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Spent brewery grains as substrate for the production of cellulases by *Trichoderma reesei* QM9414

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SUMMARY

The cellulolytic fungus *Trichoderma reesei* QM9414 can be cultivated on spent brewery grains for the production of cellulases. The levels of the cellulase components endoglucanase and exoglucanase synthesized, and the complexes filter paper cellulase and grain-hydrolyzing cellulase synthesized by the organism on spent grains were as high as 287, 182, 187, and 449 units per g available cellulose, respectively. Scaling up the spent grains fermentation system by up to 40-fold (200 g dry substrate/tray) demonstrated that cellulase production was comparable to laboratory scale (5 g dry substrate/flask) yields. Cultivation of the fungus was feasible on spent grains without pretreatment or further adjustment, although the enzyme yield was somewhat lower than that on dried grains moistened with water or *Trichoderma* medium. This suggested the possible reutilization of spent grains, with minimal pretreatment, in the cultivation of *T. reesei* QM9414 for cellulase synthesis and for future incorporation into animal feed.

INTRODUCTION

A variety of lignocellulosic wastes have been used worldwide as growth substrates for fungi and bacteria in the synthesis of cellulases. Some lignocellulosic substrates that have undergone solid and liquid fermentation by fungi include rice straw [15], wheat straw [7], apple distillery waste [5], orange peel and grape stalks [13].

Spent brewery grains are effectively utilised as cattle fodder in countries where dairy farming is practised. However, the bioconversion of spent grains by microorganisms has not been widely studied. In Singapore spent grains are not fully utilized due to the absence of extensive dairy farming. The main livestock are monogastric animals such as

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poultry, fish and pigs. We, therefore, examined microbiological conversion of spent grains for the production of cellulases as a means of improving soluble sugar and protein levels in spent grains as alternative feed sources. Earlier work showed that the fungus can grow and synthesize cellulases on the spent grains on solid medium [14].

We report here the results of experiments carried out using the cellulolytic fungus *Trichoderma reesei* QM9414 for the production of cellulases leading to hydrolysis of spent grains into sugars and proteins.

MATERIALS AND METHODS

Microorganisms and growth media

Trichoderma reesei QM9414 was obtained from the U.S. Army Natick Laboratories, Natick, MA, U.S.A. The organism was maintained on Sabouraud Agar (Oxoid) and Potato Dextrose Agar (Oxoid). The organism was grown in *Trichoderma* medium (TM) [9] containing 1% (w/v) carboxymethyl cellulose (CMC, Sigma) for use as inoculum. Spent barley grains were obtained from the brewery prior to their discharge after mashing. The grains were immediately dried and stored dry. Dried grains used in NaOH-treatment were redried before being used in experiments. For the growth of *T. reesei,* 5 g dry weight of both untreated and NaOH-treated spent grains were adjusted to 80% moisture with 20 ml TM or water as required. A similar volume of TM or water containing 0.75 g CMC (equivalent to the lignocellulose content of 5 g spent grains) was also used for the growth of *T. reesei* and cellulase production. The available cellulose of both untreated and NaOH-treated spent grains was estimated to be 15% of its total mass, on a dry weight basis [3]. The inoculum size was 1 ml per 5 g grains or 0.75 g CMC.

Enzyme extraction

Extraction procedures were standardised for both untreated and NaOH-treated grains grown with *T. reesei.* The crude cellulase enzymes were extracted from the growth medium with three volumes of 0.05 M citrate buffer at pH 4.8 to ensure maximum extraction. Crude extracts of cellulases produced by the organism grown in liquid TM containing CMC were obtained by filtration and centrifugation of the broth to remove the organism.

Enzyme assays

Enzyme assay mixtures comprised 100 mg ground spent grains or 15 mg CMC, filter paper (FP, Whatman No. 1) or Avicel (Merck) and 3 ml enzyme extract. This was buffered with 0.05 M citrate buffer at pH 4.8. Assays were performed at 50°C for 1 h. Reducing sugar levels were measured using the dinitrosalicylic (DNS) method [12]. Optical densities were measured in a Beckman DU-50 spectrophotometer at a wavelength of 550 nm using D-glucose (Merck) as the sugar standard. The enzyme activity is expressed as units/g available cellulose where one unit is equivalent to the enzyme activity which produces 1 μmol glucose from 100 mg spent grains (15 mg cellulose) per hour.

Soluble protein measurement

Total soluble proteins were measured using the method of Lowry et al. [8] using bovine serum albumin (Sigma) as the protein standard. Optical density measurements were done using a Beckman DU-50 spectrophotometer at a wavelength of 750 nm.

RESULTS AND DISCUSSION

Determination of levels of cellulase components produced on spent grains substrate

Cellulase consists of several components such as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.21.91) and beta-glucosidase (EC 3.2.1.21). Endoglucanase is also known as carboxymethyl cellulase (CMCase) as it hydrolyzes soluble derivatives such as CMC. Exoglucanase hydrolyzes crystalline cellulose such as Avicel and is also known as Avicelase. Filter paper cellulase activity, sometimes referred to as filter paperase or FPase, has been measured during the hydrolysis of filter paper and is probably cellulase activity resulting from a combination of components acting synergistically. The levels of these enzyme components and complexes can vary

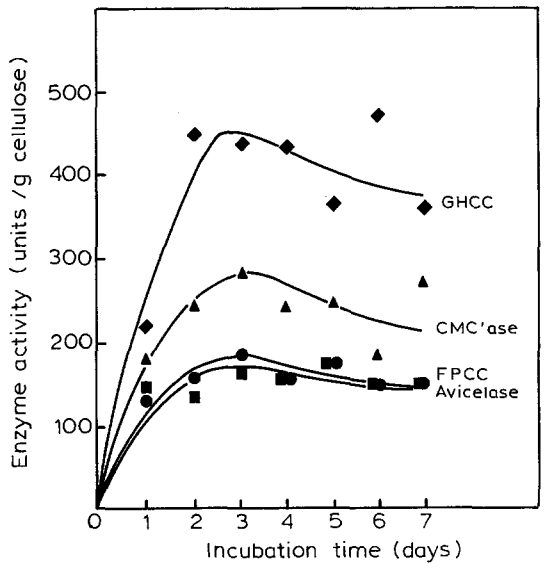

Fig. 1. Cellulase production of *T. reesei* cultivated on spent grains moistened with TM. Growth was over a period of 7 days. The cellulase complexes and components detected were grain-hydrolyzing cellulase complex (GHCC) (◆), CMCase (▲), filter paper cellulase complex (FPCC) (●) and Avicelase (■).

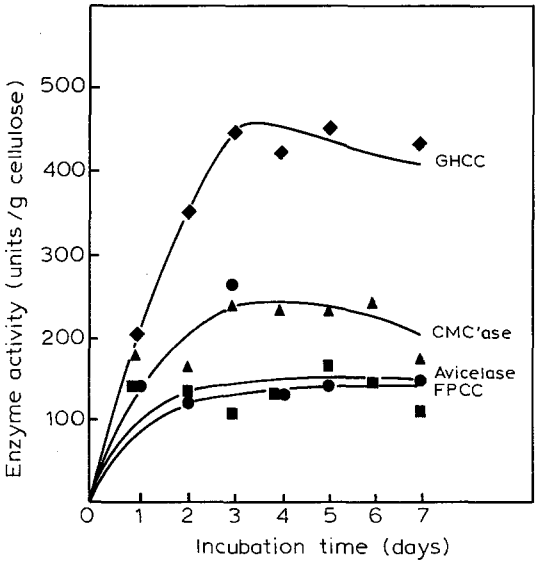

Fig. 2. Production of cellulases by *T. reesei* cultivated on NaOH-treated grains prepared in TM. Cellulases were detected in the extracts over a period of 7 days growth. The cellulase complexes and components measured were GHCC (◆), CMCase (▲), FPCC (●) and Avicelase (■).

when produced by different organisms and under different growth conditions. The grain-hydrolyzing cellulase complex is the term used here for the combination of components responsible specifically for the hydrolysis of spent grains as a whole.

The levels of the cellulase components and complexes synthesised by *T. reesei* grown on CMC, spent grains and spent grains treated by steeping them in 1% NaOH for 24 h were measured. The substrates used were standardised on the basis of their cellulose contents. The results are shown in Table 1 and Figs. 1, 2 and 3. The levels of all the components and complexes were higher when the organism was cultivated on spent grains than on CMC, suggesting greater efficiency of cellulase production on spent grains. Cellulases produced on both untreated grains and grains which had been pretreated by steeping in 1% sodium hydroxide for 24 h were almost similar (Table 1). This supports the conclusions drawn from earlier experiments which demonstrated that alkali pretreatment of grains did not improve cellulase synthesis. The levels of grain-hydrolyzing cellulase complex were the highest, suggesting the complex may be a combination of two or more cellulase components acting synergistically to hydrolyze the complex grains.

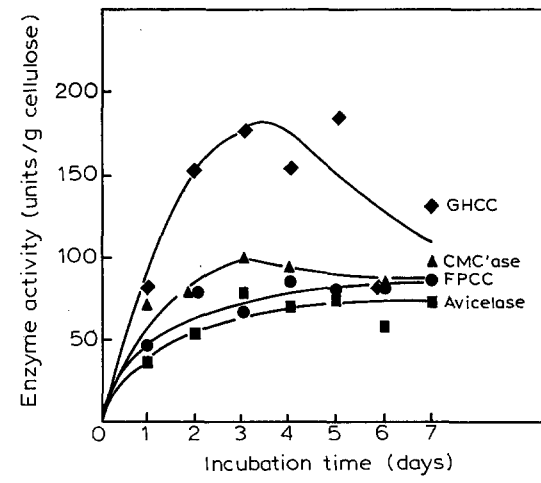

Fig. 3. Production of cellulases by *T. reesei* grown on CMC dissolved in TM over a 7-day period. The cellulases measured in the extracts consisted of GHCC (◆), CMCase (▲), FPCC (●) and Avicelase (■).

Table 1

The levels of cellulase components produced by *T. reesei* grown on untreated or NaOH-treated spent grains or on CMC

Enzyme component/complex	Enzyme units/g available cellulose produced from		
	Untreated grains	NaOH-treated grains	CMC
Grain-hydrolyzing cellulase complex	222–449	208–450	80–180
CMCase	185–287	167–240	73–102
FPase	135–187	120–147	48– 87
Avicelase	137–182	108–165	37– 78

This would have resulted in an apparently higher level of sugars.

CMCase levels exceeded those of filter paper cellulase and Avicelase under all three growth conditions. CMCase is believed to be the first component of the cellulase enzyme system to act during the hydrolysis of cellulose by cleaving the β-glucosidic bonds within cellulose [11]. CMCase may, therefore, be synthesised in relatively larger quantities to facilitate initiation of cellulose hydrolysis. The filter paper cellulase and Avicelase levels were observed to be somewhat similar when produced under each growth condition. Variations in the levels and proportions of cellulase components may be related to media and substrate compositions and other growth factors. Andreotti et al. [2] measured higher amounts of CMCase (19.2 units/ml) compared with 0.86 units/ml of FPase when *T. reesei* QM9414 was cultivated for 4 days on Solka Floc (SW40), a 40 mesh, hammer-milled, fibrous, pure cellulose pulp. It was later cited in a review by Margaritis and Merchant [10] that growth of *T. reesei* QM9414 on 1% Solka Floc for a longer period (13 days) yielded CMCase, FPase and Avicelase activities of 89, 1.1 and 2.4 unit/ml respectively. These and our data suggest that CMCase is produced in greater proportions than other cellulase components when *T. reesei* QM9414 is cultivated on different cellulosic substrates.

Scaled-up cultivation of T. reesei on spent grains prepared in aluminium trays

Cultivation of the fungus on a larger scale would be an advantage in producing cellulases and *Trichoderma*-hydrolyzed substrate for possible incorporation into animal feed.

The spent grains medium was scaled up by 10-fold, each tray containing 50 g (dry weight) grains and compared with that of the small scale flask cultures containing 5 g dry weight of substrate. The conditions of the tray substrates were similar to that of the flask cultures where the moisture levels of the grains were adjusted to 80% with TM.

Growth and enzyme production of *T. reesei* monitored over several days were somewhat better on scaling up the growth conditions by 10-fold (Fig. 4).

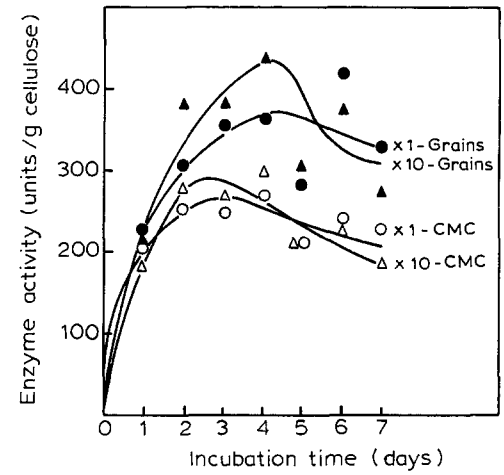

Fig. 4. Enzyme levels of *T. reesei* grown on spent grains under small scale (X1, 5 g dry substrate) and scaled-up conditions on trays (X10, 50 g dry substrate). (a) Grain-hydrolyzing cellulase complex activities were measured using spent grain as assay substrate: X1 (●), X10 (▲). (b) CMCase activities were determined using CMC as assay substrate: X1 (○), X10 (△).

The apparent improvement in cellulase levels under the large scale conditions may have been due to an increase in the available surface area which facilitated aeration and improved growth of the organism. Preliminary experiments involving scaling up by 40-fold have indicated the production of comparable levels of cellulase. These results appear promising if the system is to be applied to large scale production of cellulases and feed supplements by solid-state fermentation of spent grains.

Cultivation of T. reesei on spent grains direct from the brewery

The reutilisation of spent grains direct from the brewery with minimum treatment such as drying and adjustment of moisture would be a practical system for the cultivation of *Trichoderma* and cellulase production.

The moisture content of the spent grains from the brewery was approximately 81%. The spent grains fresh from the brewery to be inoculated without any treatment were autoclaved in portions of 25 g wet weight (equivalent to 5 g dry weight). Some of the grains were dried without prior extraction of liquid whilst others were dried after extraction of excess liquid. The dried grains were then adjusted to 80% moisture with water or TM. This was followed by sterilization and inoculation with identical volumes of seed culture.

The results indicate that the untreated grains supported growth of *T. reesei* and cellulase production. The levels of cellulases produced were, however, lower than that synthesised by the organism grown on the grains with water or TM added. The greater amount of background sugars present in the untreated grains possibly caused feedback inhibition of cellulase synthesis to a greater extent than in the liquid-extracted grains (Fig. 5). As observed in earlier experiments, cellulase synthesis by the fungus on grains moistened with water was almost comparable to that on TM-moistened grains. On comparing cellulase production by *T. reesei* grown on water-moistened grains, we observed that enzyme levels were somewhat higher on the grains which had had excess liquid extracted. This trend may again have been the result of the background sugars, and hence feedback inhibition, being reduced through the removal of excess liquid. It may be said that even though the untreated grains were not quite comparable to TM-moistened grains in the support of cellulase synthesis, this may be compromised by its practicality and economy.

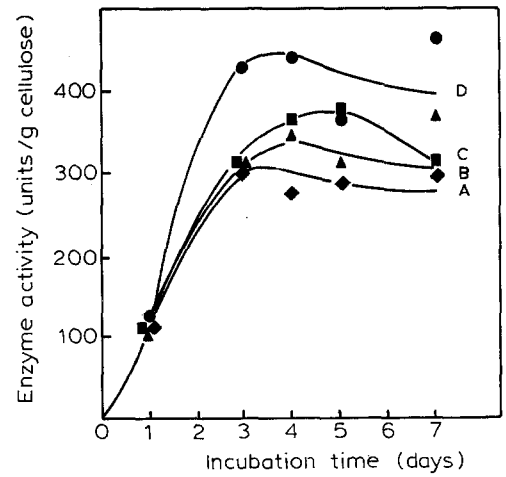

Fig. 5. Cellulase production by *T. reesei* grown on spent grains under the following conditions: A (◆): Untreated spent grains. B (▲): Dried grains. No liquid extraction. Moistened with H_2O. C (■): Liquid-extracted, dried grains. Moistened with H_2O. D (●): Liquid-extracted, dried grains. Moistened with TM.

T. reesei QM9414 produces cellulases when cultivated on lignocellulosic substrates such as wheat bran, wheat straw and waste cellophane. Wijeyaratne et al. [16] and Kim et al. [6], through solid state cultivation of *T. reesei* on wheat bran, reported respective cellulase yields of 5.8 and 65 CMCase units/g substrate/min. In solid and liquid cultivation of *T. reesei* on wheat straw, Chahal [4] observed cellulase yields of 430 FPase units/g cellulose/min whilst Acebal et al. [1] reported cellulase yields of 666 FPase units/g cellulose/min and 5761 CMCase units/g cellulose/min on milled wheat straw. Our results demonstrate that the levels of the cellulase components endoglucanase, exoglucanase, filter paper cellulase complex and grain-hydrolyzing cellulase complex were up to 287, 182, 187 and 449 units/g cellulose/h, or 4.8, 3.0, 3.1 and 7.5 units/g cellulose/min, respectively. These observations indicate that cellulase yields can vary depending on

the type of substrate, pretreatment processes and growth conditions.

Spent brewery grains are suitable as substrate for the cultivation of the *Trichoderma reesei* QM9414. Cellulases are synthesised in the process with a concomitant conversion of the cellulosic material to soluble reducing sugars and an improvement in soluble protein levels.

A range of cellulase components and complexes, viz. endoglucanase, exoglucanase, filter paper cellulase complex and grain-hydrolyzing cellulase complex, is produced in varying amounts. The system is amenable to at least a 40-fold scale-up and suggests possible reutilisation of the spent grains on an even larger scale. Our results also show that the spent grains can be used for *T. reesei* cultivation without further treatments such as liquid extraction or drying. This enhances the practicality of the process and would contribute to a reduction in preparation time and cost. There is, therefore, a possibility of reutilising spent grains through partial hydrolysis by *Trichoderma* and subsequent incorporation of the treated spent grains into animal feed.

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