whilst the organism was in the stationary phase but exoglucanase activity decreased sharply at the onset of the stationary phase. Addition of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) towards the end of growth produced complete protection toward protease inactivation, exoglucanase being stabilized even in prolonged stationary phase. The stability of exoglucanase in the presence or absence of mycelium, with and without PMSF addition, showed that endogenous cell-free and cell-associated proteases were responsible for exoglucanase inactivation in the stationary phase. Inactivation of exoglucanase depended on the physiological stage of the cultures, the enzyme remaining stable as long as the culture was in an active growth phase.

Résumé

Production et stabilisation de cellulases de Trichoderma reesei

La production d'exoglucanase et d'endoglucanase dans les cultures de *T. reesei* QM 9414 en milieu non renouvelé est associée à la croissance tant du lactose que du jus d'extraction de la pulpe de betterave. L'endo-glucanase reste stable tant que le microorganisme est dans sa phase stationnaire de croissance tandis que l'activité exoglucanasique décroît rapidement dès le début de cette phase stationnaire. L'ajout du fluorure de phenylmethylsulfonate (PMSF), un inhibiteur protéolytique, vers la fin de la croissance a exercé une protection complète contre l'inactivation par la protéase: l'exoglucanase était protégée dans ces conditions même en phase stationnaire prolongée. La stabilité de l'exoglucanase en présence ou en absence de mycelium, avec ou sans ajout de PMSF démontre la responsabilité des exo-protéases endogènes solubles et de protéases associées aux cellules dans l'inactivation de l'exoglucanase dans la phase stationnaire. L'inactivation de l'exoglucanase dépend de l'état physiologique des cultures. En effet, l'enzyme reste stable aussi longtemps que la culture est en phase active de croissance.

Resumen

Producción y estabilización de celulasas de Trichoderma reesei

La producción de exoglucanase y endoglucanasa de *T. reesei* QM 9414 en cultivo por lotes en medios con lactosa y coseta agotada de remolacha como fuentes de carbono, fue asociada al crecimiento. La endoglucanasa permaneció estable en fase estacionaria, pero la actividad de exoglucanase disminuyó drásticamente al entrar el cultivo en dicha fase. La adición del inhibidor de proteasas PMSF hacia el final de la fase de crecimiento provocó la total inactivación de las proteasas producidas, lográndose estabilizar la exoglucanasa aún en prolongada fase estacionaria. Se analizó la estabilidad de exoglucanasa en presencia y ausencia de micelio, con y sin adición de PMSF; los resultados muestran que proteasas endógenas extracelulares y asociadas a la célula son responsables de la inestabilidad de la exoglucanasa en fase estacionaria. La inactivación de la exoglucanasa depende del estado fisiológico del cultivo, ya que la enzima permanece estable mientras el cultivo se encuentra en crecimiento.

ADDENDUM: Recovery of cellulases from spent broth of sugar beet pulp fermentation by *Trichoderma reesei*

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Materials and methods

Enzyme production: *Trichoderma reesei* QM 9414 cellulases for all experiments were prepared in one batch as follows: four 2 litre Erlenmeyer flasks, each containing 400 ml culture medium, were incubated for 96 h at 30°C, pH 4, at 220 rev./min in a rotary shaker. The culture medium contained (g/l): sugar beet pulp at a particle size 35 to 100 mesh, 25; $(\text{NH}_4)_2\text{SO}_4$, 2.67; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.41; KH_2PO_4 , 1.07 and citrate buffer (pH 4.0) 0.1 M, mineral salts solution as given previously, 13.5 ml/l. The fermented broth of each flask was pooled. The inoculum for this step was obtained as follows: a) *Spore suspension*: a 50 ml spore suspension of about 0.3 mg/l spores (dry basis)/l was prepared. This was equivalent to suspend the spore contents of one agar slant in 25 ml of distilled water. b) *Propagation step*: one ml of spore suspension was added to each of four 200 ml flasks containing 49 ml culture medium. The culture conditions and medium composition were the same as for the production step except that instead of sugar beet pulp, lactose was used at 6 g/l. **Liquid-Solid separation**: the protein enriched solids (a mixture of sugar beet pulp and mycelia) were filtered through a cotton cloth; the filtrate was then centrifuged for 20 min at 10,000 g and a clear supernatant containing the crude enzyme was obtained. **Concentration**: 150 ml of filtrate containing the crude enzyme was vacuum concentrated through rotary evaporation at 30°C and at 2100 ± 660 Pa to a final volume of 21.5 ml. The concentration solution was stored at 4°C with 0.5% (w/v) of sorbic acid. **Enzyme recovery**: $(\text{NH}_4)_2\text{SO}_4$ and acetone precipitation were evaluated for enzyme recovery. Precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed standard procedures, collecting the protein precipitate by centrifuging at 15,000 g for 20 min at 5°C. Precipitation with acetone was performed at -5°C. To 50 ml of concentrated enzyme solution, acetone was added dropwise with constant agitation until a solid phase appeared. The suspension was further agitated for another 15 min and then centrifuged at -5°C and 10,000 g for 10 min. The supernatant was carefully removed and the precipitate then immediately resuspended in a 0.1 M, pH 4.8 citrate buffer. **Stability tests**: The storage stability of the protein concentrate and the resuspended enzyme precipitates was tested at 4°C, pH 4.8 and in the presence of 0.5% sorbic acid by determining exo- and endo-glucanase activities once a week over 60 and 30 d, respectively. **Analysis**: Exo- and endo-glucanase activities were defined and measured as reported in the above paper (see also Illanes and Rossi, 1980). Reducing sugars were quantitated by the dinitrosalicylic acid method as described by Miller (1959). Soluble protein was determined by Lowry's method (Legett, 1967).

Results and discussion

Vacuum concentration

Table 1 shows the effect of vacuum concentration on enzyme and protein recovery. As protein recovery was 100%, losses in activity may have been caused by denaturation of the cellulases due to surface interactions in the low pressure system at the longer process times needed for higher concentrations. With both cellulase fractions, there was a rapid decrease in activity at the beginning of the storage period (16% for exoglucanase and 5% for endoglucanase) but afterwards both activities remained unchanged after 60 days' storage.

Ammonium sulphate precipitation

Enzymes were precipitated at 67% saturation from the concentrated broth. Material balances for protein gave a 100% recovery; however only 53% and 41% of the exo- and endo-glucanase activities were recovered. Endoglucanase remained stable for 30 days but exoglucanase decreased 46% in activity over the same period.

Acetone precipitation

Enzymes were precipitated at 67% (v/v) acetone from the concentrated broth. Material balances for protein gave a 100% recovery; however yields for exoglucanase and endoglucanase were only 57% and 42% respectively. activity losses can be attributed to the strong denaturing effect of the low dielectric constant of the acetone and it being necessary to operate at -5°C to obtain good yields (Askonas, 1951; Wang *et al.*, 1979). Both exo- and endo-glucanase remained fully stable after 30 days' storage.

Table 1 Effect of vacuum concentration on cellulases and protein recovery

Concentration factor	Exoglucanase		Endoglucanase		Protein	
	activity U/ml	yield (%)	activity U/ml	yield (%)	concentration (mg/ml)	yield (%)
1.0	0.44	100	0.16	100	2.5	100
1.8	0.78	98	0.28	100	5.0	100
3.4	1.41	94	0.52	96	8.5	100
5.4	2.14	90	0.81	94	13.2	98
7.0	2.71	88	1.04	93	17.5	100

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Summary

The exo- and endo-glucanases of *Trichoderma reesei* were recovered after growth on sugar beet pulp by vacuum concentration followed by $(\text{NH}_4)_2\text{SO}_4$ or acetone precipitation. Final

respective recoveries were 53% and 57% for the exoglucanase and 41% and 42% for the endoglucanase. The resuspended acetone precipitates of both enzymes showed no loss of activity after 30 d at 4°C, pH 4.8 and in the presence of 0.5% sorbic acid. $(\text{NH}_4)_2\text{SO}_4$ -precipitated exoglucanase lost activity in the same period.

Résumé

Récupération de cellulases de milieux de fermentation sur pulpe de betterave enrichie en protéines

Les exo- et endoglucanases de *Trichoderma reesei* ont été récupérées après croissance sur pulpe de betterave sucrière, par concentration sous vide après précipitation par le sulfate ammonique ou l'acétone. Les récupérations ont été respectivement de 53% et 57% pour l'exoglucanase et de 41% et 42% pour l'endoglucanase. Les resuspensions des deux enzymes après précipitation à l'acétone n'ont révélé aucune perte d'activité après 30 jours à 4°C, pH 4.8 et en présence de 0.5% d'acide sorbique. L'exoglucanase précipitée au sulfate ammonique avait perdu toute activité dans le même laps de temps.

Resumen

Recuperación de celulasas a partir del caldo desechable de la fermentación de pulpa de remolacha azucarera enriquecida con proteínas

Las exo y endoglucanasas de *Trichoderma reesei* se recuperaron después de su crecimiento en pulpa de remolacha azucarera mediante concentración al vacío seguida de precipitación con $(\text{NH}_4)_2\text{SO}_4$ o acetona. La recuperación final fue de 53% y 57% para la exoglucanasa y de 41% y 42% para la endoglucanasa. La resuspensión de los precipitados cetónicos de ambas enzimas no mostró pérdidas de actividad después de 30 d a 4°C y pH 4.8 en presencia de ácido sórbico. La exoglucanasa precipitada con $(\text{NH}_4)_2\text{SO}_4$ perdió actividad durante este mismo periodo.