Journal of Industrial Microbiology, 9 (1992) 121–125
Elsevier



SIM 00395

# Optimized protocol for the pilot-scale preparation of fungal cellulase

V. Ramamurthy, S.P. Thacker and R.M. Kothari

*Industrial Enzymes Department, Sarabhai Research Centre, Baroda, India*

(Received 14 January 1991; accepted 8 November 1991)

*Key words: Trichoderma viride*; Cellulase production; Optimized production medium and parameters

## SUMMARY

A 25-l scale protocol is devised for the optimal secretion and recovery of fungal cellulase. Using a selected higher yielding *Trichoderma viride* SMC strain, a protocol consisted of: a) an optimized production medium rich in microcrystalline cellulose (MCC), fortified with 1% (w/v) ammonium sulphate, 0.5% (w/v) soybean flour, 0.1% (v/v) Tween-80 and other trace nutrients; b) optimized physical parameters of production, such as an inoculum containing a homogeneous suspension of $6 \times 10^7$ conidia per l, 28 ± 1 °C, pH 4.0 ± 0.5, 300 ± 20 rpm, 11000 ± 1000 l/h aeration, and 170–220 h duration; c) optimal recovery through a filter press (450 l/h rate of filtration) followed by precipitation with 2.5–3.0 volumes of acetone (15 °C and basket centrifugation (27 °C, 1700 rpm)); and d) vacuum drying (35 °C, 4–6 h). This afforded 70% recovery of cellulase in the form of white fluffy powder containing 20000 ± 2000 carboxy methyl cellulase and 1000 ± 50 units filter paperase per g activities, with raw material cost of US$ 8–10 per million carboxy methyl cellulase units. During storage for 18 months at 4 °C, ambient temperature and 37 °C, the cellulase preparation was found to retain 100, 75 and 60% of its initial activity, respectively.

## INTRODUCTION

Since cellulase is a complex enzyme system and the substrates that occur in nature are not easily hydrolyzed, progress in industrial production of cellulase has remained limited. Besides, the cost of production appeared to render many potential applications non-profitable. Hence any improvement in the economics of its production and applicability has industrial significance. Based on these objectives, we reported on the application of cellulase in improving the yield of milk [8] and in upgrading the quality of jute [9]. Since a constant source of cellulase was required for making these applications viable, attempts were made to produce cellulase efficiently. Towards this goal, we proceeded to optimize the production medium, physical parameters of production, and recovery to obtain a highly active and stable cellulase preparation. These efforts are discussed in the present communication.

## MATERIALS AND METHODS

### *Ingredients and chemicals*

Microcrystalline cellulose (MCC) and carboxy methyl cellulose (CMC, Cellulose Products of India, Ahmedabad); defatted soybean flour (SBF) and defatted groundnut cake (GNC) (Synbiotics Ltd., Baroda); corn steep liquor (CSL, Maize Products, Ahmedabad); analytical grade chemicals and commercial grade denatured alcohol and acetone (Sarabhai M. Chemicals, Baroda); milk casein (Sugam Dairy, Baroda); xylose and bovine serum albumin (Sigma, USA); Tween-80 and Whatman paper No. 1 (Durga Traders, Baroda); commercial grade ammonium sulphate (GSFC, Baroda); dodenol-OTL and unisperse-731) (Hico Products, Bombay) and metal distilled (DM) water were used. Baggasse, saw dust, rice husk and wheat bran were procured from local market.

### *Culture*

A high yielding *Trichoderma viride* SMC strain isolated upon UV-exposure as described by Shah et al. [10] was used in the present study. In brief, the mutants were isolated using the procedure wherein the culture grown in nutrient broth (28 °C, 16 h), washed twice with sterile physiological saline, was diluted suitably for exposure to UV (140 mW/cm$^2$ of radiation at 254 nm at a distance of 6 cm at 28 °C) for 1 min to give 0.2–0.5% survival. The UV exposures were carried out six times to ensure reproducible survival rates and to obtain a large number of mutants for screening their carboxy methyl cellulase and filter paperase secretion ability.

Correspondence: R.M. Kothari, Thapar Corporate Research & Development Centre, Patiala-147001, India.

*Inoculum*

The inoculum (4%) was a homogeneous conidial suspension from a 7-day-old slant maintained at 28 °C, containing $6 \times 10^7$ conidia per l inoculum medium as described by Toyama and Ogawa [14], maintained for 24 h (28 ± 1 °C, 220 ± 20 rpm) per 25 l optimized production medium (OPM).

*Optimization of the production medium*

Since the production medium of Toyama and Ogawa [14] was used during screening for selection of higher cellulase producing strains, it was the starting medium for optimization. The optimized production medium (OPM) was composed of: 2.5% (w/v) microcrystalline cellulose (MCC); 1.0% (w/v) ammonium sulphate; 0.5% (w/v) soybean flour; 0.3% (w/v) potassium dihydrogen phosphate; 0.1% (v/v) Tween-80; and 0.05% (w/v) each of magnesium sulphate and potassium chloride, adjusted to pH 4.2 and sterilized for 30 min at 121 °C.

*Experimental procedure*

A typical experiment was carried out in a sterilized 500-ml Erlenmeyer flask containing 100 ml OPM plus 4% inoculum and subjected to a rotary shaker (200 ± 20 rpm, 28 ± 1 °C) for 240–264 h. Three flasks were removed after every 24 h, microscopy carried out, the spent liquor filtered to remove mycelia and the filtrate used to assay carboxy methyl cellulase and filter paperase activities, pH, percentage residual reducing sugar(s) and protein. The experiments were scaled up to 1 and 25 l using stainless steel batch bioreactors (Alfa-Laval India Ltd., Pune).

*Analytical methods*

Cellulase was assayed by measuring carboxy methyl cellulase and filter paper hydrolyzing activity using CMC and Whatman paper No. 1 as the respective substrates [4]. One unit of cellulase is the amount of enzyme which produced 1 mg of reducing sugar(s), estimated as dextrose, per h at pH 5.0 and 50 °C.

Residual reducing sugars, and protein were estimated as described earlier [11,12].

## RESULTS AND DISCUSSION

A strategy used by us in optimizing the production medium of alkaline protease [5], amyloglucosidase [12] and bacterial α-amylase [13] was followed in optimization of the cellulase production medium in shake flask studies. Accordingly, only one parameter was examined at a time, keeping other parameters constant. Once a particular parameter was found to afford the highest enzyme activity at harvest period, it was considered as optimum and incorporated while designing the next experiment. Thus, parameters that were considered likely to affect the cellulase productivity were sequentially optimized. Using this approach, when optimization of the production medium was almost completed, a very useful method [2] for optimizing media appeared. This approach will be examined for its applicability during further scale-up to 600 l.

*Optimized physical parameters of cellulase production*

The shake flask studies pertaining to amplitude of agitation and broth volume : vessel volume indicated that at harvest (240 h period, higher (300 ± 20 rpm) rates of agitation/aeration afforded higher secretion of cellulase (480 U/ml), vis-a-vis 2 U/ml at 50 rpm and 370 U/ml at 180 rpm. At constant rate and amplitude of agitation (180 rpm, 2.5 cm), an alteration in the ratio of broth volume : vessel volume from 0.3 to 0.5 did not increase cellulase secretion.

*Choice and optimization of an inducer*

Since baggasse, wheat bran, rice husk and saw dust were readily available, and inexpensive, their utility as inducers to substitute for MCC was explored at different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, w/v) added at the beginning of cultivation. Table 1 summarises the harvest values (240 h) as a function of different inducers.

The data of such comparison clearly showed that saw dust, rice husk and wheat bran are poor inducers of cellulase activity, whereas baggasse could be reexamined for its utility at different concentrations. It is conceivable that 28% lignin in saw dust or 18% silica content in rice husk were not conducive to cellulase induction. However, why wheat bran failed to induce cellulase secretion remains a question.

TABLE 1

CMCase and FPase productivity as a function of inducer

| Inducer[a] | Concentration for optimal harvest activity (%) | Harvest activity (U/ml) | |
|---|---|---|---|
| | | CMCase | FPase |
| MCC | 2.5 | 384 | 15.4 |
| Baggasse | 2.5 | 102 | 2.7 |
| Saw dust | 1.0 | 14 | 0.3 |
| Rice husk | 0.5 | 2.0 | Nil |
| Wheat bran | 0.5 | Nil | Nil |

[a] Each inducer was used at 0.5, 1.0, 1.5, 2.0, 2.5 and 3% (w/v) concentration. For the sake of brevity the highest harvest activity obtained is reported.

TABLE 2

CMCase and FPase profiles as a function of inducer concentration

| Log (h) | CMCase (U/ml) | | | | FPase (U/ml) | | | |
|---|---|---|---|---|---|---|---|---|
| | 1% M | 2.5% M | 1% B | 2.5% B | 1% M | 2.5% M | 1% B | 2.5% B |
| 96 | 16 | 20 | 22 | 27 | 1.4 | 1.2 | 1.0 | 1.6 |
| 144 | 120 | 130 | 45 | 68 | 5.0 | 4.1 | 1.4 | 2.3 |
| 192 | 208 | 316 | 75 | 75 | 6.8 | 11.0 | 2.1 | 2.6 |
| 240 | 241 | 492 | 79 | 79 | 7.2 | 17.0 | 1.8 | 2.6 |

M = MCC; B = Baggasse. For brevity, activity at other concentrations and every 24 h are not given.

Table 2 summarises carboxy methyl cellulase and filter paperase profiles as a function of inducer concentration during fermentation. These observations have shown that although baggasse is an equally efficient inducer as MCC up to 96 h, after 96 h, it lagged considerably behind inducing the secretion of cellulase, irrespective of the concentration. Thus, 2.5% MCC showed optimal secretion.

*Choice and optimization of protein*

At 0.2% (w/v), groundnut cake (GNC), corn steep liquor (CSL), casein and soybean flour (SBF) were screened for cellulase secretion (Table 3).

These profiles showed that GNC, casein and SBF are comparable. However, casein was eliminated because of high cost. The quality of GNC available in the market is extremely variable and therefore, not a practical choice. Soybean flour (SBF) with the correct specifications is inexpensive and readily available, thus its incorporation

TABLE 3

CMCase and FPase profiles as a function of protein

| Log (h) | CMCase (U/ml) | | | | FPase (U/ml) | | | |
|---|---|---|---|---|---|---|---|---|
| | GNC[a] | CSL[b] | Casein | SBF[c] | GNC[a] | CSL[b] | Casein | SBF[c] |
| 96 | 36 | 26 | 31 | 38 | 1.8 | 1.0 | 1.9 | 2.0 |
| 144 | 119 | 132 | 123 | 108 | 4.2 | 5.1 | 4.0 | 4.0 |
| 192 | 485 | 384 | 434 | 485 | 13.2 | 9.0 | 10.4 | 13.3 |
| 240 | 526 | 414 | 520 | 524 | 17.7 | 16.5 | 16.7 | 16.7 |

[a] GNC = groundnut cake; [b] CSL = corn steep liquor; [c] SBF = soybean flour

TABLE 4

CMCase and FPase profiles as a function of quality of protein

| Log (h) | CMCase (U/ml) | | | FPase (U/ml) | | |
|---|---|---|---|---|---|---|
| | SBF[a] | SBFE[b] | Peptone | SBF[a] | SBFE[b] | Peptone |
| 96 | 40 | 20 | 11 | 1.8 | 1.2 | 0.2 |
| 144 | 118 | 77 | 30 | 5.4 | 2.5 | 0.8 |
| 192 | 460 | 360 | 49 | 11.7 | 10.0 | 1.6 |
| 240 | 530 | 428 | 60 | 17.0 | 15.5 | 1.9 |

[a] SBF = soybean flour; [b] SBFE = enzymatic hydrolysate of SBF.

in the optimized production medium was preferred. Unhydrolyzed SBF or partial hydrolysate were studied for optimal secretion (Table 4).

The observation that unhydrolyzed SBF afforded higher secretion compared to partially hydrolysed SBF or peptone (extensively hydrolyzed protein) indicated that a sustained release of amino acids was necessary for optimal secretion of cellulase. Subsequently, it was found that SBF not only afforded optimal secretion of cellulase (576 U/ml carboxy methyl cellulase, 24 U/ml filter paperase), it also reduced the harvest period from 240 to 210 h. Further efforts to reduce their harvest period by modifying several parameters (individually and cumulatively) did not improve the yield indicating that the secretion mechanism is intricate.

*Mineral constitutents of optimized production medium*

It was found that ammonium sulphate is a vital ingredient of the OPM (571 U/ml carboxy methyl cellulase in its presence and 251 U/ml in its absence) and at 1% (w/v), it afforded optimal secretion of cellulase. When 1% urea was substituted for ammonium sulphate there was negligible secretion of cellulase, indicating that a sustained release of organic nitrogen is desirable. Similarly, it was found that 0.3% (w/v) $KH_2PO_4$, and 0.05% (w/v) $MgSO_4$ and 0.05% KCl yielded optimal harvest values of cellulase. Additionally, it was found that 0.1% (v/v) Tween-80 is a useful (but not vital) ingredient, not replaceable by dodenol OTL or Unisperse-731 (data not shown).

*Scaling-up of cellulase production*

The scale-up in a 40-l fermentor, containing 25 l optimized production medium with 300 ± 20 rpm as the rate of agitation, 11000 ± 1000 l/h rate of aeration, 28 ± 1 °C, and pH 4.2 ± 0.2 afforded minimal evaporation losses, ease of operation and optimal secretion of 480 carboxy methyl cellulase and 22 filter paperase units per ml over the period 170–220 h. Further increase in the rate of agitation or aeration did not increase cellulase yields. Fermentation was terminated by lowering the temperature from 28 to 20 °C. Optimized values of each parameter were derived as a result of a series of experiments at 25-l scale, with at least one value of a particular parameter being less than optimal (viz., 24 ± 2 °C) and another value being greater than optimal (viz., 32 ± 2 °C). Throughout the fermentation period, 0.5–0.8 kg pressure per $cm^2$ was maintained. With optimized parameters, carboxymethyl cellulase yields are 530 U/ml and filter paperase 17.0 U/ml (Tables 4 and 5). In comparison, Pourquie et al. [6,7] reported 22 filter paperase U/ml through an analogous approach. However, their productivity was 140 filter paperase U/l h as compared to 74 filter paperase U/l h of the present study. This difference may be attributable to a more soluble substrate (lactose and rayon pulp) employed by Pourquie et al. [7].

TABLE 5

Harvest and recovery parameters and profiles

| Optimal parameter | Profiles |
|---|---|
| Fermentation duration (h) | 210–220 |
| CMCase and FPase (U/ml) | 480–520 and 15–17 |
| Clarification | Through filter press; 2 plates of 60 × 60 cm |
| Filtration rate (l/h) | 400–450 |
| Recovery upon clarification (%) | 85–90 |
| Precipitating agent | 2.5–3.0 vol. acetone |
| Precipitation conditions | 15 °C, 4 h |
| Product recovery conditions | Basket centrifugation (1700 rpm, 27 °C, 20 l/h) |
| Vacuum drying | 35 °C, 4–6 h |
| Minimal cellulase % recovery– | |
| CMCase | 60 |
| FPase | 75 |
| Product appearance | White, fluffy |
| Activity (U/gm): CMCase; FPase | 20000 ± 2000; 1000 ± 50 |
| Storage stability after 18 months at 4, 27 and 37 °C | 100, 75 and 60%, respectively |

*Recovery of cellulase*

The recovery profiles summarised in Table 5 depict the range of observations made during 15 production trials, under comparable conditions. The use of different precipitating agents provided valuable data to devise an optimal protocol of cellulase recovery. The use of acetone provided an ease of precipitation, facilitated filtration, rapid drying and highest specific activity. In contrast, 50% (optimal) ammonium sulphate afforded 45% recovery of carboxy methyl cellulase and 90% recovery of filter paperase with less specific activity vis-a-vis 30% recovery of carboxymethyl cellulase and 40% recovery of filter paperase with the highest specific activity by 3 vol of ethanol. Cellulase thus obtained, exhibited significant hemicellulase activity (xylose being one of the products of hydrolysis).

The present raw material cost of US$ 8–10 per million carboxy methyl cellulase units seems to be acceptable for improving the quality of jute since the improved jute can be sold profitably. These cost calculations are obtained from 25-l data. However, we anticipated that costs can be reduced for larger scale operations. Cost

reductions are possible when membrane technology is optimized for concentration prior to precipitation so that the quantity of acetone used would be substantially reduced.

Although, extensive literature exists on the biosynthesis, factors affecting biosynthesis, purification, physical properties and desirable characteristics for enhanced production efficiency of cellulase [1,3], details regarding stepwise protocols for production, optimal recovery and data on stability as a function of storage have remained conspicuously scant. The present study (Table 5) addresses some of these deficiencies.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge financial assistance and pilot plant facilities provided for this work by Dr. V. Srinivasan, Director, Sarabhai Research Centre, Baroda, India.

## REFERENCES

1 Bisaria, V.S. and Mishra, S. 1989. Regulatory aspects of cellulase biosynthesis and secretion. (Stewart, G.G. and Russell, I., Eds.) CRC Press Inc. Boca Raton, FL Critical Rev. Biotechnol. 9: 61–103.

2 Emborg, C., Jepsen, P.K. and Biedermann, K. 1989. Two level factorial screening of new plasmid/strain combinations for production of recombinant DNA products. Biotech. Bioeng. 33: 1393–1399.

3 Goksoyr, J. and Eriksen, J. 1980. Cellulases. In: Economic Microbiol. (Rose, A.H., Ed.) Academic Press, NY 5: 283–330.

4 Mandels, M., Hontz, L. and Nystrom, J. 1974. Enzymatic hydrolysis of waste cellulose. Biotechnol. Bioeng. 16: 1471–1493.

5 Nehete, P.N., Shah, V.D. and Kothari, R.M. 1985. Profiles of alkaline protease production as a function of composition of the slant, age, transfer and isolate number and physiological state of the culture. Biotechnol. Lett. 7: 413–418.

6 Pourquie, J. and Desmarquest, J.P. 1989. Scale up of cellulase production by Trichoderma reesei. (Coughlan, M.P., Ed.) Proc. Workshop Prod. Charact. Appl. Cellul. Hemicellul. Lignin Degrading Enzyme Systems, Elsevier, London, U.K., 283–292.

7 Pourquie, J., Warzywoda, M. Chevron, F., Thery, M., Lonchamp, D. and Vandecasteele, J.P. 1988. Scale up of cellulase production and utilization. (Aubert, J.P., Beguin, P. and Millet, J., Eds.) Biochemistry and Genetics of Cellulose Degradation, Academic Press, London, FEMS Symp. 43: 71–86.

8 Ramamurthy, V., Kothari, R.M. and Bhojan, J. 1987. Application of fungal cellulase in improving the milk yield. Biotechnol. Lett. 9: 369–372.

9 Ramamurthy, V., Sinha, S.N. and Kothari, R.M. 1987. Effective and economical enzymatic upgradation of jute. Biotechnol. Lett. 9: 593–596.

10 Shah, D.N., Shah, V.D., Nehete, P.N. and Kothari, R.M. 1986. Isolation of *Bacillus licheniformis* mutant for stable production profiles of alkaline protease. Biotechnol. Lett. 8: 103–106.

11 Shah, D.N., Shah, N.K. and Kothari, R.M. 1987. Isolation of a transglucosidase-nonproducing mutant of *Aspergillus awamorii* yielding improved quality and production of amyloglucosidase preparations. J. Ind. Microbiol. 2: 175–180.

12 Shah, N.K., Shah, D.N., Upadhyay, C.M., Nehete, P.N., Kothari, R.M. and Hegde, M.V. 1989. An economical, upgraded, stabilized and efficient preparation of amyloglucosidase. J. Biotechnol. 10: 267–276.

13 Shah, N.K., Upadhyay, C.M., Nehete, P.N., Kothari, R.M. and Hegde, M.V. 1990. An economical, upgraded, stabilized and efficient preparation of alpha amylase. J. Biotechnol. 16: 97–108.

14 Toyama, N. and Ogawa, K. 1975. In "Symposium on enzymatic hydrolysis of cellulose" (Baily, M., Enari, T.M. and Linko, M., Eds.), SITRA, Aulanko, Finland p. 375–383.