BIOTECHNOLOGY TECHNIQUES
Volume 6 No.5 (sep/oct 1992) pp.429-432
Received as revised 21st May

EFFICIENT LEACHING OF CELLULASES PRODUCED BY *Trichoderma harzianum* IN SOLID STATE FERMENTATION

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SUMMARY

Recovery of cellulases from solid state cultures of *Trichoderma harzianum* was efficiently achieved by hydraulic pressing. Pressing of fermented solids yielded carboxymethyl-cellulase (CMCase) extraction efficiency of 71 % and a ratio of leachate to fermented solids of 0.58 (v/w). Addition of water to pressed solids and second pressing improved the efficiency (95 %) with simultaneous increase in the ratio to 1:16 (v/w). The overall extraction of filter paper activity was lower (85 %) than that of CMCase. This technique is simple and its extraction efficiency is similar to that obtained in multiple-contact counter-current systems. The hydraulic press in its individuality was not used earlier to leach the product from fermented solids.

INTRODUCTION

The presence of the product in concentrated form (Lonsane *et al.*, 1985) and the consequent decreased cost of down-stream processing as well as waste obviating the need for waste treatment (Lonsane and Ramesh, 1990) are the major reasons which explain the growing interest in solid state fermentation (SSF) processes (Steinkraus, 1984; Hahn-Hägerdal, 1986). In spite of recently research and development efforts carried out on SSF, the commercial exploitation arising from these studies is almost negligible (Vaccarino *et al.*, 1989). One of the reasons is the lack of data on down-stream processing with possibilities for scaling-up (Lonsane *et al.*, 1992). The technique preferably should not involve high capital and operating expenses (Lonsane and Krishnaiah, 1992). The leaching of the product from fermented solids is an important unit operation in SSF due to its economic implications (Lonsane and Krishnaiah, 1992). Indiscriminate selection of a leaching technique can result in a dilute leachate, requiring vacuum concentration (Ramakrishna *et al.*, 1982). The product concentration in the leachate and the ratio of leachate to fermented solids thus assume critical importance. An efficient technique based on hydraulic pressing is reported in this paper. This method has not been used before except for its combination with a multiple-contact, counter-current, leaching system for obtaining slightly higher recoveries of fungal rennet at the end of each contact stage (Thakur *et al.*,1990).

MATERIALS AND METHODS

Enzyme production process. Cellulases were produced by SSF technique at pilot plant scale in Zymotis, the fermenter designed by ORSTOM, using *Trichoderma harzianum* CCMF-470 (Roussos, 1987). The fermentation medium (pH 4.4) contained (g): sugar cane bagasse 80, wheat bran 20, $(NH_4)_2SO_4$ 9.7, urea 2.4 , KH_2PO_4 5.0 and tap water 117. The moist medium was transferred to cloth sacks, autoclaved at 110°C for 60 min, cooled to about 30°C, inoculated with spore suspension at a rate of $3x10^7$ conidiospores/g substrate dry matter (SDM) given a final moisture content of 74%. Inoculated moist solids (42 kg) were charged into Zymotis fermentor and allowed to ferment at 29°C for 60 h. The temperature control was achieved by forced aeration of the solids by humidified air (aeration rate : 300 l/h/kg during first 16 h; 600 l/h.kg after 16 h) and cooling water circulation through the heat exchangers of the fermenter. Other experimental details, the design of Zymotis fermentor, methodology for inoculum preparation in disc fermenter and the source of the culture have been reported elsewhere (Roussos, 1987; 1991; González-Blanco *et al.*, 1990).

Hydraulic pressings. The fermented solids, at the end of fermentation, were removed from Zymotis fermentor and the lumps were broken. A 500 g sample was transferred to the cylindrical stainless steel (SS) perforated basket of the hydraulic press (Type 45 T, Pinette Emidecau S.A., 71 Chalon/Saone, France) and pressed at 220 bar pressure for 1 min. The leachate was collected in a container and the solid residue left in the basket was mixed throughly with tap water in quantity equal to that of the leachate from first pressing. The mass was again pressed similarly to obtain second leachate. In preliminary experiments, the hydraulic pressure was varied at 220 and 230 bars while the time of pressing at 220 bar pressure was varied at 1, 2 and 2.5 min. The leachates were passed through gauge cloth for separation of finer solid substrate particles before subjecting to the assays.

Analytical aspects. The activities towards carboxymethyl-cellulose (CMCase; 1,4-β-D-glucan 4-glucanohydrolase; endo 1,4-β-D glucanase; EC 3.2.1.4) and Whatman filter paper No. 1, 1 cm x 5 cm, 50 mg, (FPase; 1,4-β-D-glucan cellobiohydrolase; exo-1,4-β-D glucanase; EC 3.2.1.91) were determined by the methodology of Mandels *et al.* (1976). The enzyme concentration is expressed as International Units (IU) which denote the μmol of glucose released per min of the reaction. The enzyme titres are calculated per ml of leachate or g of fermented moist solids.

RESULTS AND DISCUSSION

Hydraulic pressing of fermented solids: Preliminary experiments on the effects of pressures (220 and 230 bars) and pressing time (1, 2 and 2.5 min) showed that the volume of the leachate in all the cases was in the range of 80-81% of the total water originally present in the fermented solids. Consequently, the pressing of the solids at 220 bar for 1 min was adopted for use in further studies.

The leaching patterns of the enzyme in two stages hydraulic pressings are showed in Table 1. The overall enzyme recoveries achieved are 85 and 95 % for FPase and CMCase activities respectively. These are similar than those of 80-86% reported with the use of other leaching techniques (Ramakrishna *et al.*, 1982; Kumar and Lonsane, 1987; Ramesh and Lonsane, 1988; Hang and Woodams, 1989). Such high recoveries are possible due to removal of larger proportion of water from the solids during hydraulic pressing. This facility is not possible with other leaching techniques and thus imparts commercial significance to the method of hydraulic pressing. The bagasses employed in

our SSF system have ability to absorb about 5 times of their weight of water. In fact, it is this characteristic of the solids which make them suitable for SSF system, the absorbed liquid meets the water requirement of microorganisms during the course of fermentation. However, this water-absorbing capacity of bagasse proves a drawback in product leaching operation. In the present case, the fermented solids (500 g moist weight; 142 g SDM with 358 ml water) yielded 290 ml leachate when pressed in the first stage. Thus, only 68 ml water was held up in the solids and the ratio of absorbed water to solids is thus 0.48 (v/w). This water probably can not be removed under the present experimental conditions. This has been proved by the data from second stage hydraulic pressing. Water, 290 ml, was added to the solids from first pressing and it gave same quantity of leachate when pressed in second stage.

A difference in the recovery of CMCase and FPase activities in the first stage has been observed (Table 1) and might be due to lower solubility of FPase activity. This is confirmed by the data of the second stage hydraulic pressing wherein water was added to the leached solids from the first stage. The recovery of FPase activity in second stage is higher than that of CMCase activity (Table 1).

Table 1 . Leaching patterns of cellulases from fermented solids by hydraulic pressing in two stages

Pressing stage	Recovered volume (ml)	CMCase activity			FPase activity		
		IU/ml leachate	Total activity, IU	Recovery %	IU/ml leachate	Total activity IU	Recovery %
First	290	82	23780	71	5,6	1624	59
Second	290	26	7714	23	2,5	725	26
Overall	580	54	31494	95	4,1	2349	85

Total enzyme activities were 33 300 and 2750 IU for CMCase and FPase activities respectively. It was calculated on the basis of 66.6 and 5.5 IU of CMCase and FPase activities respectively per g of fermented solids. In each stage 500 g of solids were pressed. The leaching recovery in each stage was 80.9 % of the total water present.

The ratios achieved at each stage of hydraulic pressings are given in Table 2. The overall ratio of leachate to fermented solids was 1.16 (v/w) which is lower than that from many other leaching techniques (Ramakrishna *et al.*, 1982) and is nearly equal to that obtainable in multiple-contact counter-current leaching (Kumar and Lonsane, 1987). Moreover, one has to use 4 to 5 contact-stages in the latter technique to achieve about 85% extraction efficiency (Hang and Woodams, 1989; Lonsane and Krishnaiah, 1992). The concentration of the product in this absorbed water is equal to that in the leachate from that pressing stage. Addition of less water during second pressing stage results in a higher enzyme concentration in leachate as well as decrease of extraction efficiency. The ratios of the product concentration in the fermented solids and the leachate (Table 2) showed that performance of the method of hydraulic pressing is just below the supercritical fluid extraction technique (Kumar *et al*,.1991) which has utility only for low-volume high-value products (Lonsane and Krishnaiah, 1992). Such high ratios are possible as no water, i.e. solvent, is added to the fermented solids in the first hydraulic pressing stage.

Table 2. Ratios achieved in two stage hydraulic pressing of fermented solids for enzyme recovery.

Pressing stage	Leachate to fermented solids ratio (v/w)	IU/ml leachate: IU/g fermented solids	
		CMCase activity	FPase activity
First	0.58	1.23	1.02
Second	0.58	0.40	0.45
Overall	1.16	0.82	0.75

Calculated on the basis of 66.6 and 5.5 IU of CMCase and FPase activities respectively per g of fermented solids and leachate data from Table 1.

Practical aspects: The hydraulic press used in the present studies is a commercially available laboratory scale unit which is commonly employed in sugar mills to extract the juice from sugar-canes for estimation of sucrose (Roussos, 1987). The cylindrical SS basket for holding the material to be pressed has conical shaped perforations (1 and 3 mm diameter at inner and outer sides of the basket) so as to avoid the need for filter cloth. It has two pistons which press the material from bottom and top sides. The selection of this type of press was mainly guided by the suitability in scale-up of the hydraulic pressing at pilot and industrial scales. Similar hydraulic presses of industrial capacities are routinely employed in fruit processing industries. Thus, excellent adaptability as well as feasibility of this technique at industrial scale is possible and the scale-up of the leaching operation may not pose any problem in SSF processes.

ACKNOWLEDGEMENTS: The authors thank Mme Laure Hannibal for the technical assistance. B.K.L. is thankful to Department of Biotechnology , Government of India, New Delhi for the award of Overseas Associateship (Short-term), Council of Scientific and Industrial Research , New Delhi for deputation terms and ORSTOM, Center Montpellier, for excellent facilities.

REFERENCES

González-Blanco, P., Saucedo-Castañeda, G., Viniegra-G., G. (1990) *J. Ferment. Bioeng.*, 70, 351-354.

Hahn-Hägerdal, B. (1986) *Enzyme Microb. Technol.*, 8, 322-327.

Hang Y.D. and E.E. Woodams (1989) *MIRCEN Journal*, 5 , 379-382.

Kumar, P.K.R., Lonsane, B.K. (1987) *Process Biochem.*, 22, 139-143.

Kumar, P.K.R., Uday Sankar K, Lonsane, BK. (1991) *The Chem. Engineer. J.*, 46, 853-858.

Lonsane, B.K, Krishnaiah, M.M. (1992) *In* Doelle, H.W., Mitchell, D.A., Rolz, C.E. (eds) Solid substrate cultivation. Elsevier Science Publishers, Essex, England, in press.

Lonsane, B.K., Ramesh, M.V. (1990) *In* Neidleman, S.A., (ed) *Advances in Appl. Microbiol.*, vol. 35, Academic Press, New York, pp.1-56.

Lonsane, B.K., Ghildyal, N.P., Budiatman, S., Ramakrishna, S.V. (1985) *Enzyme Microb. Technol.*, 7, 258 - 265.

Lonsane, B.K., Saucedo-Castañeda, G., Raimbault, M., Roussos, S., Viniegra-González, G., Ghildyal N.P., Ramakrishna, M., Krishnaiah, M.M. (1992) *Process Biochem.*, in press.

Ramakrishna, S.V., Suseela, T., Ghildyal, N.P., Jaleel, S.A., Prema, P., Lonsane, B.K., Ahmed, S.Y. (1982). *Indian J. Technol.*, 20, 476-480.

Ramesh, M.V., Lonsane, B.K. (1988)*Chem. Mikrobiol. Technol Lebensm* 11, 155-159.

Roussos, S. (1987). Thèse d'Etat, Université de Provence, France, ORSTOM Eds No. 857-3, Paris.

Roussos, S., Olmos, A., Raimbault, M., Saucedo-Castañeda, G., Lonsane, B.K. (1991) *Biotechnol. Techniques*, 5, 415-420.

Steinkraus, K.H. (1984) *Acta Biotechnol.*, 4, 83-88.

Thakur, M.S., Karanth, N.G., Krishna, N. (1990) *Appl. Microbiol. Biotechnol.*, 32, 409-413.

Vaccarino, C., Lo Curto, R., Tripodo, M.M., Patané, R., Laganà, G., Schachter, S. (1989) *Biol. Wastes*, 29, 279-287.