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Production of Cellulase Systems by Selected Mutants of *Trichoderma reesei* in Solid-State Fermentation and Their Hydrolytic Potentials

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ABSTRACT

Three mutants of *Trichoderma reesei* were grown in solid-state fermentation (SSF) in flasks and in a pan bioreactor. Mutant strain MCG 80 proved to be best at producing an optimal cellulase system using lignocellulosic material (wheat straw [WS]) as substrate. This preparation exhibited a β-glucosidase activity (βGA) to FPA (FPA) ratio of about 1.0, which is indicative of a high potential for hydrolysis of cellulose. The yields of cellulase systems and the ratio of βGA to FPA produced in flasks were comparable to that of the pan bioreactor. The cellulase system of *T. reesei* MCG 80 having a ratio of βGA to FPA close to 1.0 gave the most complete (88–95%) hydrolysis of 5% delignified wheat straw (DWS). On the other hand, the cellulase system of cocultures of *T. reesei* QMY-1 and *Aspergillus phoenicis* failed to produce high hydrolytic yields in spite of having a very high ratio of βGA to FPA (3.04). This failure was owing to the fact that coculture contained the relatively poor-quality cellulase system of the dominant organism, *A. phoenicis*. The resulting fermented WS can be used, as a source of enzyme (unextracted), for hydrolysis of wheat straw, and it gives increased yields of reducing sugars compared to analogous extracted enzyme preparations. The hydrolytic potential of two commercial enzymes tested were considerably lower than those of the cellulase systems produced on WS. It is evident that a complete cellulase system having a βGA-to-FPA ratio close to 1.0 and high hydrolytic potential can be produced on lignocellulosic feedstocks in SSF.

Index Entries: Cellulase; hydrolytic potential; solid-state fermentation (SSF); *Trichoderma reesei*; wheat straw.

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INTRODUCTION

In the hydrolysis of lignocellulosic feedstocks, the cellulase system is part of a complex lignocellulolytic enzyme system that hydrolyzes glucosidic bonds of crystalline cellulose to glucose. The lignocellulolytic enzyme system comprises ligninases, hemicellulases, pectinases, endo-1,4-β-glucanases, exo-1,4-β-glucanases (cellobiohydrolase), and β-glucosidase. The latter three types of enzymes are collectively referred to as the cellulase system. Production of an efficient and inexpensive cellulase system is at the cutting edge in the effort to establish a biotechnological route to complete transformation of cellulose into glucose. A high-quality and complete cellulase system is now realizable using solid-state fermentation (SSF) techniques *(1,2)*. SSF is a preferred technique in cellulase production because of the several advantages it has over the submerged or liquid state fermentation cultivation method *(3,4)*.

The levels of cellulases produced by several cellulolytic microorganisms are comparable with the parent strain, *Trichoderma reesei* QM6a *(5,6)*. Now, several *T. reesei* mutants have been developed for the production of effective and affordable cellulase systems. The driving force has been to obtain high filter paper activity (FPA)/unit volume and/unit of substrate. The term FPA used in this article represents the synergistic action of endo-and exoglucanases. However, the efficiency of hydrolysis of cellulose by a cellulase system is also governed by the quantities of both the FPA and the β-glucosidase activity (βGA). More importantly, a critical factor is the ratio of βGA to FPA, which should be close to 1.0 *(7,8)*. This ratio is particularly important in preventing catabolic repression of the endoglucanase and cellobiohydrolase by ensuring that cellobiose does not accumulate during the course of the reaction. Therefore, a cellulase system having a βGA-to-FPA ratio close to 1.0 is called a "complete" cellulase system, since it establishes a threshold enzyme ratio for the elimination of accumulated cellobiose during hydrolysis of cellobiose. It has been estimated that in cellulose hydrolysis (10–400 g/L), a cellobiose concentration of 0.01–0.1 g/L almost completely inhibits cellobiohydrolase *(9)*.

Although the literature abounds with filter paper and β-glucosidase production values *(10,11)* and a few cellulase quality assessment studies *(12,13)*, very little work is available on the hydrolytic potential of cellulase systems produced by different mutants of *T. reesei* using varied conditions and practical substrates. Hydrolytic potential is defined as the extent to which a given substrate can be hydrolyzed by a set loading of enzyme over a defined period of time and reaction conditions.

In the present article, we report the biochemical characteristics and hydrolytic potentials of cellulase systems of selected mutants of *T. reesei* when cultured individually and also in cocultures with *Aspergillus phoenicis* (a βGA producer) in SSF in flasks and in a specially designed pan bioreactor *(14)*.

MATERIALS AND METHODS

Microorganisms

T. reesei mutants QMY-1 (NRRL 18760), QM 9414 (NRRL 6165), and MCG 80 (NRRL 12368) were cultivated individually and in cocultures with *A. phoenicis* (NBL 1956) in Mandels' medium *(15)*. The genealogy of the *T. reesei* mutants is as follows: QMY-1 is a direct descendant of QM 9414 obtained in our laboratory by D. S. Chahal, whereas MCG 80 is a direct descendant of *T. reesei* Rut. C-30. *A.*

phoenicis was selected for its ability to produce high levels of β-glucosidase *(16,17)*. *T. reesei* MCG 80, QM 9414, and *A. phoenicis* were obtained from J. L. Swezey (ARS Patent Culture Collection, USDA, Peoria, IL). All the test organisms were maintained on agar in Petri plates with Mandels' medium having delignified wheat straw (WS) as carbon source.

Substrate and Enzyme Production

WS has the following approximate chemical composition: cellulose (39%), hemicelluloses (36%), lignin (10%), and silica (6%) *(18)*.

Ground WS (20 mesh) was treated with 4% NaOH (w/w) to a moisture content of 70% on dry-wt basis and led overnight. The treated substrate without washing was autoclaved at 121°C for 30 min. Twenty grams (dry-wt basis) of the treated and autoclaved WS were used for each pan bioreactor, whereas each Erlenrmeyer flask contained 4 g (dry-wt basis) of WS. The nutrient elements of Mandels' medium were calculated by weight of the polysaccharide (cellulose and hemicelluloses) content of WS and added to the substrate, and the final pH was adjusted to 6.5. Five and 25 mL of 36-h grown mycelium of the mutants were inoculated on the surface of each flask and pan, respectively. The moisture content of the substrate after inoculation was about 75% (dry-wt basis). The cultures were held in a humidified incubator (Norco 4100), and samples were harvested after 20 d of cultivation.

Enzyme Extraction and Analysis

Water was added to the fermented substrate to a final volume of 100 mL. The substrate was agitated in a rotary shaker at 200 rpm for 30 min and then centrifuged at 11,000*g* for 30 min. The supernatant was used for enzyme analyses and cellulose hydrolysis. FPA and βGA were determined by the method of Mandels et al. *(19)*, approved by the International Union of Pure and Applied Chemistry (IUPAC Protocol) *(20)*.

Hydrolysis of Cellulose

To examine the hydrolytic potential of cellulase systems, hydrolysis was performed on delignified wheat straw (DWS) using cellulase systems obtained from WS without urea in the cultivation medium. WS was delignified by the method of Toyama and Ogawa *(21)*. The extracted cellulase system was supplied at the rate of 20 IU FPA/g DWS. Crude unextracted enzyme (CUE) as found in the resulting fermented WS was also used for the hydrolysis of DWS. The CUE is that portion of the resulting fermented WS in SSF that was not subjected to extraction. The quantities of CUE to supply 20 IU FPA/g DWS were calculated based on the FPA of the extracted enzyme/g fermented substrate. The required enzyme units were added to 5 and 10% DWS in citrate buffer (0.1*M*, pH 4.8) in Erlenmeyer flasks and incubated at 45°C for 60 h with agitation at 200 rpm.

Analysis of Hydrolysates

Samples were quantitatively analyzed for dominating sugars, such as glucose, cellobiose, xylose, and arabinose, with a Beckman 344 HPLC system, degassed deionized water as mobile phase, an Aminex HPX-87P cohlmn heated to 85°C, and an Altex 156 Refractive Index Detector. In the hydrolysate, glucose and cellobiose

(a dimer of glucose) are products of cellulose, whereas xylose and arabinose (pentoses) are from the residual hemicelluloses in the DWS.

RESULTS AND DISCUSSION

Comparison of Cellulase Systems Produced from *T. reesei* Mutants and Their Cocultures with *A. phoenicis*

Economic production of fuel ethanol from cellulose calls for the need to compare different cellulase-producing microorganisms in terms of their ability to produce sufficient quantities of the complete cellulase system to hydrolyze cellulose to glucose efficiently. *T. reesei* occupies a dominant role in the continued search for an efficient cellulase system. This study assesses different mutants evolving from *T. reesei* QM6a (the original strain) for the efficiencies of their cellulase systems.

Table 1 shows the comparison of characteristics of cellulase systems produced from three mutants of *T. reesei*: QM 9414, QMY-1, and MCG 80 alone and in cocultures with *A. phoenicis* in SSF for 20 d with Mandels' medium with or without urea. Urea in the medium not only supplies nitrogen, but also offsets drastic pH drop during growth in SSF. When the *T. reesei* mutants were cultivated individually in Mandels' medium without urea, the MCG 80 appeared to have an edge in both FPA and βGA over all the mutants and had a ratio of βGA to FPA of 1.0. The QMY-1, which is a good producer of cellulase system in SSF with a βGA-to-FPA ratio close to 1.0 *(2)*, failed to achieve this ratio when urea was eliminated from the medium. The QM 9414 also produced low yields of cellulase system with a low ratio of βGA to FPA in the medium without urea. The addition of urea, however, improved the cellulase system of *T. reesei* QMY-1 in the pan bioreactor, and it was comparable to that of MCG 80, except that the ratio of βGA to FPA was a little low (0.71).

Coculturing the *T. reesei* mutants with *A. phoenicis* in SSF did not improve cellulase system production, but instead had a detrimental effect on the FPA yields. The apparent growth of the cocultures indicated that *A. phoenicis* outgrew the *T. reesei* mutants in SSF. *A. phoenicis* alone produced low FPA and high βGA. Therefore, its domination in cocultures resulted in the production of low FPA and high βGA. Thus, a ratio of βGA to FPA of more than 3.0 was obtained.

Commercial enzymes from Genencor and Iogen were also analyzed in our laboratory. These two enzyme sources were added in our study to compare their hydrolytic potentials with those obtained from our cellulase systems produced in SSF. These commercial enzymes, when tested in our laboratory, showed very high FPA and βGA/mL. However, the ratio of βGA to FPA for the Genencor cellulase was 0.32, whereas that of Iogen cellulase was 0.6 (Table 2), which are low with respect to hydrolytic potential and from the point of view of our definition of a "complete" cellulase system.

Hydrolytic Potential of Different Cellulase Systems

For this experiment, the cellulase system of MCG 80 was selected for hydrolysis of DWS, because it had the desired ratio of βGA to FPA of 1.0. The other cellulase systems were selected because of the following qualities: the cellulase system of QMY-1 produced on medium without urea had a low ratio of βGA to

Table 1
Comparison of Cellulase Systems Produced with Three Mutants of *T. Reesei*

Mutant bioreactor[a]	FPA IU/mL	FPA IU/g subt.	FPA IU/g cellulose	βGA, IU/mL	βGA/FPA
QM 9414, no urea in flask	3.75	94	235	1.11	0.30
QMY-1, no urea in flask	3.58	90	224	1.33	0.37
MCG 80, no urea in flask	5.33	133	333	5.33	1.00
QM 9414 + AP, no urea in flask	1.01	25	63	3.78	3.70
QMY-1 + AP, no urea in flask	1.08	27	68	2.80	2.60
MCG 80 + AP, no urea in flask	1.02	26	64	4.10	4.00
A. phoenicis (AP)	1.02	26	64	3.24	3.17
QMY-1 + 0.1% urea on pan	9.55[b]	129	322	6.80	0.71
QMY-1, no urea in pan	6.35[b]	86	214	2.15	0.34
MCG 80, no urea in pan	9.25[b]	125	312	9.55	1.03
QMY-1 + AP, no urea in pan	2.04	28	69	6.96	3.41
Genencor[c]	354.57	–	–	114.71	0.32
Iogen[c]	329.26	–	–	197.46	0.6

[a]Cultural conditions: WS was pretreated with 4% (w/w) NaOH and fermented in Mandels' medium.

[b]The enzyme activity (IU/mL) is high, because in pan bioreactor, the enzymes were extracted fiom 10 g (dry-wt basis) in 100 mL H_2O, whereas it was 4 g (dry-wt basis) from the flask.

[c]Enzymes supplied by Trichromatic Techno-Chem, Québec, Canada, for evaluation.

FPA and that of cocultures of QMY-1 and *A. phoenicis* had a very high ratio of βGA to FPA (3.41). The commercial enzymes were tested because they showed low ratios of βGA to FPA (0.32 and 0.60), and it is assumed that these enzymes were produced by submerged (liquid) state fermentation.

The data presented in Table 2 clearly indicate that the cellulase system of MCG 80 gave the highest hydrolytic potential (88–95%), whereas other enzyme systems gave low hydrolytic potentials (varying from 33.5–51.8%). The low hydrolytic potential of QMY-1 reported here is probably owing to the low ratio of βGA to FPA (0.34) produced in a medium without urea. In an earlier study, when urea was added to the cultivation medium of QMY-1, the ratio of βGA to FPA was close to 1.0, and over 90% hydrolytic potential was recorded *(1)*.

Table 2
Comparison of Hydrolytic Potential of Different Cellulase Systems

Cultrual condition	Type of fermentation	FPA, IU/mL	βGA, IU/mL	Ratio βGA/FPA	Hydrolytic potential, %[b] Extracted enzyme	Hydrolytic potential, %[b] Unextracted enzyme, CUE
T. reesei QMY-1 without urea	SSF in pan bioreactor	6.34	2.15	0.34	51.8	69.1
T. reesei MCG 80 without urea	SSF in pan bioreactor	9.25	9.55	1.03	88.0	95.3
T. reesei QMY-I + AP without urea	SSF in pan bioreactor	2.04	6.96	3.41	33.5	49.8
Genencor	Not known	354.60	114.71	0.32	44.2	nd[c]
Iogen	Not known	329.26	197.46	0.60	41.3	nd[c]

[a]As given in Table 1.
[b]Hydrolytic potential = 100 (Rs/Wt), where Rs is total reducing sugars and Wt is weight of DWS. Hydrolysis conditions: 5 g DWS, 20 IU/g DWS load based on FPA, and the rest of the conditions are as explained in Materials and Methods.
[c]nd = Not determined.

It is worth noting that the CUE gave higher hydrolytic potential in all the three cases (Table 2). The higher hydrolytic potential could be owing to the fact that the use of CUE could have been an additional source of cellulose and cellulase. The use of fermented WS as such (CUE) has two major advantages: (1) It would eliminate the need to extract enzyme, and (2) unutilized cellulose in the production phase will have a second chance for conversion into glucose. Both factors contribute to reducing the cost of ethanol production from lignocellulosic feedstocks.

The composition of hydrolysates obtained with these cellulase systems indicated that in the case of MCG 80 when the ratio of βGA to FPA was 1.0, the cellobiose concentration was low and therefore did not inhibit the hydrolysis (Figs. 1–3). The hydrolytic efficiency of CUE of *T. ressei* MCG 80 went up as high as 95%, even though the cellobiose concentration in the hydrolysate was as high as 0.8 g/L in 5% DWS digestions (Fig. 1). This calls to question the earlier suggestion of Klyosov *(9)* that cellobiose concentrations of 0.01–0.1 g/L completely inhibit cellobiohydrolase in cellulose hydrolysis concentration of 10–400 g/L. At 5% DWS, CUE from MCG 80 produced less cellobiose in the hydrolysate compared to its analogous extracted enzyme (Fig. 1). This was probably a result of availability of adsorbed enzyme in the CUE during hydrolysis. The cellobiose content was higher in the hydrolysates of the QMY-1 samples compared to the MCG 80 samples (Figs. 1 and 2). The plausible explanation is that MCG 80 had higher βGA than QMY-1 (Table 2), since β-glucosidase has a critical role in preventing cellobiose accumulation during hydrolysis of cellulose.

In the case of the hydrolysate from the cellulase system of coculture of QMY-1 with *A. phoenicis* (Fig. 3), at a ratio of βGA to FPA >1.0, no cellobiose in the hydrolysate was noticed. Even with no inhibition of cellulases because of the absence of cellobiose, the hydrolytic potential was still very low, although the quantity of FPA loading was 20 IU/g in all cases. Low hydrolytic potentials in the cellulase systems

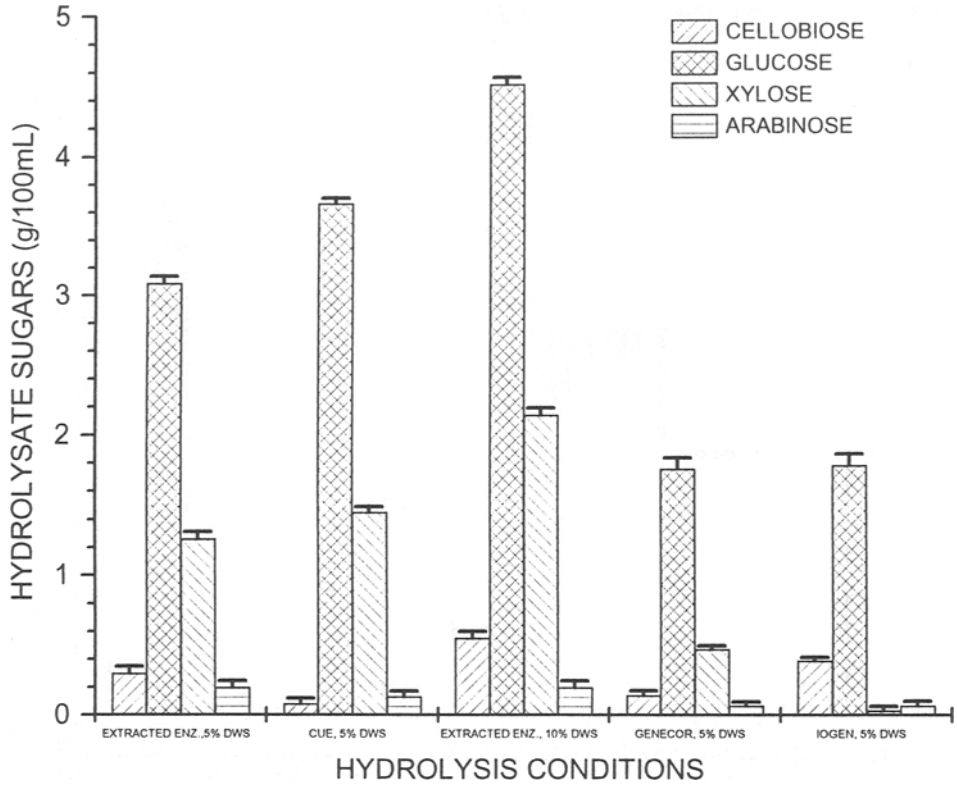

Fig. 1. Hydrolysis of DWS with cellulase system from *T. reesei* MCG 80 and commercial enzymes; Genencor and Iogen after 60 h of incubation.

of cocultures and commercial enzymes could be attributed to the fact of the poor-quality of cellulase systems produced under certain cultural conditions as reported earlier *(2)*. It may, therefore, be noted that equal enzyme loadings from different sources could result in different hydrolytic potentials (Table 2), suggesting the presence of a quality attribute not apparent when enzyme loadings are based on FPA alone.

High sugar concentration in the hydrolysate will also contribute to lower the overall cost of ethanol production. Thus, an efficient cellulase system should have the capability to hydrolyze a high concentration of cellulose. When the substrate concentration for hydrolysis was increased from 5 to 10%, there was an increase on total sugar concentration in the hydrolysate in all the cases (Table 3). Further examination of the data indicated, however, that there was a decrease in percentage of hydrolysis with an increase in substrate concentration. This decrease was lowest (13%) in the case of QMY-1 compared to that of other cellulase systems (about 25%). It is evident from the data (Table 3) that some cellulase systems, like that of QMY-1, can tolerate higher substrate concentration than others (MCG 80) at the same enzyme loading of 20 IU/g.

In fact, when the cellulase system of QMY-1 had the ratio of βGA to FPA of about 1.0, it hydrolyzed 10% DWS to the extent of 89–100% *(1)*. The cellulase system of the coculture was the least tolerant to the high substrate concentration. This

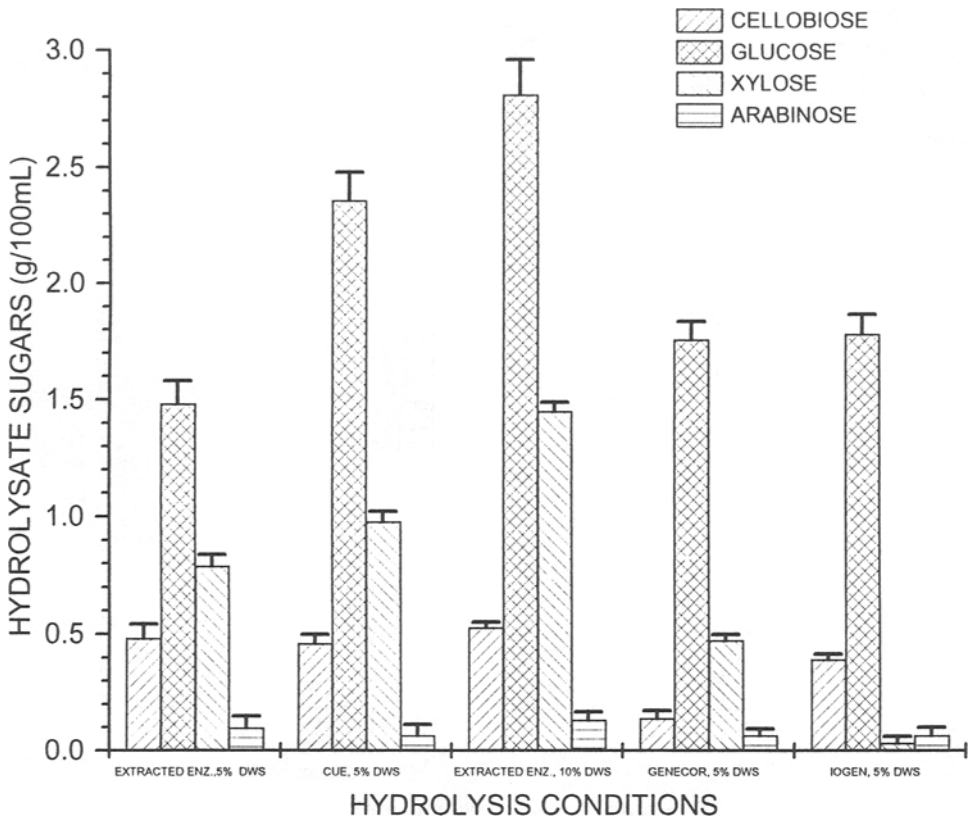

Fig. 2. Hydrolysis of DWS with cellulase system from *T. reesei* QMY-1 after 60 h of incubation. (Results of commercial enzymes from Fig. 1 are also represented here for comparison.)

again might be owing to the poor quality of the cellulase system as already explained. It appears that a cellulase system should also have high tolerance to substrate concentration for a viable hydrolysis process.

CONCLUSION

It is evident from the study that a complete cellulase system having a ratio of βGA to FPA close to 1.0 and high hydrolytic potential can be produced with suitable mutants of *T. reesei* in a pan bioreactor in SSF. Such a complete cellulase system does not require an external source of β-glucosidase for cellulose hydrolysis. Coculturing of *T. reesei* mutants with *A. phoenicis* increased yields of βGA in the cellulase system, but proved to be detrimental to the FPA production in SSF.

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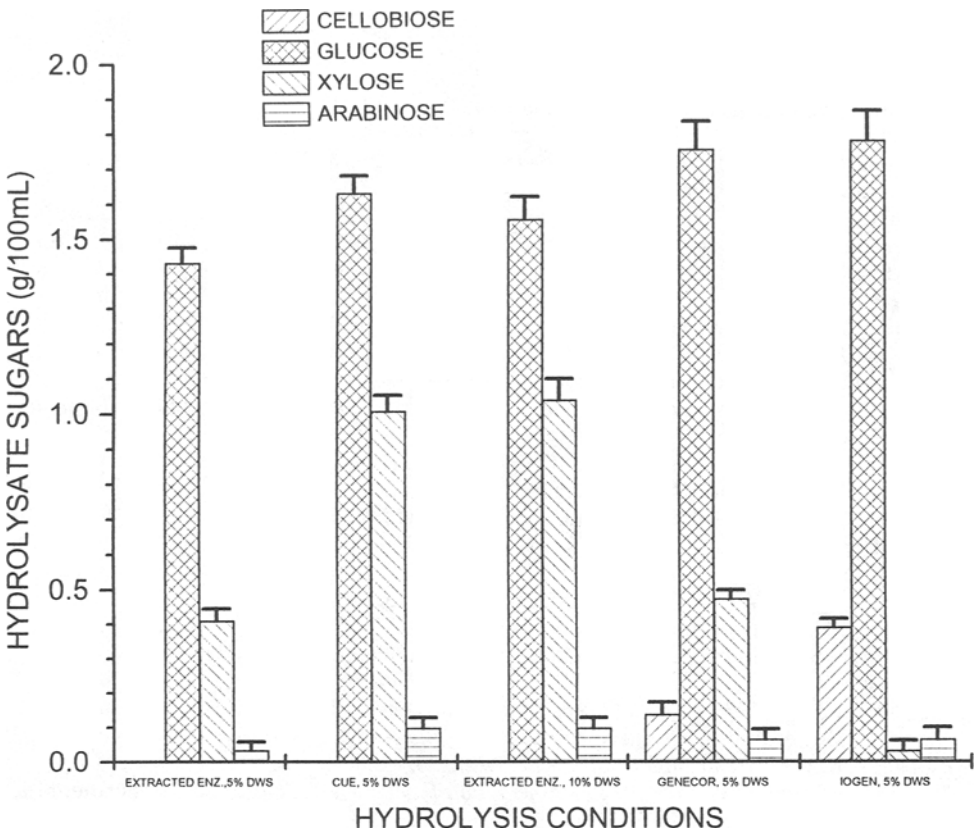

Fig. 3. Hydrolysis of DWS with cellulase system from coculture of *T. reesei* QMY-1 and *A. phoenicis* after 60 h of incubation. (Results of commercial enzymes from Fig. 1 are also represented here for comparison.)

Table 3
Effect of Different Cellulase Systems and Substrate Concentration on the Hydrolysis of DWS

Cellulase system[a]	5% DWS		10% DWS		Decrease in hydrolysis with increase in substrate conc., %
	Total sugars,[b] g/100 mL	Hydrolysis, %	Total sugars,[b] g/100 mL	Hydrolysis, %	
T. reesei MCG 80	4.84	88	7.4	67	24
T. reesei QMY-1	2.85	51.8	4.9	45	13
T. reesei QMY-1 + *A. phoenicis*	1.87	34	2.7	25	25

[a]Extracted cellulase was used for hydrolysis.
[b]Total sugars = (cellobiose + glucose + xylose + arabinose).

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