SUGARCANE USED IN SOLID STATE FERMENTATION FOR CELLULASES PRODUCTION

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Cellulases production and applications

Cellulases, the enzymes capable of hydrolyzing cellulosic compounds, find extensive use in extraction of green-tea components, modification of food tissues, removal of soybean seed coat, improving cattle feed quality, recovering juice as well as other products from plant tissues and as component of digestive aid (Toyama, 1969; Lonsane and Ghildyal,1991). Cellulases can be produced by submerged or solid state fermentations.

The latter technique is generally preferred as it offers many advantages such as two-three times higher enzyme production as well as protein rate, higher concentration of the product in the medium, direct use of air-dried fermented solids as source of enzyme which lead to elimination of expenses on downstream processing, employment of natural cellulosic wastes as substrate in contrast to the necessity of using pure cellulose in submerged fermentation (SmF) and the posibility of carrying out fermentation in nonaseptic conditions (Chahal 1983; Toyama and Ogawa, 1978; Pamment *et al.*, 1978; Deschamps *et al.*, 1985; Allen, 1983; Sternberg, 1976). The biosynthesis of cellulases in SmF process is strongly affected by catabolic and end product repressions (Gallo *et al.*, 1978; Ryu and Mandels, 1980) and the recent reports on the overcoming of these repressions to significant extent in solid state fermentation (SSF) system (Ramesh and Lonsane, 1991 a, b), therefore, are of economic importance.

The amenability of SSF technique to use upto 20-30% substrate, in contrast to the maximum of 5% in SmF process, has been documented (Pamment *et al.*,1978). It is, therefore, not surprising that cellulases to the extent of 45 tonnes/annum and worth about 170 $x10^6$ yen were produced by SSF in Japan as early as in 1975-76 (Yamada,1977). About 7.2 tonnes of cellulases were exported to West Germany and Australia from Japan in 1967-68 (Toyama,1969), thereby indicating its leading status in cellulase production by SSF system.

An important potential application of cellulases is in the production of glucose, ethanol, high fructose syrup and other feedstocks from agro-industrial cellulosic residues and wastes (Emerts and Katzen, 1980; Lonsane and Ramakrishna, 1989). Extensive R&D efforts have been put up in last 20 years to produce the enzymes by microbial fermentations and thousands of publications as well as patents are available on the production and application of the cellulases (Ryu and Mandels, 1980; Mandels, 1982; Frost and Moss, 1987). However, no commercial exploitation has emanated from these efforts because of high cost of cellulases even when these are produced by SSF system. For example, the cost of the enzyme was shown to comprise nearly 50% of the outlay required to produce sugar from corn stover (Perez *et al.*1980).

Lignocellulosic residues/wastes solid substrat

The agro-industrial lignocellulosic residues/wastes form a most important renewable reservoir of carbon for a variety of vitally important chemical feedstocks and fuel in the overall economy of any country. Their unlimited availability and environmental pollution potential, if not disposed-off properly, dictate renewed efforts for their efficient and economic utilization. It is well known that the selection of appropriate and highly potent microorganism, use of cheaper and efficient substrate, selection of bioreactor, employment of standardized process parameters, characteristics of the enzyme produced, extent of downstream processing as well as waste treatment, inoculum development technique, degree of colonization of the substrate, and efficiency of each unit operations of the process are of vital importance in determining the economics of the process (Kumar and Lonsane, 1989; Ramesh and Lonsane, 1990; Mitchell and Lonsane, 1991; Roussos *et al.* 1991 a,b,c; Lonsane and Krishnaiah, 1991).

Criteria for cellulolytic microorganisms selection

Efforts were, therefore, initiated for screening of potent and most appropriate microorganisms for cellulase production in SSF system. A large number of cultures from various fungal genera and species were screened and *Trichoderma harzianum* CCM F-470 was selected based on four different criteria, i.e., rapid apical growth which leads to higher degree of colonization of the substrate, good sporulation capability which is vital for uniform distribution of the culture in the moist solid medium during inoculation , rapid growth of the culture which facilitates fermentation in non-aseptic conditions and higher enzyme production ability (Roussos and Raimbault, 1982). The kinetics and the ratios of two different cellulosic enzyme activities on various solid substrates in column fermenter were investigated in the continuation of these renewed efforts. These results are reported in the present communication due to their importance in effecting economy in the enzyme production cost. The data also allow the production of tailor-made activities of different cellulosic enzymes which may prove useful in efficient hydrolysis of different lignocellulosic materials.

Natural Microflora of sugarcane 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 use in the fermentation process.

The pretreatment of the substrate also leads to many 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, yeasts 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 large inoculum (3×10^7 spores / g SDM) which probably allowed preferrential growth of *T. 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).

Table 1. Natural microflora of sugarcane bagasse.

Sample	Moisture %	Microbial load / g material		
		Total bacteria	Total fungi	Cellulolytic fungi
Immediately as it exit 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

Untreated and pretreated substrates

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, cryomiling, 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 occurance 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.

The results of the comparative enzyme production on untreated and pretreated substrates in column fermenters are depicted in table 2. The CMCase and FPA fractions were present right 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 sugarcane bagasse in the sugar mill and institute premises before employing it for fermentation it the present studies. These enzymes, in contrast, were totally absent upto 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 the 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, the peak values of the enzymes in case of pretreated substrate were higher by about 2.7 and

Table 2. Comparative production of cellulases in column fermenter by *T. harzianum* on treated and untreated substrates

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.
The enzyme titres are expressed as IU / g SDM.

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

Attribute	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

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.

The microscopic examination of the samples from both the fermentations, involving untreated and pretreated substrates, revealed extensive contamination by bacteria and yeast in the former case right from the begining 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. The contamination of the order of 10^{11} cells of bacteria and yeast was also recorded by Pepe (1984) when sugar beet cosset 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.

Many times higher production of the 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 substrate becomes more amenable to microbial growth and leads to improved production of the 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.

Sugarcane bagasse alone as substrate for *T.harzianum* growth

The manufacture of sucrose from cane sugar in the tropical countries results in the generation of large quantity of bagasse which are generally used as fuel in the sugar mill. In another usage, the bagasses are depithed are the fibres thus obtained are used in the manufacture of paper. The sugarcane bagasse forms an excellent substrate in SSF processes.

The results of the growth and metabolism of *T.harzianum* on bagasse in column fermenter for 64 h under SSF system revealed that the conidiospores started germinating at about 10 h and the spore germination was 100 % by 20 h. The mycelial cells enveloped the substrate particles more or less fully by about 30 h. The moisture content of the medium during the course of fermentation was quite stable and ranged between 70.5 - 72.9 % (Fig. 3). Similar was the case for the kinetics of pH changes which were in the range of 5.9 - 6.3 during first 52 h fermentation. This is the fermentation period which led to maximum enzyme titres. Further continuation of fermentation beyond 52 h resulted in increasing the pH of the medium to 6.9 at 58 and 64 h fermentation.

The data on the production of CMCase and FPA fractions indicated that no CMCase was produced upto 24 h, in contrast to the production of FPA at slower rate right from the start of fermentation (Fig. 4). The rates of production of these enzymes were, however, faster between 24 to 44 h and about 80 % of the total enzyme was formed during this period. The peaks in enzyme titres were achieved ar 52 h for both the enzyme and their levels decreased if

Table 4. Large scale production of cellulases by *T.harzianum* in Zymotis and laboratory scale column fermenter run in parallel

Fermentation time, 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. The enzyme titres are expressed as IU/g SDM

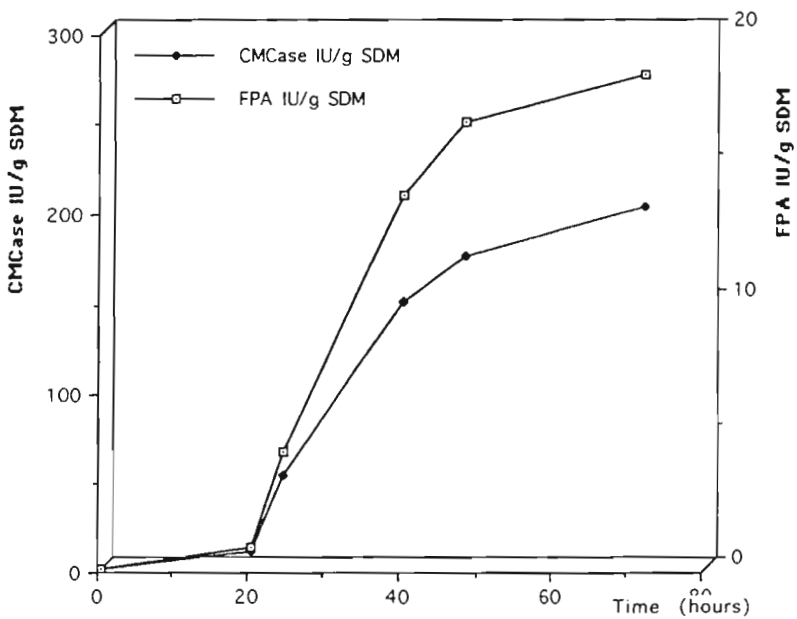

Figure 4. CMCase and FPA cellulase production by *T. harzianum*

the fermentation was continued further (Fig. 4). The ratios of CMCase and FPA ranged between 8.3-10.0 during 44-52 h fermentation.

Combination of bagasse and wheat bran as substrate

The use of sugarcane bagasse and wheat bran at the ratio of 80 : 20 in the column fermenter and under the fermentation parameters such as 20 g medium in the column, 72 % initial moisture content, 28 ± 1°C incubation temperature and aeration at 5 l air / h / column in the production of cellulolytic enzymes by *T. harzianum* showed entirely different patterns in the enzyme synthesis during initial period of fermentation. The CMCase and FPA fractions were not formed upto 20 h fermentation. Subsequently, the rate of production of these enzyme activities was at faster rate between 28-48 h (Fig. 6). The peak in enzyme titres was attained at 48 h in both the cases. The ratios of these activities were about 6 upto 28 h but these changed to 11.0-12.3 in the subsequent period.
The kinetics of moisture content of the medium showed that it increased gradually during the entire course of fermentation and was 4-5 % higher at the end of fermentation as compared to the initial value at 0 h (Fig. 7). The kinetics of the pH changes are interesting. The pH decreased sharply from 5.8 to 4.8 between 12-28 h, the period which involves active growth of the culture. The bagasse appears to have strong buffering action as the pH was not reduced to a value less than 4.8. Subsequently, the pH increased to 6.3 between 28-65 h fermentation, the period which corresponds to enzyme biosynthesis and its release in the medium. In contrast, the pH was nearly stable at the initial value of 5.8 during 0-12 h, the period which involves the germination of the conidiospores. The data indicate that there is no need for pH control during the fermentation as the above kinetic changes in the pH seems to be helpful.

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 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 enzyme production phases and its utility in monitoring the fermentation as stressed earlier (Roussos *et al.*, 1991 a). 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 ar 1 : 0.08, an absence of any contamination during the fermentation due to 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

The 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 were at peak values at 48 h in both the fermenters except for that of CMCase in Zymotis at 46 h. The production of both these components of the cellulolytic enzyme were initiated ar 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 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-Castaneda *et al.*, 1991).

The moisture content of the medium during entire period of fermentation was similar in both the 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 same in both 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

The 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 and 4). The production of such low enzyme at larger scale in Zymotis indicates some lacunae or deficiency at 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 parameter and Zymotis rules out the possibility of any role played by fermentation parameters in giving lower yields at larger scale. The temperature, medium compositions, inoculum quality, inoculum ratio are also similar in both the 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 % as compared to those in the laboratory scale column fermentation under standardized parameters (Table 3 and 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 is 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 achived 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 pretreatment-cum-autoclaving 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.

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