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INCREASED SACCHARIFICATION OF KALLAR GRASS USING ULTRAFILTRATED ENZYME FROM SPOROTRICHUM THERMOPHILE

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ABSTRACT

The local wild type strain of *Sporotrichum thermophile* when grown on untreated lignocellulose was found to produce a greater level of B-glucosidase component along with other cellulase/xylanase components than most of the reported wild type potent strains. Culture filtrate obtained, when grown on 4% *Leptochloa fusca* (kallar grass) was used as such and after concentration by ultrafiltration technique for saccharification purpose. Concentrated enzyme titre was increased to 1.2 and 4.0 U/ml for FP-ase and B-glucosidase, respectively. There were losses in the enzyme titre obtained through ultrafiltration possibly due to adsorption on to the ultrafiltration membrane. Enzyme preparations used, saccharifide 5% kallar grass to 70, 55, 75 and 60% (theoretical basis) from cellulases of *S. thermophile* concentrate, dilute, *T. reesei* alone and in supplementation with B-glucosidase from *A. niger*, respectively. Analysis by HPLC revealed slightly higher glucose yield from *S. thermophile* enzyme preparations, whereas higher level of xylose was attained from *T. reesei* preparations. Rest of the sugars pooled as Oligo-sugars were found in almost similar concentrations.

INTRODUCTION

Initially, it was emphasized on production of increased cellulase titre from microbial sources for efficient degradation of cellulose. However, cellulase activity in vivo is not mediated by a single enzyme. Rather it is a complex of several different enymes which act in concert (Eveleigh, 1987; Lamed and Bayer, 1988). Hydrolysis of lignocellulosic substrates requires a complete spectrum of cellulases and hemicellulases for its conve rsion into mono-meric sugars. Most of the potent strains are misssing one or the other enzyme component required for effective conversion of the substrate (Saddler et al, 1985). Although *Trichoderma reesei* strains are the most promising cellulase producers still they lack a proportionate B-glucosidase, required for the ultimate conversion of cellulose into glucose (Reese, 1977; Mandels, 1982). *S. thermophile* has been reported to produce a substantial amount of B-glucosidase along with the other cellulases (Canevascini et al, 1979; Grajek, 1987). Thermophilic cellulolytic organisms due to their inherent characteristics are thought to be ideal for efficient bioconversion of lignocellulosic substrates (Romanelli et al, 1975; Coutts and Smith, 1976; Bhat and Maheshwari, 1987). Moreover, use of thermostable cellulases from these organisms might make the saccharification process cost attractive. Durand, 1984; Chahal, 1985; Grajek, 1986; Margaritis and Merchant, 1983 and 1986).

In the present studies dilute culture filtrate from *S. thermophile* was concentrated by ultrafiltration. This strain had been shown to produce a complete system of cellulase/xylanase titre (Latif et al, 1989) when grown on kallar grass. In order to elucidate the spectrum of cellulases, concentrated enzyme prepa ration and commercial cellulases were used for saccharification purpose.

MATERIALS AND METHODS

Enzyme Source:

The enzyme filtrate from *S. thermophile* was obtained by cultivation on 4% Kallar grass using mineral medium of Eggins and Biosaline research sub-station (BSRS) near Lahore, during peak periods of growth. The enzyme was harvested after 6 days of incubation in an orbital shaker (Gallenkamp Co. U.K) at 45°C. The enzyme filtrate after centrifugation was refrigerated. Commercial enzyme prepartions from *T. reesei* VTT-D-79125 (Novo Baegsvard-Denmark) and *Aspergillus niger* (Miles Kalli) were used along with the local enzyme for comparison.

Ultrafiltration:

Refrigerated enzyme filtrate (500 ml) of *S. thermophile* was passed through ultrafiltration membrane (cut out size 20,000 daltons, Amicon Co. USA) fixed in an ultrafiltration assembly (RA 2000 Amicon Co. USA). The enzyme filtrate was passed at a back pressure of about 10 lb/in^2, developed at the ultrafiltration membrane. The filtrate was collected in a separate flask, while the concentrate was allowed to recycle in the ultrafiltration jar, till it was one fifth of the total volume. Recovery of the enzyme titre was estimated from concentrate as well as filtrate.

Enzyme Assays:

For determining activities towards FP-ase, method of Mandel et al (1976) with slight modification from Saddler et al (1985) was performed. Whatman No. 1 filter paper of 1x6 cm size was rolled in to 1 ml of 0.05 M citrate buffer, pH 5.0. To this was added 0.5 ml of suitably diluted enzyme. The enzyme unit was calculated for diluted enzyme to give reducing sugars in the range of 0.4- 0.8 using 0.1% glucose as a standard. The reducing sugars were estimated, according to Miller (1959).

B-glucosidase activity was determined by the modified method used by Rajoka and Malik (1984). 0.2 ml of suitably diluted enzyme was added to 0.2 ml of (0.05 M citrate buffer) pH 5.0 and 0.2 ml of 15 mM p-nitrophenyl B-glucoside. The assay was carried out for 10 min at 50°C. The release of p-nitrophenyl was determined by adding 3 ml of 2% Na_2CO_3 and the absobance was read at 410nm.

Extracellular Protein:

These values were determined from the enzyme filtrate by Lowry's (1957) method using bovine serum albumin as standard.

Saccharification:

The enzyme filtrate from *S. thermophile* (dilute and concentrate) was used as such in 20 ml volumes in 100 ml flasks. The pH was adjusted to 5.0 with 1 N.HCl. Commercial enzyme preparation of *T. reesei* cellulase alone and with supplementation of B-glucosidase from *A. niger* contained FP-ase and B-glucosidase in the ratio of 30:20 and 33:54 units per gram of substrate, respectively. The freeze dried enzyme from commercial preparations were solublised in 0.05 M citrate buffer pH 5.0. Alkali pretreated Kallar grass (Rajoka and Malik, 1984) was used as a substrate at 5% concentration. Sodium azide (0.02%) was added as a biocide, while aluminium foil was capped on the conical rection flasks for minimising the Vapor losses. The incubation was carried out at 50°C in a water bath shaker (GFL Co. W. Germany) at 200 rpm for 70 hours.

Sugar Analysis:

At different intervals of time 0.5 ml of sample was withdrawn from the hydrolysates, taking care that the slurry density of the withdrawn samples was the same as that in the remaining sample. The samples in microfuge tubes were centrifuged for 1-2 minutes at 3000 rpm. The reducing sugar concentrations were determined by Dinitrosalicylic acid (DNS reagent). The sugar composition was determined by HPLC (Gilson's France). Ion exchange column, aminex HPX-87H (Biorad Co. USA) with was used an 0.01 N H_2SO_4 aseluent. The flow rate was kept at 0.6 ml/min at a column temperature of 85°C. The sugars were detected on a refractive index detector from Shimadzu Co, Japan, while the column oven was also from the same company. The recorder unit (Gilson's Co) was kept at a chart speed of 5 mm/min. The standard sugars of D-glucose, D- xylose and cellobiose (all GLC grading sugars Co) were run to standardize the condition. Samples after suitable dilutions were injected (25ul) through a rheodyne injection valve loop.

The saccharification yield from DNS method was calculated using the equation:

$$\text{Saccharification \% (based on total polysaccharides)} = \frac{\text{Reducing sugars formed x 0.9 x 100}}{\text{carbohydrates in straw}}$$

$$\text{Glucose Yield \% (based on total cellulose)} = \frac{\text{Glucose sugar formed x 0.9 x 100}}{\text{cellulose in straw}}$$

RESULTS AND DISCUSSION

Concentration of Enzyme Filtrate:

Crude enzyme filtrate of *S. thermophile*, when concentrated up to five folds by volume through ultrafiltration, was of dark brown in colour. The concentrated enzyme showed an increase of 2.4, 3.6 and 2.5 folds for FP-ase, B-glucosidase and extracellular proteins, respectively, over the dilute enzyme. A part of cellulase titre was found to be present in the filtrate during ultrafiltration process. Fig 1. shows that the filtrate contained FP-ase to a greater extent than B- glucosidase. The cumulative enzyme activities present in the concentrate and filtrate suggested a loss of small portion of enzyme, possibly due to adsorption on the ultrafiltration membrane. The increased adsorption for the culture filtrate obtained at 4% substrate concentration, was ascribed to viscosity and pigmentation from substrate (kallar grass). In fact, there was appearance of a gummy substance which possibly blocked the

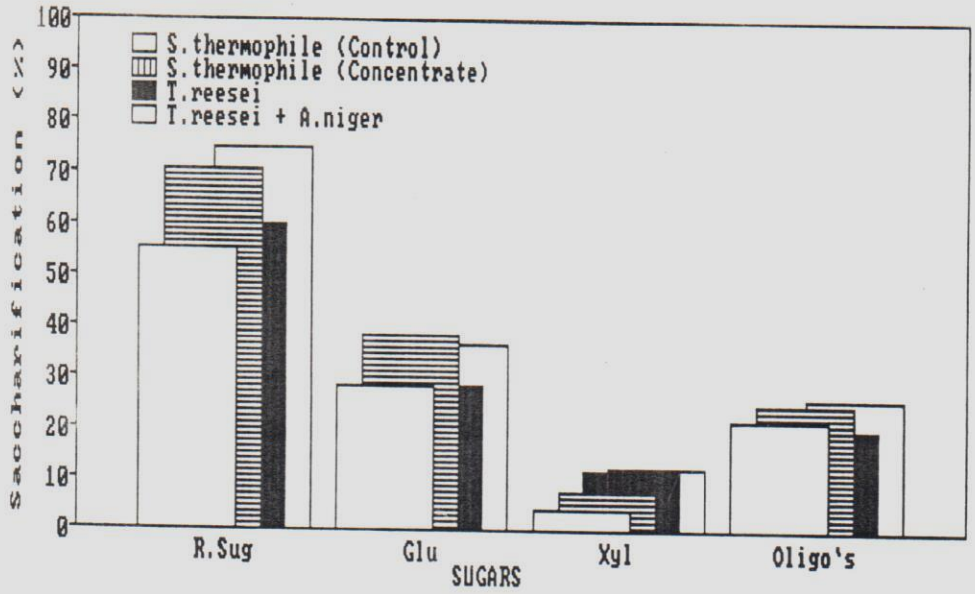


Figure 1: Percentage recovery for the cellulases and extracellular protein after ultrafiltration of crude extracts from *S. thermophile* is presented. The recovery was estimated in the concentrate well as the filtrate.

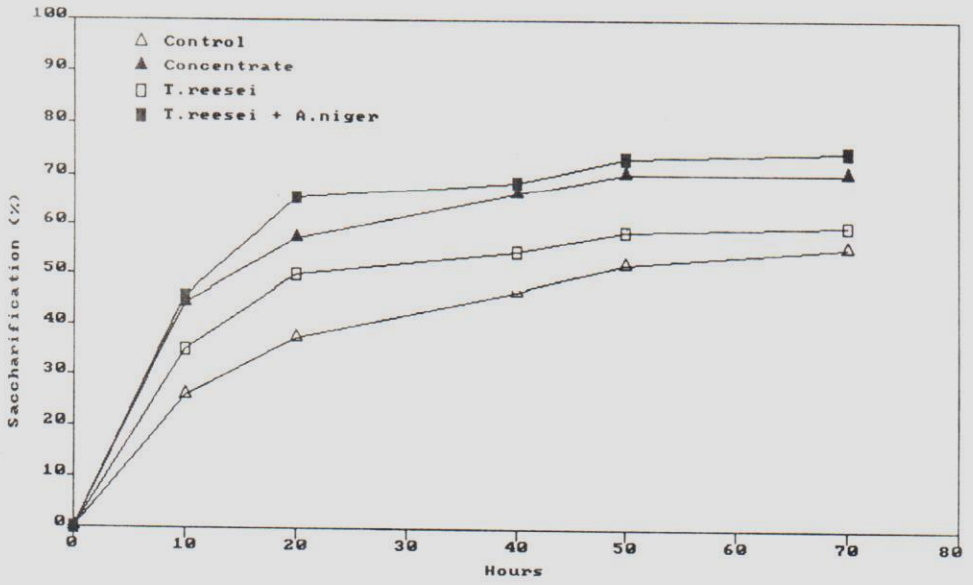


Figure 2: Time course saccharification yield from 5% Kallar grass using enzyme titre from *S. thermophile* (Concentrated and its control); *T. reesei* alone and in supplementtation with *A. niger*. The saccharification was carried out for 70 hours at a pH of 5.0 and 50°C.

membrane pores and thus, appreciable amount of cellulase was lost (Fig. 1). Markanen and Eklund (1975) found that the culture filtrate from *T. viride* was partially denatured when passed through ultrafiltration membrane (cut out size, 20,000). Fahnrich and Irrgang (1982) concentrated the enzyme by ultrafiltration for effective saccharification. The ultrafiltration technique was adopted for concentrating the enzyme filtrate in order to use it at higher strengths towards increased amounts of substrate.

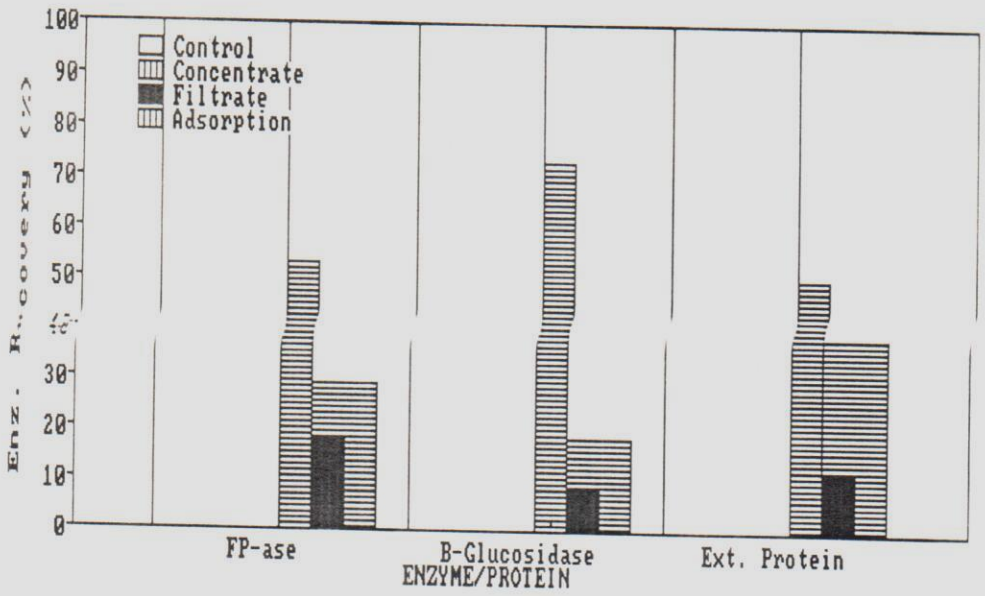


Figure 3: Saccharification yield of 5% Kallar grass using different enzyme sources after 70 hours of incubation at 50o C. Reducing sugars were determined by DNS method while sugar composition was evaluated from HPLC.

Effect of Cellulase Concentration on Saccharification:

The time course study shows a high saccharification rate up to 10 hours, which slowed down thereafter up to 50 hours, after which the increase was minimal (Fig. 2). The concentrated enzyme filtrate from *S. thermophile* saccharified 5% Kallar grass up to 70% (theoretical basis) after 70 hours of incubation (Fig. 3) Saccharification yield by the concentrated enzyme preparation, although 5% less than that obtained from *T. reesei* cellulase supplemented with B-glucosidase from *A. niger*, contained a slightly higher amount of glucose. Their was a considerable difference in the cellulase titre, especially for B-glucosidase used per gram of substrate from all the preparations (see legend to Fig. 3). These results were attributed to the concentrated enzyme system of *S. thermophile* with a potent B-glucosidase component as also reported by (Grajek, 1986; 1987). *T. reesei* cellulase used alone showed higher reducing sugar yields than the control by *S.thermophile*, however,

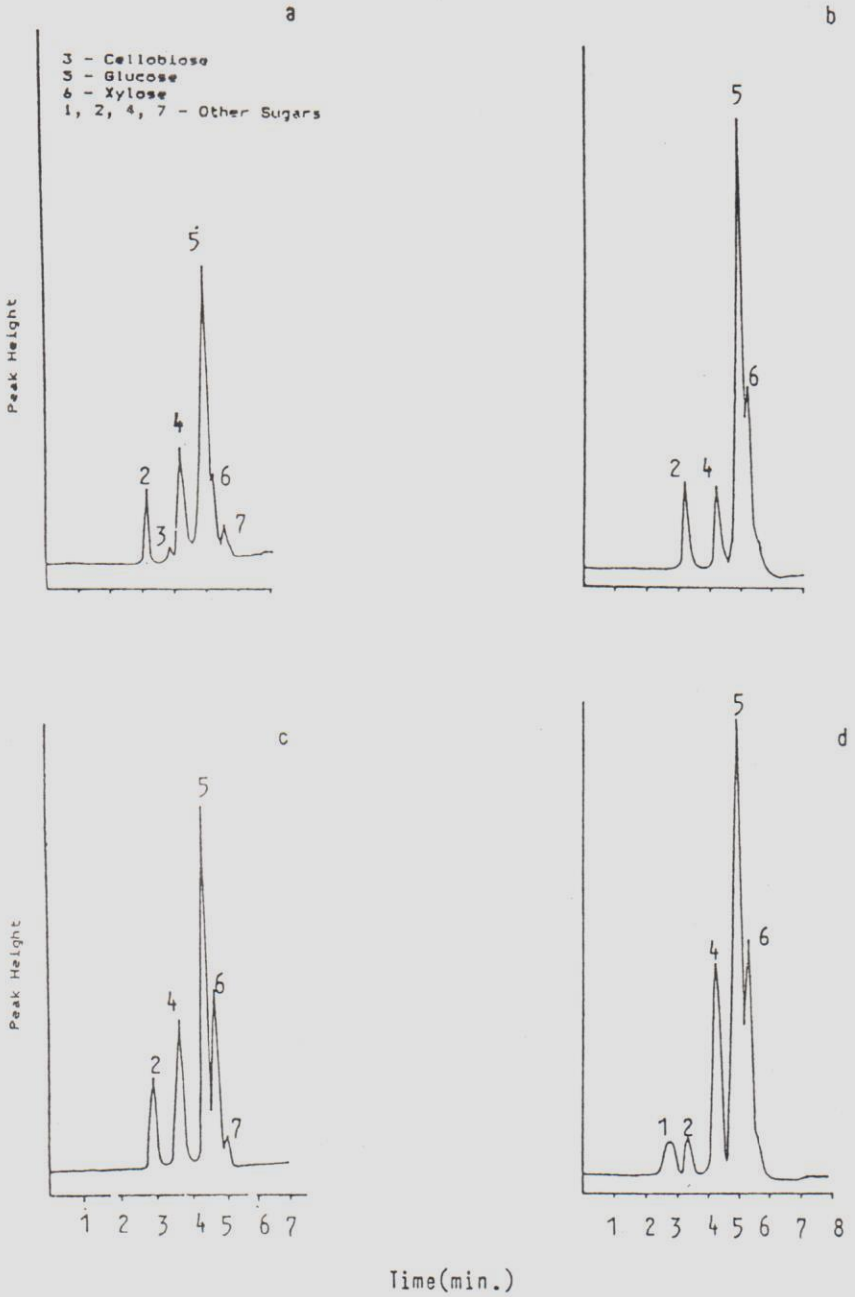
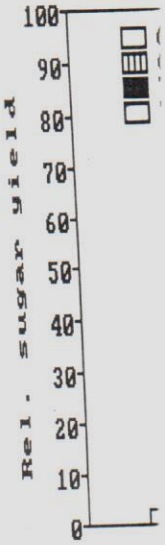


Figure 4: Seperation of sugars by HPLC after hydrolysis.
a) S. thermophile (control), b) S. thermophile (concentrate), c) T. reesei (without supplementation) and d) T. reesei (with supplementation from A. niger). Conditions for HPLC were as follows: Column; Aminex HPX-87H cation exchanger; Mobile phase, 0.001 N H2SO4; Column temperature, 85o C; Flow rate, 0.6 ml/min; Injection volume, 20ul; Attenuation x16.



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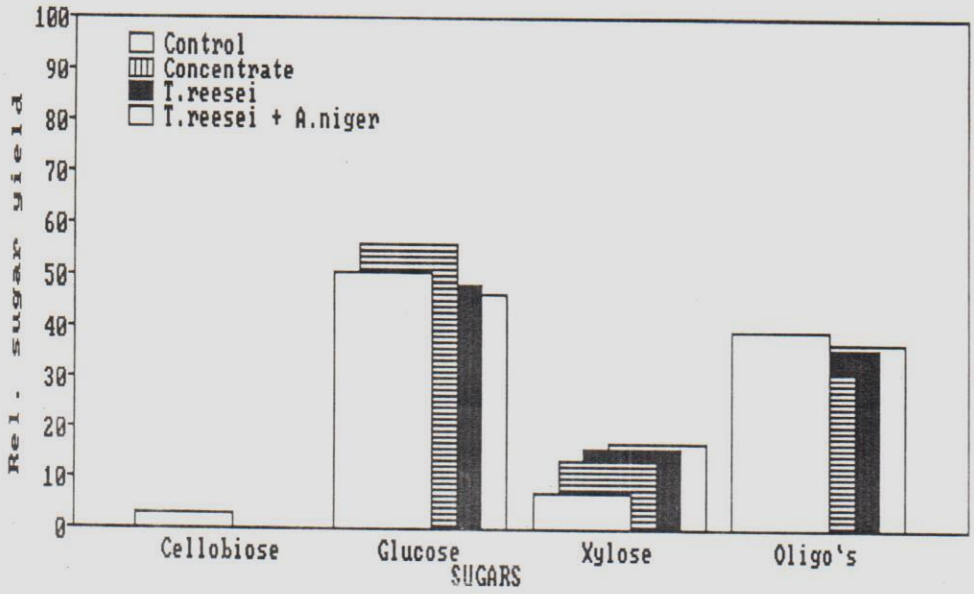

Figure 5: Percent relative yield for sugars in the hydrolysate after 70 hours of incubation from different enzyme sources.

the glucose content was of similar level. Analysis by HPLC showed that in fact, the composition of sugars from the hydrolysates showed 6-7 peaks by the HPLC (Fig 4). Three of the major peaks namely cellobiose, glucose and xylose were determined from the standards, whereas rest of peaks were pooled as other sugars/oligo-saccharides (Fig. 4). Cellobiose was obtained to a small level from the control of *S. thermophile*, only. Yields for xylose from supplemented *T. reesei* were about 2 and 3 fold higher than the concentrate and control from *S. thermophile*, respectively.

Fig. 5 shows the composition of various sugars in the hydrolysates. The higher level of glucose from the enzyme prepa rations of *S. thermophile* elucidates a higher B- glucosidase component in the enzyme system (see legend to Fig. 3). However , due to a low xylanase and endo-glucanase relatively lower saccharification yields were obtained for *S. thermophile* than *T. reesei* cellulase preparations. The relative yield for oligo-sugars was about the same except the concentrate which showed a lower level in the hydrolysate. This suggested incomplete saccharification, which can be attributed to time factor and the presence of weaker cellulase compenents resulting in incomplete synergistic effect. However, in comparison to much of the reported work on *T. reesei* this preparation from strain VTT-D-79125 contained FP-ase : B- glucosidase in the ratio of 1:0.66 and thus along with a potent endoglucanase and xylanase it formed a complete system and did not show characteristics of end-product inhibition. This is in agreement for some of the *Trichoderma* mutants derived (Sternberg et al, 1976; Rabinovich et al, 1979; Sinitsyn et al, 1982). The enzyme system from *S. thermophile* on the

converse lacking in comparable endo-glucanase and xylanase (not reported here) compensated in retrospect with high B-glucosidase which enhanced the potency of the enzyme system. Moreover, the ability of this thermophilic organism to be effective for saccharification at dilute enzyme concentrations and the thermostable nature of the enzymes needs further exploitation.

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