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Bioprocessing of Sweet Sorghum with *In-Situ*-Produced Enzymes

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ABSTRACT

Enzyme-assisted ensiling (ENLAC), using *in situ*-produced enzymes from *Gliocladium* sp. TUB-F-498, preserved 80% of the sugar content of sweet sorghum, and facilitated its extraction by countercurrent diffusion. The *in situ* enzyme was produced on the extracted sweet sorghum pulp by an 8-d solid substrate fermentation (SSF) with a yield of 4.6 cellulase and 400 IU/g dry wt xylanase. Two percent of the fermented substrate had cellulase and xylanase levels equivalent or superior to levels found in the commercial enzymes Celluclast and Viscozyme Novo at the 0.025% application level in ENLAC.

The *in situ*-production of enzymes on recyclable substrates may reduce bioprocessing costs significantly. In this ENLAC process, the cost of the *in situ* enzymes is estimated to be about $0.12/MT substrate, compared to $9.5/metric ton (MT) for the commercial enzymes, a cost reduction of nearly 80-fold.

Index Entries: Bioprocessing of sweet sorghum; *in situ* enzyme production; fungal cellulases and hemicellulases.

INTRODUCTION

The use of enzymes in bioprocessing of natural materials is limited by the high price of commercial enzymes. A possible alternative is the production of necessary enzymes *in situ* on process residues by solid substrate fermentation (SSF) with single or mixed cultures of lignocellulolytic fungi *(1,2)*. In this article, an example is given for the production and use of *in situ*-produced enzymes in the bioprocessing of sweet sorghum by enzyme-assisted ensiling (ENLAC) *(3)*. In this process, the lignocellulolytic enzymes necessary for increasing sugar yield are produced on the extracted sweet sorghum pulp by SSF with lignocellulolytic fungi. The process is illustrated in Fig. 1. About 2–3% of the extracted pulp may be recycled as *in situ* enzyme, and the rest may be used as animal feed.

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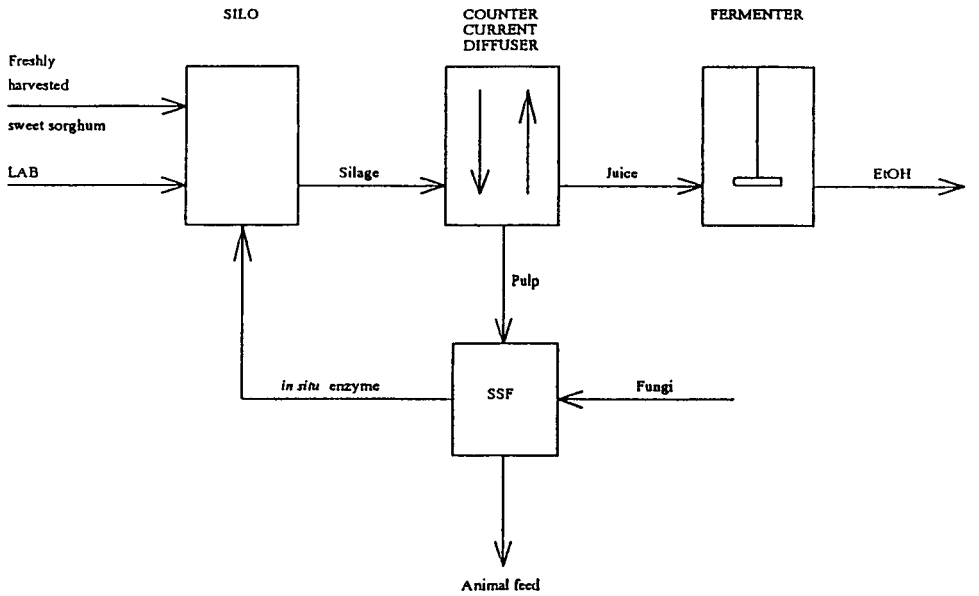

Fig. 1. Scheme of integrated bioprocessing of sweet sorghum for ethanol production.

MATERIALS AND METHODS

Substrate

Sweet sorghum, Honey-Marion variety, was ensiled immediately after harvesting and chopping to about 0.5–1.0 cm in size, with one or more of the following additives: Commercial lactic acid bacteria (LAB) (Silaferm, Monor, Hungary), consisting of 95% *Lactobacillus plantarum* and 5% *Streptococcus faecium*, were added at the level of 10^5 colony forming units (CFU)/g substrate. Vicozyme (Novo, Denmark), a commercial plant cell-wall-degrading enzyme preparation, was added at the level 0.075% (wet basis). *In situ* enzyme, the product of SSF by fungi, was added at the level of 1–10% (dry matter [DM] basis).

After ensiling for 60 d at 25°C in 100-L model silos, the silage was extracted with water in a sugar industry-type countercurrent diffuser (Fig. 1) to obtain a sugar and lactic acid-containing juice. The extracted residue was dewatered by pressing to approx 30% DM content, and this material served as substrate for SSF.

Inoculum

The fungi used in this study were selected in a screening program from about 400 lignocellulolytic fungi in the culture collection of the Technical University of Budapest (TUB) (G. Szakacs). The selected fungi were compared with the reference strain *Trichoderma reesei* Rut C30, a leading cellulase-producing mutant.

Inoculum was prepared by growing and sporulating the fungi on potato dextrose agar (Difco Labs, Detroit, MI) for 10 d at 30°C and then washing the spores with 0.1% Tween-80 containing water, to have a spore suspension of 10^9 CFU/ mL. This spore suspension was used for inoculating the substrate.

Table 1
Conservation of Sugar Content in ENLAC

Treatment	Reducing sugar g/kg DM[a]		DM, g/kg
	15 d	60 d	
Untreated sweet sorghum	201.1	25.0	212.7
Control silage	235.9	47.5	253.3
Silage with LAB[b]	232.2	106.7	265.7
Silage + Viscozyme[c] + LAB[b]	336.6	280.6	237.0
Silage + *in situ* enzyme[d] + LAB[b]	350.1	290.5	230.0

[a]Reducing sugar content of freshly harvested sweet sorghum: 364 g/kg DM; DM content 314 g/kg.
[b]LAB: Lactic acid bacteria added at 10^5 CFU/g sorghum.
[c]Viscozyme, Novo: Added at the level of 0.075%.
[d]*In situ* enzyme: pulp fermented with *Gliocladium* TUB F-498, added at the level of 2.0% (w/w).

SSF

The extracted sweet sorghum was supplemented with 2.5% $(NH_4)_2SO_4$ to adjust the C:N ratio to 10:1. The final DM content was adjusted to 30% by the addition of a basal salt solution *(4)*. The prepared substrate was loosely packed (9.6 g DM/100 mL) into a 250-mL double plastic cup, the inner one having perforations for air access, and the outer one providing protection from contamination. The cups filled with substrate were sterilized at 121°C for 20 min, and then inoculated with the prepared spore suspension of the respective fungus to a final concentration of spores 10^7 CFU/g DM.

The inoculated substrate was incubated at 30°C in a 99% relative humidity chamber for 8 d. The cellulase (as filter paper activity) and xylanase activity, the fungal biomass level, and DM loss of the fermented substrate were determined as described earlier *(1)*.

RESULTS AND DISCUSSION

Conservation of Sugar Content in ENLAC

The main effect of enzyme-assisted ensiling on sweet sorghum is the conservation of the sugar content (Table 1). Although 93% of the sugar content was lost from sweet sorghum 60 d after harvest, the loss was only 23% in the Viscozyme + LAB-treated silage and 20% in the *in situ* enzyme + LAB-treated silage. Ensiling without additives or only with LAB led to considerable sugar loss. The *in situ* enzyme was at least equivalent to the commercial enzyme in conserving the sugar content of sweet sorghum. The increased reducing sugar level in the enzyme-treated silages was partially owing to the hydrolysis of cell-wall components (mainly hemicellulose) and partially owing to the increased release of sugars from cells made permeable by the enzyme treatment.

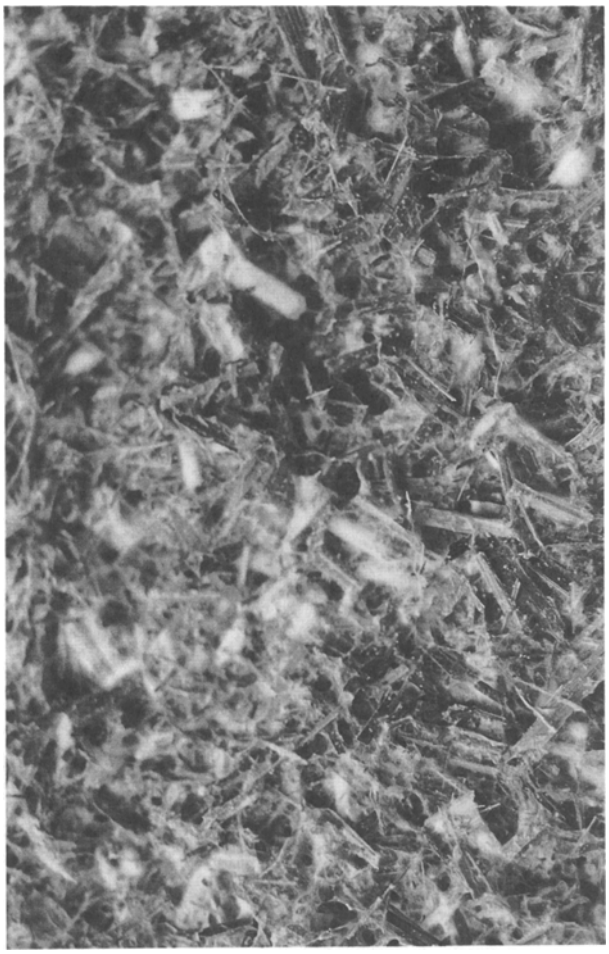

Fig. 2. Two days' growth of *Gliocladium* TUB F-498 on extracted sweet sorghum silage.

In Situ Enzyme Production in SSF

The growth of *Gliocladium* TUB F-498 on extracted sweet sorghum silage is shown in Fig. 2. This fungus rapidly colonized and penetrated the substrate in 2–3 d. The kinetics of growth and enzyme production by *Gliocladium* TUB F-498 is shown in Fig. 3. Enzyme production followed growth by about a 2-d delay. Satisfactory enzyme production is reached in 7–8 d, but newer experiments indicate that the fermentation may be prolonged for 10–14 d for maximum enzyme production.

Other fungi differed in their ability for growth and enzyme production on this substrate. The most successful fungi from an extensive screening program have been compared for cellulase and xylanase production in an 8-d SSF in Table 2. The enzyme activities of the four wild strains, isolated from nature, were near equal or superior to that of *T. reesei* Rut C30, one of the most productive mutant strains so far developed. With genetic manipulations and/or optimization of the

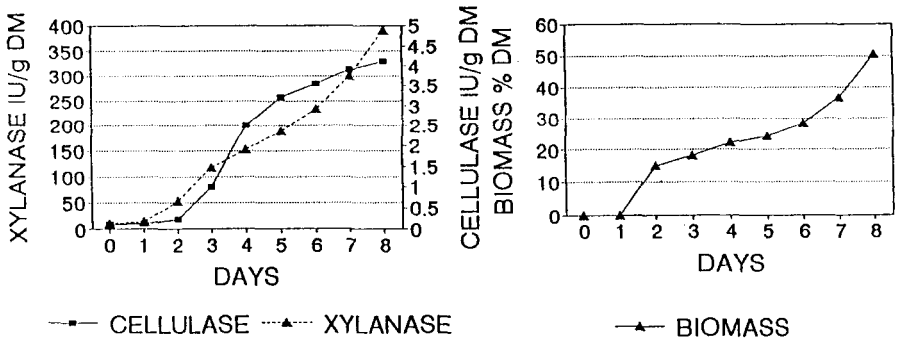

Fig. 3. Kinetics of growth and enzyme production by *Gliocladium* sp. TUB F-498.

Table 2
Comparison of Fungi for Enzyme Production in SSF[a]

Strain	Cellulase, IU/g DM	Xylanase, IU/g DM
Trichoderma reesei Rut C30	3.1	240
Trichoderma hamatum TUB F-105	2.7	217
Trichoderma sp. TUB F-482	3.4	142
Trichoderma sp. TUB F-486	2.9	141
Gliocladium sp. TUB F-498	4.6	400

fermentation, the enzyme production potential of these wild strains could be increased significantly.

Estimation of the Economy of *In Situ* Enzyme Production and Use

The main advantage of the use of *in situ* enzymes is the very significant reduction of production costs because of the availability of a recyclable substrate generated in the bioprocessing, the very low production cost of SSF, and avoidance of the expensive isolation and purification of enzymes. The substrate cost is practically zero, it has been pretreated in the ENLAC process, and it is practically sterile after diffusion.

The estimated unit costs of commercial and *in situ* enzymes, calculated for the cellulase component only, are given in Table 3. In Table 3, a currently available firm quotation from Novo, Denmark was used for a commercially available and widely used cellulase preparation. The estimate for the *in situ* enzyme is based on the estimated cost of composting and ensiling—$3–6/MT—the only large-scale solid-state fermentation processes in the US. Since the fermentum is used directly as an enzyme source, no further processing costs are incurred. The cost estimates in Table 3 serve only as guidelines for arriving at future realistic process enzyme costs. The point of these estimates is that we cannot use commercial enzymes for bioprocessing at present prices. "Real" alternatives, such as on-site or *in situ* submerged or solid-state fermentation processes, must be used for enzyme production. To illustrate this point further, the cost estimates for sweet sorghum bioprocessing by ENLAC, using commercial or *in situ* enzymes, are given in

Table 3
Comparison of Commercial and *In Situ* Enzyme Costs

Commercial cellulase, Novo Celluclast 1.5 L
Bulk price: \$12.00/L; activity: 80 IU/ mL
Unit price:
$\$12.00 \cdot (L/1000\ mL) \cdot (1\ mL/80)(1\ mL\ IU) = 1.5 \times 10^{-4}\ (\$/IU)$
In situ enzyme by TUB-F498
Production cost: ~\$6.00/MT (estimated from the production cost of compost)
Activity: 4.5 IU cellulase/g DM fermentum
Enzyme content of fermentum at 30% DM:
Cellulase: $1.35.10^6$ IU/MT
Cost of cellulase:
$(6.00/1.35.10^6) \cdot (\$/MT) \cdot (MT/IU) = 4.4.10^{-6}\$/IU$

Table 4
Enzyme Costs in ENLAC

Recommended level of enzymes used
0.025% Celluclast 1.5 L (\$12/L, 20,000 IU/MT)
0.025% Viscozyme 120 L (\$26/L)
Commercial enzyme cost in process:
Celluclast:

$$250\ mL/MT \times (12/1000)\ (\$/ML) = \$3.0/MT$$

Viscozyme:

$$250\ mL/MT \times (26/1000)\ (\$/ML) = (\$6.5/MT/L)$$

Total enzyme cost: \$9.5/MT
In situ enzyme cost in process
A 2.0% addition of the *in situ* enzyme source to the substrate would provide 27,000 IU/MT cellulase and 900,000 IU/MT xylanase, more than provided by the commercial enzymes at the 0.025% level.
Since the cost of the *in situ* enzyme is ~\$6.00/MT, the total enzyme cost in the process would be:

$$6.00\ (\$/MT) \times 0.02 = \$0.12/MT$$

Table 4. The cost saving is significant compared with commercial enzymes currently used in silage additives. Even greater savings might be achieved by using *in situ* enzymes in lignocellulose bioconversion processes, where the needed enzyme loads are much higher than in ensiling.

CONCLUSIONS

In situ enzymes may be an economical replacement for commercial enzymes, especially in agrobiotechnological processes, such as ensiling, upgrading animal feed, bioconversion of crop and forestry residues, and so forth, where purified

enzymes are not needed, and where the substrate for SSF is readily available from the bioprocess itself. The use of *in situ* enzymes may make thus far uneconomical enzymatic bioprocessing feasible and profitable.

ACKNOWLEDGMENTS

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