MICOL. NEOTROP. APL. 4 : 83-98, 1991.

SCALE-UP OF CELLULASES PRODUCTION BY *TRICHODERMA HARZIANUM* ON A MIXTURE OF SUGAR CANE BAGASSE AND WHEAT BRAN IN SOLID STATE FERMENTATION SYSTEM

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ESCALAMIENTO DE LA PRODUCCION DE CELULASAS DE *TRICHODERMA HARZIANUM* EN UNA MEZCLA DE BAGAZO DE CAÑA DE AZUCAR Y SALVADO DE TRIGO POR FERMENTACION EN MEDIO SOLIDO

Accepted for publication October 15, 1991

SUMMARY

Studies on the scale-up of an efficiently developed laboratory scale process for production of cellulases by *Trichoderma harzianum* in solid state fermentation system indicated a number of technical and economical considerations. The microbial load of sugar cane bagasse, which has undergone storage in the sugar mill yard as well as the institute premises, was significantly higher than that of the fresh material exiting from the sugar cane crushing section. The use of such highly contaminated substrate, without sterilization, in solid state fermentation resulted in drastic reduction of cellulases production in column fermenter. The combination of sterilization

and pretreatment of the substrate by heat resulted in complete control of contamination during entire fermentation of 68 h and significant increase in the production of the cellulolytic enzymes. The culture produced 204.4 and 16.1 IU of carboxy-methyl cellulase (CMCase) and filter paper activity (FPA) fractions/g substrate dry matter (SDM), under standardized conditions in laboratory scale column fermenter. The scale-up trials in Zymotis (static fermenter), charged with 41.4 kg moist medium and the parallel fermentation in column bioreactor, however, resulted in lower enzyme production. Similar productivities in Zymotis and parallel column fermenter eliminates any doubt about the design and operation of Zymotis. The data analysis indicated that the autoclaving substrate pretreatment at larger scale involved insufficient heat transfers due to the use of 60 cm medium depth as against that of 10 cm at laboratory scale. The employment of trays with about 10 cm medium depth, as is generally practiced in tray fermentation processes, appears to be the most practical and economic solution for overcoming these limitations.

Key words: Scale-up, cellulases, *Trichoderma harzianum*, solid state fermentation, sugar cane bagasse.

RESUMEN

El escalamiento del proceso de producción de celulasas de *Trichoderma harzianum* por fermentación sólida (FS), satisfactorio a nivel de laboratorio, requiere la consideración de diversos puntos técnico-económicos. La carga microbiana del bagazo de caña de azúcar que ha sido almacenado durante mucho tiempo, aumenta significativamente respecto a la del material fresco. La utilización de un substrato contaminado y sin esterilizar en FS, trae como consecuencia una fuerte disminución en la producción de celulasas a nivel laboratorio. El tratamiento térmico del substrato es un control eficaz de la contaminación durante la fermentación (68 h) y favorece el aumento en la producción de celulasas. Bajo condiciones óptimas en el laboratorio, se obtuvo una producción de 204.4 y 16.1 UI de carboxi-metil celulasa (CMCasa) y actividad papel filtro (APF)/g substrato materia seca (SMS), respectivamente. La comparación de los ensayos de escalamiento, usando el Zymotis (fermentador estático) cargado con 41.4 kg de material y las columnas testigo de 20 g realizada en el laboratorio, demostró en ambos casos una disminución en la producción de enzimas. La productividad similar obtenida tanto en el Zymotis como a nivel laboratorio, elimina cualquier duda sobre el diseño y la operación del Zymotis. El análisis de los resultados indicó que el tratamiento térmico fue deficiente a mayor escala, debido al uso de camas de 60 cm de altura en el autoclave, mientras que en los ensayos de laboratorio la altura fue de 10 cm. El uso de charolas con camas de 10 cm de altura, parece ser la vía más práctica y económica para dar solución a los problemas encontrados en este trabajo.

Palabras clave: Escalamiento, celulasas, *Trichoderma harzianum*, fermentación en medio sólido, bagazo de caña de azúcar.

INTRODUCTION

The global shortage of fossil fuel, its non-renewable nature and ever increasing consumption, and the steady increase in the demand of stocks for diverse chemical as well as biotechnological industries (Mandels and Weber, 1969) have prominently focussed the attention of the planners in almost all countries of the world in the last three decades. One of the major alternatives, that has emerged from the thorough analysis of the global situation, is to convert the lignocellulosic byproducts and wastes from agro-industrial ventures as well as the biomass to fuels and feedstuffs (Reese, 1976). The ease in the production of renewable biomass, the abundance of lignocellulosic byproducts/wastes and the potential of these materials in causing environmental pollution also collectively dictate the vital necessity of their efficient utilization. In spite of extensive efforts throughout the world in the last three decades to evolve an efficient process (Enari and Markkanen, 1977; Ghose, 1977; Gong and Tsao, 1979; Mandels, 1982), the hydrolysis of these substrates by cellulases has not yet been industrialized mainly due to the prohibitive cost of the enzyme (Perez *et al.*, 1980; Toyama, 1976).

A thorough analysis of the literature data with a relook at the whole situation indicated the possibility of reducing the cost of the enzyme by various means and by adopting a solid state fermentation (SSF) system. The renewed efforts were therefore initiated at ORSTOM in the last decade (Roussos *et al.*, 1991a). Different problematic aspects of the fermentation process were investigated with a view to develop industrially feasible technology for production of cellulases. These include: 1) screening of a large number of cultures and selection of *Trichoderma harzianum* CCM F-470, 2) study of its physiology, 3) standardization of fermentation parameters, 4) comparison of submerged fermentation and SSF processes, 5) attempts to carry out the fermentation in non-aseptic conditions, 6) evaluation of various substrates, 7) kinetic studies of the changes in fermentation parameters as well as of the enzyme biosynthesis, 8) importance of different ratios of cellulolytic activities, 9) evolution of a simple substrate pretreatment method for the substrates, 10) development of strategy for large inoculum production, 11) evolving of the most efficient technique for recovery of the product from

fermented solids, 12) evaluation of the process in static as well as agitated bioreactors, and 13) efficient management of the spent solids resulting from the process (Deschamps *et al.*, 1985; Roussos, 1981, 1987; Roussos and Hannibal, 1984; Roussos and Raimbault, 1982; Roussos and Sedha, 1983; Roussos *et al.*, 1985a, b, c; Roussos *et al.*, 1991a, b, c; Vidaud, 1980; Vidaud *et al.*, 1982).

The standardized laboratory scale process that has emerged from these efforts consists of (1) autoclaving of the substrate at 121°C for 20 min after moistening to 50% level by mineral salt solution, (2) inoculum preparation and use of conidia at a rate of 3×10^7 / g substrate dry matter (SDM), (3) initial moisture adjustment to 70-72%, (4) aerobic fermentation with circulation of humidified air for 48 h, (5) pressure leaching of cellulases, (6) product concentration by vacuum evaporation, and (7) ensiling of the spent solids for feed purposes. It is essential to determine the feasibility of the laboratory scale process for exploitation at large scale and to obtain data for its economic evaluation. The scale-up trials were, therefore, undertaken and the results are reported in the present communication.

MATERIALS AND METHODS

Microorganism

Trichoderma harzianum Rifai, strain CCM F-470 (CCM= Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia) was employed. The methodology for its preservation and maintenance was as described earlier (Roussos *et al.*, 1991a).

Microbiology of sugar cane bagasse

Samples of sugar cane bagasse as it exits from a sugar mill and after storage in the sugar mill yard were collected aseptically for subjecting to the determination of microbial loads. Total bacteria, total fungi and cellulolytic fungi present on the substrate were estimated by standard methods (Roussos, 1987).

Pretreatment of the substrate

A mixture of sugar cane bagasse and wheat bran at a ratio of 80:20 was moistened with a mineral-salt medium to 50% moisture (Roussos *et al.*, 1991a). One part of this was used as such, without any pretreatment or

autoclaving, in column fermenter, while another part was transferred in 100 g moist weight quantity in a beaker for pretreatment autoclaving at 121°C for 20 min and fermentation in the column.The fermentation parameters used include pH 4.5, moisture content of the medium at 67% and fermentation at 25°C. The sugar cane bagasse was chopped to a particle size of 1-5 mm before using in the medium preparation in all cases. The autoclaving pretreatment of the medium at larger scale was done by transferring 6 kg wet quantity of the medium in cloth sacs for autoclaving at 121°C for 60 min.

Inoculum preparation and inoculation

The composition of the inoculum preparation medium, the methodology for large scale inoculum preparation in disk fermenter D2, the procedure for harvesting spores from agar surfaces and the design, as well as operation of the inoculum fermenter have been as described elsewhere (Roussos *et al.*, 1991c). The moist solids, with or without pretreatment, were mixed thoroughly at 30°C with the conidiospore suspension to provide 3 x 10^7 spores / g SDM. The moisture content of the medium was raised to 74% by using tap water before charging the medium in desired quantities in the fermenter. All subsequent operations, in all cases, were in non-aseptic conditions.

Cellulases production at laboratory scale

The column fermenter of size 210 mm lenght x 22 mm diameter with capacity to hold 18 g moist medium was used. The fermentation was carried out under optimized parameters, i.e, 74% moisture content of the medium, 5.8 initial pH and 28±1°C temperature. Humidified air was passed continuously through the medium at a rate of 4 L h^{-1} per column throughout the fermentation period of 68 h. The design and control system of the column fermenter has been previously described (Raimbault and Alazard, 1980).

Scale-up in Zymotis

The fermentation in Zymotis was carried out under the optimized conditions as reported above for laboratory scale process. The unit was charged with 12 kg SDM (41.4 kg moist medium) and the column fermenter was also run in parallel. The design and control system of Zymotis has been reported in an earlier work (González-Blanco *et al.*, 1990).

Downstream processing

The fermented solids, at the end of fermentation, were removed from the bioreactor and mixed thoroughly for uniformity as per the methodology

described earlier (Roussos *et al.*, 1991a). The procedure for recovery of the enzyme from the fermented solids in case of column and Zymotis bioreactors has been described elsewhere (Roussos *et al.*, 1991a, b).

Analytical methods

The production of Carboxy-Methyl Cellulase (CMCase) and Filter Paper Activity (FPA) fractions of the cellulolytic enzymes, moisture (%) and pH of the media were determined in the samples collected at intervals during entire period of fermentation. The details of the analytical methods used have been previously described (Roussos *et al.*, 1991a). The microbial counts of the samples of sugar cane bagasse were determined by using standard procedures (Roussos, 1987).

RESULTS AND DISCUSSION

Microbiology of sugar cane bagasse

The microbial loads on fresh bagasse and after storage for 15 days in the normal conditions of storage in the sugar mill yard are presented in Table 1. The data indicate tremendous increase in the microbial population in the bagasse stored for 15 days. For example, the total bacterial, total fungal and cellulolytic fungal counts were about 715,917 and 2218 times over those of the fresh bagasse. Such high microbial counts will be disastrous in the fermentation process without sterilization. It is, therefore, essential to sterilize the substrate before using it in the fermentation process.

Table 1. Microbial load on sugar cane bagasse.

Sample	Moisture (%)	Microbial load / g material		
		Total bacteria	Total fungi	Cellulolytic fungi
Immediately as it exits from sugar mill	44.4	2.63×10^6	1.08×10^4	2.66×10^3
After 15 days storage in sugar mill yard	59.6	1.88×10^9	9.90×10^6	5.90×10^6

The necessity for pretreating the lignocellulosic residues and wastes to improve the accessibility of cellulose to microbial attack has been well established (Tanaka and Matsuno, 1985). A number of different physical and chemical pretreatments, either individually or in combination, have been developed and include ball milling, compression milling, grinding, cryomilling, gamma ray dosage, microwave irradiation, steam explosion, rapid depressurization and autohydrolysis by various chemicals such as acids, alkalies, solvents, gaseous ozone, etc. (Tanaka and Matsuno, 1985). Most of these pretreatment methods are impractical at larger scale and highly cost-intensive due to various reasons, such as longer pretreatment time, high energy requirement, need for using specific equipments or machinery and occurrence of undesirable side reactions.

The possibility of combining sterilization of the substrate and its pretreatment was, therefore, conceived in the present studies. The moist solids with 50% moisture were transferred in 100 g moist weight quantity in beaker for autoclaving at 121°C for 20 min.

Results of the comparative enzyme production of untreated and pretreated substrates in column fermenters are shown in Table 2. The CMCase and FPA

Table 2. Comparative production of the enzyme in column fermenter by *Trichoderma harzianum* on treated and untreated substrates (the enzyme titres are expressed as IU/g SDM).

Fermentation time (h)	Medium (pH)		CMCase production		FPA production	
	A	B	A	B	A	B
0	6.2	5.4	1.1	0	1.1	0
6	6.2	5.4	0.7	0	1.1	0
21	4.8	5.3	0.1	0	2.8	0
30	5.9	4.2	45.9	31.6	7.1	3.4
44	7.4	5.1	35.4	124.6	5.4	10.9
48	7.7	5.1	14.1	125.8	3.5	12.8
67	8.3	5.5	7.6	12.4	2.8	11.2

A= Untreated substrate.
B= Treated substrate.

fractions were present from 0-21 h in the medium without any pretreatment probably due to their presence in the substrate used for fermentation. These might have been formed during the storage of sugar cane bagasse in the sugar mill and institute premises before employing it for fermentation in the present studies. These enzymes, in contrast, were totally absent up to 21 h in the medium based on pretreated substrate. Obviously, the enzymes initially present on the substrate were destroyed during autoclaving of the moist medium. The enzyme production beyond 21 h was, however, at faster rate in both media. The peaks in enzyme production were achieved at 30 h in untreated medium as compared to those at 48 h in the pretreated medium. However, peak values of the enzymes in case of pretreated substrate were higher by about 2.7 and 1.8 times as compared to those on the untreated substrate. These values at 48 h fermentation were higher by about 8.9 and 3.7 times in case of the pretreated substrate.

Microscopic examination of samples from both fermentations, involving untreated and pretreated substrates, revealed extensive contamination by bacteria and yeast in the former case right from the beginning of the fermentation. The growth rate of some of these contaminants, especially the bacterial cultures, was much faster than that of *T. harzianum* as the samples from latter phases of fermentation showed many bacterial cells and very few fungal mycelia or spores. Moreover, the mycelial cells of *T. harzianum* were noticed to be lysed probably by the contaminants or their products. A contamination of the order of 10^{11} cells/g SDM of bacteria and yeast was also recorded by Pepe (1984), when sugar beet pulp was used without any autoclaving in large fermenter for protein upgradation.

The changes in the pH of the media, based on the use of untreated and pretreated substrates, showed interesting pattern (Table 2). The pH dropped to 5.9 by 30 h and then increased to 8.3 by 67 h fermentation in case of untreated substrate. The general trend in pH drop and rise was similar in the medium based on pretreated substrate but the values were 4.2 and 5.5 at 30 and 67 h, respectively. It is interesting to note that the initial pH was much higher in case of the medium involving untreated substrate as compared to that of the medium based on pretreated substrate.

A higher production of enzymes by the culture in the medium based on pretreated substrate indicates that the heat treatment of the substrate in moist condition modifies it physically for imparting better accessibility of the

cellulose to microbial attack. Consequently, the substrates become more amenable to microbial growth and leads to an improved production of enzymes. The particle size reduction due to chopping of the bagasse might also have resulted in exposing larger surface area of the substrate to heat action, and thus is partially responsible in making the substrate more accessible to the microorganism.

The pretreatment of the substrate also leads to many other advantages. For example, it is found to be efficient in killing a larger microflora present naturally on the substrate. No contamination of the medium by bacteria, yeast and other fungi was observed during the entire course of fermentation in the cases when the medium is based on pretreated substrate, in spite of the use of non-aseptic conditions beyond the moist medium autoclaving stage. The contamination control was probably aided by the use of a large inoculum (3×10^7 spores/g SDM), which probably allowed preferential growth of *Trichoderma harzianum* and imparted it the status of dominance. Other beneficial changes due to pretreatment of the substrate are: 1) reduction in crystallinity of the cellulose due to formation of amorphous celluloses, 2) gelatinization of starch present in the substrate, 3) swelling of the substrate, 4) hydration of the substrate, 5) homogeneous distribution of mineral-salt media and a horde of other benefits (Tanaka and Matsuno, 1985).

Typical fermentation data at laboratory scale

The production of cellulases by *T. harzianum* in pretreated moist medium in laboratory scale column fermenter of 18 g working capacity under standardized parameters indicated that the maximum enzyme production was achieved at 48 h (Table 3). The peak values of enzymes were 204.4 and 16.1 IU/g SDM of CMCase and FPA fractions, respectively, thereby leading to the ratio of 1:0.08 at 48 h. These values are much higher than those in the pretreated moist medium under non-standardized parameters (Table 2), thereby indicating the efficacy of parameter standardization performed in this process (Roussos, 1987). The continuation of the fermentation under standardized parameters beyond 48 h, however, resulted in a reduction in the titres of the enzymes, which was more drastic in case of FPA fraction. The moisture content of the medium during the course of fermentation ranged between 68.3-73.9% (Table 3). The pH of the medium decreased gradually in the initial 28 h fermentation from the initial value of 5.8 to 4.5. In the subsequent fermentation period, it started increasing and reached the value of 6.3 at 48 h. This confirms the trend of pH changes during growth and

Table 3. A typical fermentation data on the production of cellulases by *Trichoderma harzianum* at laboratory scale in column fermenter under standardized parameters.

Atribute	Unit	Value
Peak value in CMCase	IU/g SDM	204.4
Peak value in FPA	IU/g SDM	16.1
CMCase: FPA at peak level	ratio	1:0.08
Peak enzyme production time	h	48
Range of moisture content of the medium during fermentation	%	68.3-73.9
Lowest pH in growth phase (at 28 h)	-	4.5
Highest pH during enzyme synthesis and liberation (at 48 h)	-	6.3
Contamination during fermentation	-	absent

enzyme production phases and its utility in monitoring the fermentation as stressed earlier (Roussos *et al.*, 1991a). The microscopic examination of the fermenting solids at different intervals during the entire fermentation period has not revealed any contamination by bacteria, yeast and fungi other than *T. harzianum*. The growth of the culture was found to be uniform throughout the solid mass in the fermenter.

The higher production of the enzymes at 48 h without the need for maintaining aseptic conditions during fermentation, the ratio of CMCase: FPA fractions at 1:0.08, an absence of any contamination during the fermentation due to a combination of substrate pretreatment with autoclaving of

the medium, the cheapness of the substrate and the homogeneous growth of the culture in the medium (probably due to uniform distribution of the spore inoculum during inoculation) collectively indicate the high potential of the system for economic exploitation at industrial scale. Hence, the scale-up trials were undertaken.

Scale-up in Zymotis

Data on the production of cellulases in Zymotis charged with 41.4 kg moist medium and in laboratory scale column fermenter run in parallel, along with the changes in pH as well as moisture (%) of the media are presented in Table 4. The titres of CMCase and FPA fractions reached peak values at 48 h in both fermenters, except for that of CMCase in Zymotis at 46 h. The production of both these components of the cellulolytic enzyme were initiated at 30 h and their accumulation increased steadily till the peak values were attained. The ratios of CMCase and FPA fractions at peak levels were 1:0.075 and 1:0.077 in Zymotis and parallel column fermenter, respectively. The ratio increased to 1:0.11 at 48 h in Zymotis due to increase in FPA production between 46-48 h but no change in CMCase titre. The production of both of these enzymic fractions was higher and also at a faster rate in Zymotis as

Table 4. Large scale production of cellulases by *Trichoderma harzianum* in Zymotis and laboratory scale column fermenter run in parallel. The enzyme titres are expressed as IU/g SDM.

Fermentation (h)	Moisture content of the medium (%)		pH of the medium		CMCase production		FPA production	
	A	B	A	B	A	B	A	B
0	71.0	71.3	5.5	5.6	0	0	0	0
10	72.6	70.5	5.6	5.5	0	0	0	0
22	71.5	70.4	4.8	5.3	0	0	0	0
26	71.4	71.5	4.2	4.7	0	0	0	0
30	72.4	71.6	4.5	4.4	9.3	3.1	1.2	0.8
34	72.6	72.1	5.0	4.7	29.9	15.1	3.4	3.2
46	73.3	73.1	5.7	5.6	74.1	62.2	5.6	4.4
48	72.7	76.6	5.8	5.8	74.2	71.8	8.1	5.5

A= Zymotis.
B= Laboratory scale column fermenter.

compared to those in the parallel column fermenter (Table 4), thereby indicating that the conditions were more favorable at the large scale than those at smaller scale fermentation. Similar results were also reported earlier for other products in SSF system (Lonsane *et al.*, 1991; Saucedo-Castañeda *et al.*, 1991).

Moisture content of the medium during entire period of fermentation was similar in both fermenters and ranged between 71.0-73.3 and 70.4-73.1 % in Zymotis and parallel column fermenter, respectively (Table 4). The pH of the medium at the start and also at the end of fermentation was the same in both of these fermenters. However, the drop in pH during the initial growth phase and the increase in pH during the subsequent enzyme production phase were faster in Zymotis (Table 4). This probably explains the faster rate of enzyme production, as well as its accumulation in Zymotis as compared to those in parallel column fermenter.

Comparison of enzyme production at laboratory and large scales
Production of CMCase and FPA fractions at larger scale in Zymotis was 36.28 and 50.03% of those produced by the culture in column fermenter at laboratory scale under standardized parameters (Tables 3-4). The production of such a low enzyme at larger scale in Zymotis indicates some deficiency in the larger scale fermentation.

The close similarity in the profiles of fermentation parameters, such as moisture content and pH of the medium, in the laboratory scale column fermentation under standardized parameters and Zymotis rules out the possibility of any role played by fermentation parameters in giving lower yields at larger scale. The temperature, medium composition, inoculum quality, inoculum ratio were also similar in both cases. The deficiency in the performance of Zymotis or its design features in obtaining lower enzymes at larger scale is also ruled out as the production of the enzyme in Zymotis and column fermenter, which was run in parallel to Zymotis, were also 35.15 and 34.27%, respectively, as compared to those in the laboratory scale column fermentation under standardized parameters (Tables 3-4). In addition, the enzyme titres in Zymotis and the parallel column fermenter were also similar (Table 4).

The analysis of the whole process and process methodology indicate that the only difference between laboratory scale column fermentation in Zymotis

and the parallel fermentation in column was the change in the substrate pretreatment method. The substrate pretreatment was carried out by charging the moist medium in 100 g quantity in a beaker for autoclaving at 121°C for 20 min in case of laboratory scale column fermentation under standardized parameters, in contrast to the use of 6 kg moist solid medium in cylindrical aluminium vessel at 121°C for 60 min. The depth of the medium in the aluminium vessel during autoclaving was much higher (60 cm) as compared to that in the beaker (10 cm) with the use of 100 g moist medium in the laboratory scale process. In fact, it is for this reason that the autoclaving time was extended from 20 to 60 min in the larger scale process for giving more time for heat transfer.

The results, however, indicate that the heat transfer during autoclaving at larger scale is less than that achieved at laboratory scale. Probably the temperature achieved at the centre of the moist medium held in the cloth sac during autoclaving at large scale was less than 121°C or the heating of each particle of the medium was not for 20 min at 121°C. The autoclaving of the medium has also been specified as problematic unit operation during scale-up of submerged fermentation processes (Bank, 1984). It is felt that the same productivity would be possible to achieve at larger scale either by increasing the autoclaving temperature or time. Both of these approaches are, however, energy and cost intensive. Use of perforated aluminium trays for substrate autoclaving pretreatment and the bed depth of about 10 cm, as generally employed in tray fermentation processes (Lonsane *et al.*, 1985; Ghildyal *et al.*, 1981), may provide a simple and economic approach to overcome the problem.

ACKNOWLEDGEMENTS

The authors thank Mme. Laure Hannibal and Mme. Nathalie Pujet for the technical assistance. B.K.L. is thankful to Council of Scientific and Industrial Research, New Delhi, India, for deputation terms and ORSTOM, Centre Montpellier, for excellent facilities as well as the associateship.

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