation broths were subjected to bench-scale spray drying using the Yamato model GA-31 dryer. Enzyme activity recovery studies of dried products included filter paper activity, activity on carboxymethylcellulose (endocellulase), β -glucosidase and β -xylosidase activities as well as starch degrading ability (for amyloglucosidase). These parameters were evaluated as a function of the drying conditions (inlet and outlet temperatures) and sample preparation.

EXPERIMENTAL

Commercial enzyme preparations

The following cellulase preparations were studied: cellulase 150L from a selected strain of Trichoderma reesei, Genencor Inc.; Celluclast 1.5L (lot CNN3000) from T. reesei, NOVO Ltd.; cellulase C-0901 (lot 54F-0241) from P. funiculosum, Sigma Chem. Co.; Cellulysin (lot 405843) from T. viride, Calbiochem; Maxazyme C1 (ref. 5756) from T. reesei, Gist-

Brocades USA, Inc.; and cellulase "Onozuka" RS from a mutant strain of *T. viride*, Yakult Honsha Co., Ltd. The β -glucosidase enzymes G-8625 (lot 36F-4043) from almonds and Novozym 188 (batch DCN-01) from *Aspergillus niger* were obtained from Sigma Chem. Co. and NOVO Ltd., respectively. Cellobiase (batch DCN-01) from *A. niger* was also obtained from NOVO Ltd. Amyloglucosidase (lot 124F-0369) from *Rhizopus* genus mold was obtained from Sigma Chem. Co.

In-house cellulase enzyme preparations

Fourteen-liter fermentations of the Rut-C30 mutant of *T. reesei* were prepared according to the method of San Martin et al. [14] and 12-liter fermentations of *Acidothermus cellulolyticus* were grown according to Mohagheghi, et al. [15], for fresh enzyme spray drying. In both cases, the fermentation broth was freed of cells using a Ceba continuous centrifuge (30,000 rpm) and concentrated ten-fold at 4°C with a Millipore Pelicon ultraconcentrator equipped with a 10,000 M_r cutoff membrane cassette.

Spray drying equipment and operation

All spray drying studies were conducted on a Yamato model GA-31 bench scale dryer with electric heat control. Most drying tests were controlled at inlet temperatures of 90, 120, 150 or 180°C (corresponding to outlet temperatures of 30, 50, 65, 80, and 110°C), with filtered, compressed air (1.6 to 1.8 kg/cm³) as the atomizer gas. The hot-air aspirator flow rate was controlled at 0.38 cfm. This setting was found to provide the optimum velocity for the cyclone collection system. Surface temperature thermocouples (Omega Engineering Inc., no. C03-K) were cemented on the collection cyclone to indicate enzyme powder contact temperature. On most runs, this was found to be 20°C lower than the indicated outlet temperature. A modified air distribution plate was fabricated to allow installation of a non-standard, ultrasonic nozzle (Heat Systems-Ultrasonics, Inc., Sonimist® nozzle # HSS-700-3). Operation of the ultrasonic nozzle required conversion to bottled nitrogen (98%) for the atomization gas and delivery to the nozzle at 60 psi. Samples were pumped to the dryer at a flow rate of 2 mL/min in 20-100 mL aliquots per run after adjustment to 5% w/v.

Analytical HPLC

Enzyme preparations were subjected to analysis by Ion Mediated Partition (IMP)/HPLC to determine possible monosaccharide and polysaccharide contents. IMP chromatography was also used to screen for preservatives, such as glycerol, sorbitol, ethylene glycol and others. The HPLC system used has been described earlier [16].

Chemical analysis of enzyme preparations

All enzyme preparations were analyzed for protein, reducing sugar and salt content. These are presented as weight-percent relative to starting material (usually powders). In the case of liquid enzyme preparations, the solids content of aliquots were determined by both lyophilization and oven drying at 60°C for 24 h. Protein content was

determined by the modified Lowry method of Markwell et al. [17] and by the dye binding method of Bradford [18]. Reducing sugars were determined by the alkaline-DNS (3,5-dinitro salicylic acid) method described by Miller [19]. Salt content was determined as conductance equivalence based on a NaCl series as standards. A Beckman model RC-16C conductivity bridge with a 1 cm cell was used for this determination.

Enzyme assays

Assays for cellobiase, endo- β -1,4 glucanase (CMCase), and saccharifying cellulase (FPU) activities followed the methods of Sternberg et al. [20] and Mandels et al. [21] as modified in a recent IUPAC report [22]. Here, one cellobiase unit equals that amount of enzyme converting 2.0 μmol glucose per minute from cellobiose, one CMCase unit equals that amount of enzyme liberating 1.0 μmol glucose (or reducing sugars as glucose) from carboxymethylcellulose per min, and one filter paper unit of activity equals that amount of enzyme complex yielding 1.0 μmol glucose per minute from 50 mg filter paper strips. Under the recommended conditions of the CMCase and filter paper activity assays, enzyme/substrate ratios must be adjusted so that 0.5 mg and 2.0 mg glucose is released per hour at 50°C, respectively. β -glucosidase was determined according to the method of Wood [23] as aryl- β -glucosidase by the hydrolysis of p-nitrophenyl- β -glucopyranoside (Sigma Chem. Co.). The concentration of p-nitrophenol was estimated from the extinction at 410 nm under alkaline conditions induced by the addition of 2 M Na_2CO_3 . One unit of activity is defined as that amount of enzyme that catalyzes the cleavage of 1.0 μmol substrate per min. β -xylosidase activities were determined in a similar way using p-nitrophenyl- β -xylopyranoside as substrate [24]. Glucoamylase activity was determined using soluble potato starch (Sigma Chem. Co.) as substrate by the method given in Tucker et al. [25]. Here one unit of activity is given as that amount of enzyme which releases 1.0 μmol of glucose per min from a 1% starch solution at 55°C.

Spray drying methods

The enzyme preparations available as dry powders were spray dried as a 5% w/v solution. The liquid preparations were diluted to give approximately this concentration after evaluating the solids content of the original sample. The NOVO liquid enzyme preparations required ultrafiltration/dialysis using an Amicon PM10 membrane to remove the glycerol (included as preservative by NOVO) and excess glucose before spray drying. Several spray drying adjuvants (0.5% w/v) were studied. These included fumed silica (Sigma Chem. Co.), maltodextrin® G-250 (Grain Processing Co.), 1.0 M NaCl, and maltose (Sigma Chem. Co.).

Enzyme powder storage and activity retention

Spray dried powders of Genencor cellulase 150L and NOVO Celluclast 1.5L were stored under similar conditions with filter paper and beta-glucosidase activity checked every two weeks for 2 months. Storage conditions were under nitrogen or air at 25°C.

Table I
SELECTED PROPERTIES OF ENZYMES SUBJECTED TO SPRAY DRYING

Enzyme type	Source	Solids ^a mg/mL	Protein content g Lowry protein /g preparation	Sugar content g sacchar- ides/g prep.	Salt content g NaCl equiv./g prep.	IFPU/g ^b Lowry protein	CMCU/g Lowry protein	β GU/g Lowry protein	β XU/g Lowry protein
cellulase	Genencor	165	0.196 (0.052) ^c	-0-	0.0036 ^c	558	14,500	1470	22
cellulase	NOVO	487[49]	0.171 (0.042) ^c	0.004 ^c	0.0064 ^c	417	7,700	121	150
cellulase	Sigma	powder	0.50	0.022	0.190	583	10,800	8077	145
cellulase	Calbioch.	powder	0.37	0.50	0.066	325	13,100	1063	225
cellulase	Gist-Bro.	powder	0.29	0.744	0.039	565	12,800	660	466
cellulase	Rut-C30	44 ^d	0.023 (0.001) ^c	-0-	0.012 ^c	556	17,800	289	453
cellulase	A. cell.	56 ^d	0.020 (0.001) ^c	-0-	0.0006 ^c	127	650	30	4
cellulase	Yakult	powder	0.52	0.568	0.374	366	9,573	395	40
β -gluco.	Sigma	powder	0.94	-0-	0.133	NA ^e	NA	6500	NA
β -gluco.	NOVO	391[34]	0.140 (0.020) ^c	0.172 ^c	0.055 ^c	NA	NA	1789	NA
cellobiase	NOVO	397[44]	0.147 (0.020) ^c	0.167 ^c	0.056 ^c	NA	NA	1677 (14,490) ^f	NA
amylgluc.	Sigma	powder	0.42	0.133	0.053	19,788 ^g	NA	NA	NA

^aDetermined by air drying at 60°C for 48 h. Spray drying of NOVO preparations was performed after samples were subjected to Amincon PM10 ultradialysis (values shown in square brackets only). ^bEnzyme activities are defined in text. ^cComponents of liquid forms of enzyme are given as g/mL preparation (Note: obtain units/ml or units/gram of original preparation from the product of the protein content and specific activity). Parenthetical values from dye-binding protein assay illustrate possible protein assay-specific bias in specific activity data. ^dSpray dried as shown (represents a 10 fold concentration from fermentation broth). ^eNA is not analyzed. ^fAssayed as cellobiase activity (see text). ^gAssayed as starch degrading activity.

Table II
SPRAY DRYER NOZZLE COMPARISON USING GENENCOR CELLULASE 150L

Nozzle type	Drying Temperature °C	IFPU/g protein	CMCU/g protein	β -GU/g protein	β -XU/g protein
Standard ^a	150	528	13,639	1,450	19
ultrasonic ^b	150	465	12,750	409	0.70

^aFor purposes of comparison, data shown for standard nozzle were taken from Figures 1 - 4.

^bThe Sonimist® model 700-3 ultrasonic spray nozzle (Heat Systems-Ultrasonics, Inc.) was adapted to the Yamato GA-31 spray dryer by design of a new hot-air distributor plate.

RESULTS AND DISCUSSION

Table I illustrates many important characteristics of 10 commercial and 2 in-house hydrolytic enzyme preparations. Of the liquid cellulase preparations studied, the Genencor 150L enzyme had the highest overall filter paper activity (110 IFPU/mL preparation), with NOVO Celluclast 1.5L, Rut-C30 and A. cellulolyticus producing 71, 13 and 2.5 IFPU/mL, respectively. The Sigma cellulase appeared to have the highest activity (292 IFPU/g) of the powder preparations, with Yakult-Honsha RS, Gist-Brocades C1 and Calbiochem producing 190, 164 and 120 IFPU/g preparation, respectively. Also, the Sigma enzyme demonstrated the highest beta-glucosidase activity, even higher than the NOVO 188 preparation which is sold as beta-glucosidase. All enzymes showed relatively low, but measurable, beta-xylosidase activity. Note that these values are independent of Lowry protein content values given in Table I; however, activity values are given in Table I as units/gram Lowry protein (specific activity) to allow comparison with materials recovered from the spray dryer receiver and cyclone where a partial fractionation of components may occur. Table I also shows several important trends in cellulase content: the Sigma enzymes appear to contain a very high salt content; the Gist-Brocades, Yakult-Honsha and Calbiochem enzymes contain a very high sugar content (mostly glucose); and after diafiltration with an Amicon PM10 membrane, the NOVO preparations lose more than 90% solids (HPLC analysis shows that half of this is glycerol and glucose).

The characteristics described above proved important in interpreting spray drying performance. The NOVO preparations required diafiltration to remove high concentrations of glycerol added as preservative before spray drying was possible. The following conditions were found to give good collection of dried product: sample at a concentration of 5% w/v, aspirator rate of 0.38 to 0.40 cfm, inlet air temperature of 120 to 150 °C and a sugar content below 0.5% w/v. Adjuvants added to samples before spray drying included fumed silica, maltodextrin®, salts and monosaccharides. No adjuvant examined significantly improved the poor performance of problem preparations (i.e., product burning or sticking to glassware); however, addition of fumed silica was found to produce very low beta-glucosidase activity recoveries while having no effect on filter paper activity recovery.

Most cellulase preparations examined showed IFPU/g and CMCU/g recoveries of 90 to 100% in the inlet air temperature range 90 to 180°C. The beta-glucosidase and beta-xylosidase activity recoveries were high only in the 120 to 150°C range, with many beta-xylosidases showing poor activity recovery under all conditions. The amyloglucosidase examined showed very good activity recovery (95%) in the range 120 to 150°C. These activity recoveries are somewhat higher than those found for Aspergillus species β -galactosidase (66%, [13]) and Saccharomyces fragilis β -galactosidase (86%, [26]).

Studies of enzyme activity retention upon storage of spray dried preparations showed that NOVO Celluclast 1.5L retained nearly 100% of initial filter paper and beta-glucosidase activities after two months at 25°C under air, regardless of spray drying temperatures used (which

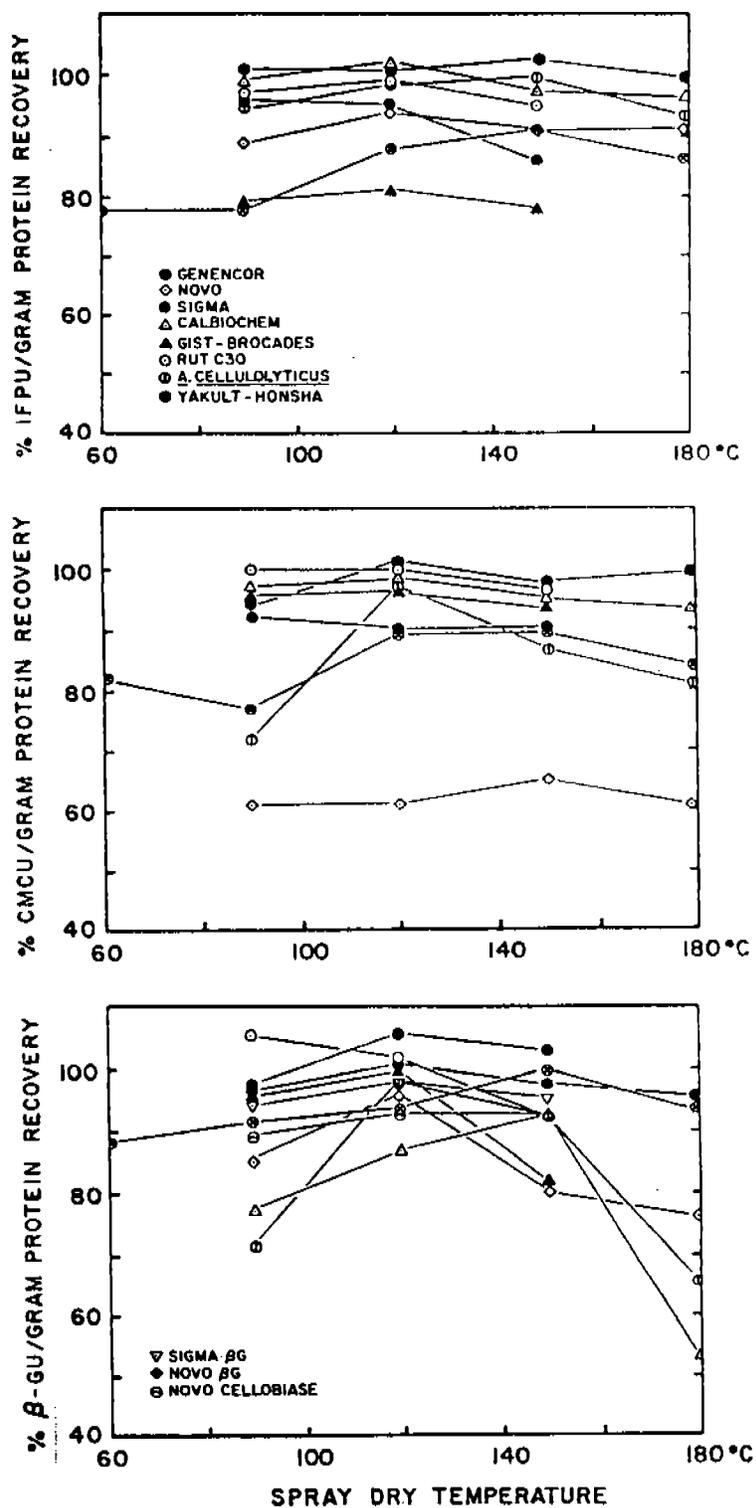


Figure 1. Comparison of the percentage of international filter paper (IFP), carboxymethylcellulase (CMC), and beta-glucosidase (β -G) units per gram protein recovered from starting material plotted at various inlet air temperatures for different cellulase preparations.

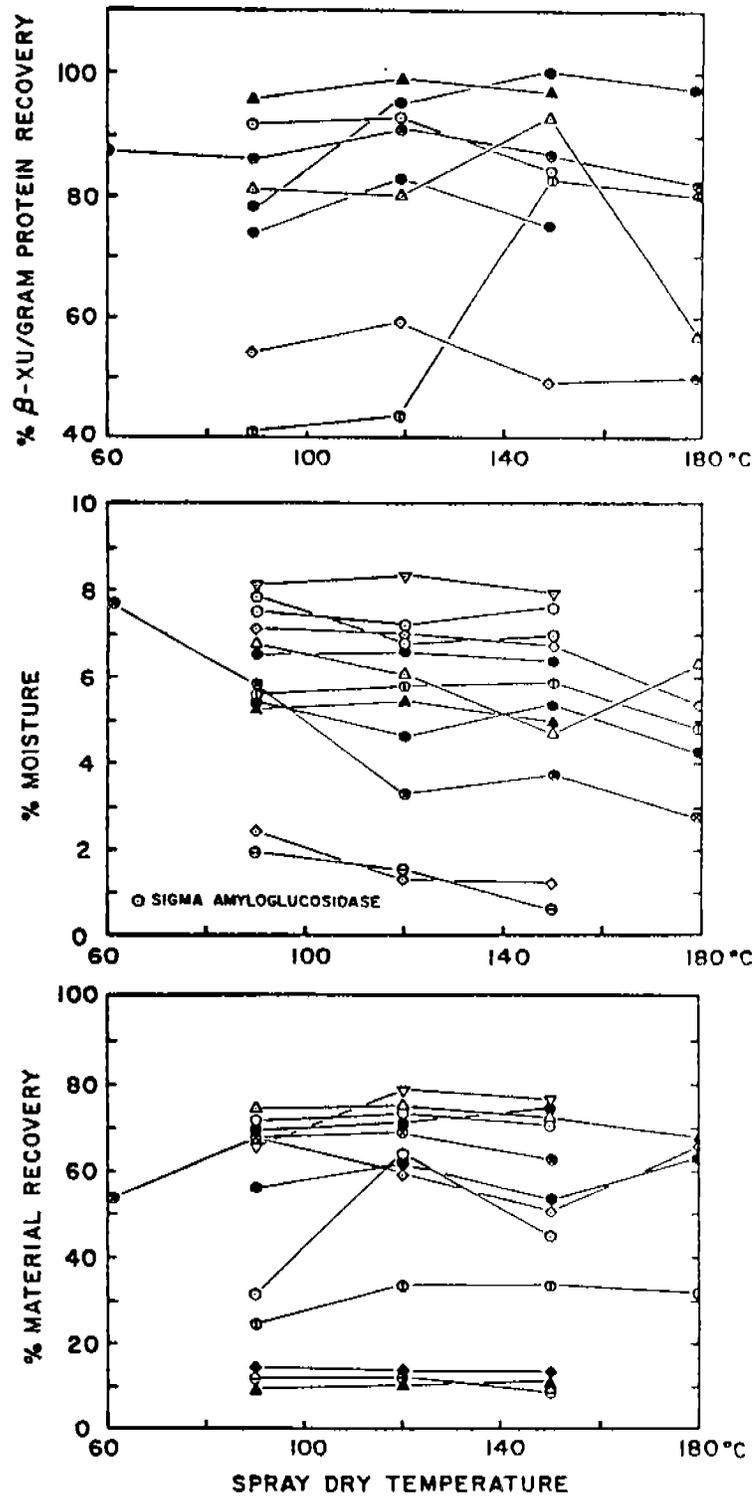


Figure 2. Comparison of the percentage of beta-xylosidase (β -X) units per gram, moisture and material recovered from starting material plotted at various inlet air temperatures for different cellulase preparations.

influences the moisture content of the powder). Early results showed that storage under nitrogen instead of air had no effect. The Genencor cellulase 150 L, however, demonstrated a 60% loss in activity under similar storage conditions. A possible explanation for this difference may lie in the trace quantities of glycerol entrained in the NOVO powders after spray drying.

The ultrasonic nozzle adaptation to the Yamato dryer provided some insight into possible ultrasonic drying of cellulase component enzymes. Table II shows that the high energy nozzle produced no effect on the recovery of overall filter paper activity, but had a devastating effect on the beta-glucosidase and beta-xylosidase activities. This observation is supported by the well known relative instability in solution of these classes of enzymes.

FUTURE DIRECTION

The cellulase spray drying research is now completed and the methodology will be used for the preparation of enzymes used in-house.

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**IIa. ACIDOTHERMUS CELLULOLYTICUS:
A NEW AEROBIC CELLULOLYTIC THERMOPHILE**

Ali Mohagheghi, Karel Grohmann, and Michael E. Himmel

SUMMARY OF CHARACTERISTICS
(an excerpt from Mohagheghi, et al. [1])

Twelve isolates of thermophilic, acidophilic, cellulolytic bacteria were obtained from three different primary enrichment cultures from acidic