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KINETICS AND RATIOS OF CARBOXY-METHYL CELLULASE AND FILTER PAPER ACTIVITIES OF THE CELLULOLYTIC ENZYMES PRODUCED BY *TRICHODERMA HARZIANUM* ON DIFFERENT SUBSTRATES IN SOLID STATE FERMENTATION

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CINETICA Y RELACIONES DE LAS ACTIVIDADES CARBOXI-METIL CELULASA Y PAPEL FILTRO PRODUCIDAS POR *TRICHODERMA HARZIANUM* CULTIVADO EN MEDIO SOLIDO SOBRE DIFERENTES SUBSTRATOS

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SUMMARY

Studies on the production of cellulolytic enzymes by *Trichoderma harzianum* on different substrates in a solid state fermentation system revealed that the highest enzyme activity was produced in the shortest time on a mixture of sugar cane bagasse and wheat bran at 80:20 (w/w) ratio. These substrates, when used individually, produced less enzyme activity and required longer fermentation time. The data revealed the possibility of obtaining tailor-made ratios of carboxy-methyl cellulase (CMCase) and filter paper activities (FPA) by using a specific substrate and stopping

the fermentation at appropriate time. The relationship between pH changes of the medium and growth pattern as well as enzyme production could also be used as a yard-stick for monitoring the course of fermentation and the extent of enzyme biosynthesis. The use of sugar beet pulp and wheat bran resulted in poor enzyme production but the protein content of the medium was higher. Therefore, it may be economical to exploit these substrates for protein enrichment as the culture was found to preferentially utilize simple carbohydrates over the proteins as carbon source. The data on the whole and the literature reports on diverse metabolic production by *T. harzianum* indicate that it may prove to be an excellent fungus for commercial exploitation with socioeconomic benefits.

Key words: Cellulases production, solid state fermentation, *Trichoderma harzianum*, sugar cane bagasse, wheat bran, sugar beet pulp.

RESUMEN

En este estudio se presenta la producción de celulasas del hongo filamentoso *Trichoderma harzianum*, cultivado en diferentes substratos celulósicos por fermentación sólida. Los resultados indicaron que la producción más alta de celulasas se obtuvo empleando bagazo de caña de azúcar mezclado con salvado de trigo en una proporción 80:20 (p/p). Cuando se utilizaron ambos substratos individualmente, se produjeron menos celulasas y se requirió más tiempo de fermentación. Por otro lado, los resultados demostraron la posibilidad de obtener diferentes relaciones de actividades carboxi-metil celulasa (CMCasa) y papel filtro (APF), utilizando substratos específicos y deteniendo la fermentación a diversos tiempos. La variación del pH y la producción de enzimas estuvieron bien correlacionadas. El pH resultó ser un excelente parámetro para seguir la biosíntesis de celulasas durante la fermentación. El uso del bagazo agotado de remolacha y del salvado de trigo como substratos para el crecimiento de *T. harzianum* reveló, para ambos casos, que la producción de celulasas fue baja, mientras que la producción de proteínas fue bastante elevada. Por esta razón, desde el punto de vista económico, sería más atractivo explotar estos substratos preferentemente para su enriquecimiento proteico con *T. harzianum*. Contar con este tipo de información sobre el uso de diferentes substratos lignocelulósicos para el desarrollo de este hongo es de sumo interés, ya que *T. harzianum* puede también producir enzimas, antibióticos y otros metabolitos de interés comercial.

Palabras clave: Producción de celulasas, fermentación sólida, *Trichoderma harzianum*, bagazo de caña de azúcar, salvado de trigo, bagazo de remolacha.

INTRODUCTION

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 enzymes 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 possibility of carrying out fermentation in non-aseptic 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, 1991a, b), therefore, are of economic importance. The amenability of SSF technique to use up to 20-30% substrate in contrast to the maximum of 5% in SmF process, has been documented (Pamment *et al.*, 1978). It is, accordingly, not surprising that cellulases, to the extent of 45 tonnes/annum and worth about 170 x 10^6 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 (Emert 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 stubble (Pérez *et al.*, 1980). The agro-industrial lignocellulosic residues/wastes form the 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 an 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; Lonsane and Ramesh, 1990; Mitchell and Lonsane, 1991; Roussos *et al.*, 1991 a,b,c; Lonsane and Krishnaiah, 1991).

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* (strain 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.

MATERIALS AND METHODS

Microorganism

Trichoderma harzianum Rifai, strain CCM F-470, was obtained from the

Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia. It was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured once in every four months (Roussos *et al.*, 1991 a,c).

Inoculum preparation

The spores from the freshly grown culture on PDA slants (28±1°C, 6 days) were suspended in 0.01% sterile solution of Tween 80 by adding 10 ml solution to the slant. The spores were scrapped from the surface of the agar aseptically by means of the inoculation loop, and 1 ml suspension was used to inoculate 20 ml of cassava flour agar medium in Erlenmeyer flask.

The cassava flour medium contained (g/L): cassava flour 40, KH_2PO_4 2, $(NH_4)_2SO_4$ 4, urea 1, $CaCl_2$ 1, agar 15 and distilled water 1000 ml. The pH of the medium was adjusted to 5.6 using 5N HCl. The medium was heated on the boiling water for solubilizing the agar and 20 ml of the medium was transferred to 250 ml capacity Erlenmeyer flask. After cotton plugging, the flasks were autoclaved at 121°C for 20 min, and the medium was allowed to cool to about 45°C before inoculating it in the liquid state with 1 ml spore suspension obtained as described above. The content was mixed thoroughly and allowed to solidify undisturbed. The flasks were incubated at 28±1°C for 6 days to obtain heavy sporulation on the surface of the agar. The spores were harvested from the agar surface by adding 100 ml sterile water (containing 3 drops of Tween 80) to the flask and keeping it on magnetic stirrer, using a triangular iron bar to scrap the surface. The resulting dark green coloured spore suspension was removed from the flask aseptically, diluted suitably to determine the spore count by haemocytometer and used as inoculum for the column fermenter. The detailed methodology has been previously reported (Roussos *et al.*, 1991c).

Solid substrates

Four different substrates, either individually or in combination, were employed. These include a mixture of wheat straw and wheat bran or sugar cane bagasse and wheat bran at 80:20 ratio, as well as the individual use of the wheat bran, sugar beet pulp and sugar cane bagasse. These substrates were obtained from the local market or local industries. In all the cases, the moist solid medium involving the desired solid substrate, either alone or in combination, was prepared by adding the mineral salt medium to get 50% moisture content and thorough mixing. The mineral salt medium used contained (g/100 g initial dry matter): $(NH_4)_2SO_4$ 9.7, urea 2.4, KH_2PO_4 5.0 and

tap water 117. The sterilization method consists of transferring 100 g moist medium (50% moisture) to 500 ml capacity beaker, covering it by aluminium foil and autoclaving at 121°C for 20 min.

Solid state fermentation technique
The autoclaved medium was cooled to about 35°C and inoculated with the spore suspension at a rate of 3 x 10^7 spores/g substrate dry matter (SDM) initially present in the medium. Additional sterile water was added to achieve 75% moisture content in the medium. After thorough mixing, the content was transferred in 18 g wet weight quantity in the column fermenter of the size 210 mm length x 22 mm diameter, with gentle packing. The fermentation was carried out at 28±1°C and the medium was aerated at a rate of 5 L humidified air $h^{-1}column^{-1}$. The design and control system of the column fermenter have been previously described in details (Raimbault and Alazard, 1980).

Downstream processing
The fermented solids at the end of fermentation, were removed from the column and the moist weight was taken. The fermented solids were then mixed thoroughly with spatula and 2.5 g portion was dried in the oven at 105°C for 24 h to obtain dry matter (%). 100 ml water was added to another portion of 2.5 g well mixed fermented solids, mixed for 2 min in Ultra-Turrax (Janke & Kunkel, Germany) at 20,000 rotations/min and the resulting extract was separated. The pH of the extract was measured. The extract and its appropriate dilutions were homogenized before using in analytical determination.

Analytical methods
The reducing sugars were estimated by DNS method (Miller, 1959) while the anthrone method (Dubois *et al.*, 1956) was used for determination of the total carbohydrates. In both cases, glucose was used as standard. The proteins were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The fermenting solids were examined under microscope to study the morphology of the culture at different fermentation times.

The CMCase (1,4-β–D-glucan 4-glucanohydrolase; endo 1,4-β–D glucanase; EC 3.2.1.4) activity towards carboxymethyl-cellulose and the FPA (1,4-β–D glucan cellobiohydrolase; exo-1-4-β–D glucanase; EC 3.2.1.91) activity towards Whatman filter paper No. 1 (1 x 5 cm, 50 mg) were determined as

per the methodology reported by Mandels *et al.* (1976). The enzyme concentration is expressed as International Units (IU), which denotes the micromoles of glucose released per min of the reaction. The enzymes titres are calculated for 1 g SDM.

RESULTS AND DISCUSSION

Wheat bran alone as substrate

The studies on the fermentation of wheat bran at 28±1°C, pH 6.0, 60% initial moisture and 7 L h^{-1} $column^{-1}$ aeration rate (Roussos, 1987), revealed many interesting results. The fermentation in column bioreactors under these optimum conditions for 64 h indicated that the germination of the conidiospores of the culture in the medium is rapid and homogeneous. The mycelial cells started colonizing on the substrate by 24 h. The branching of the mycelial cells was extensive and rapid, thereby resulting in formation of the compact mass of the medium by 40 h. The pH of the moist solid medium was found to decrease gradually to about 4.1 up to 48 h of fermentation (Fig. 1). However, it increased slightly when the fermentation was continued beyond 48 h. The protein content of the medium increased steadily from the initial value of 0.22 to 0.33 g/1 g SDM at 50 h fermentation (Fig. 1).

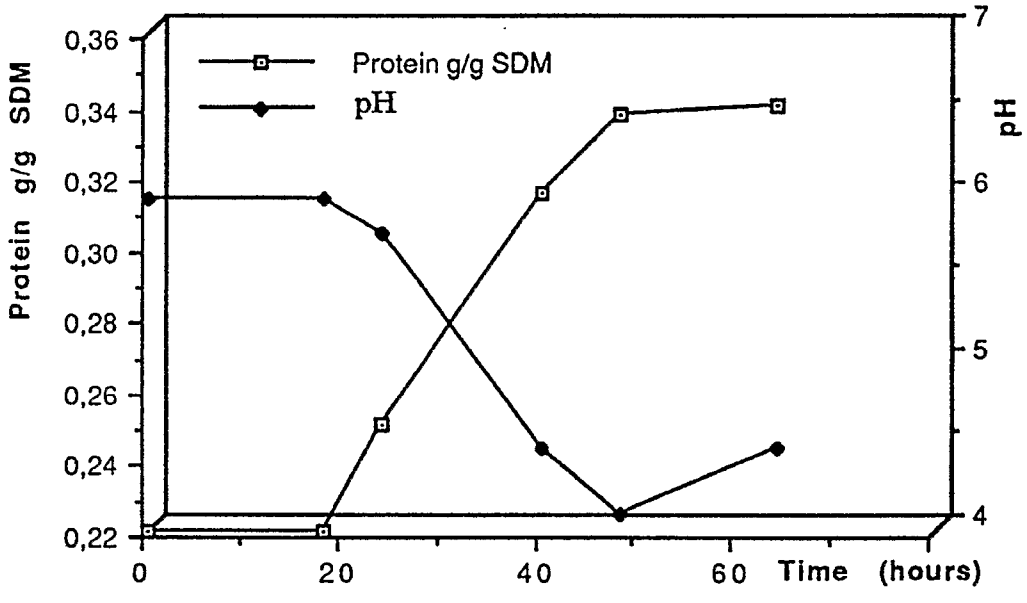

Fig. 1. Kinetics of pH and protein levels of the medium based on wheat bran alone as carbon substrate.

The production of CMCase and FPA by the culture was initiated at about 20 and 5 h fermentation, respectively (Fig. 2). The rate of biosynthesis of these enzymes was, however, very slow up to 25 h. It increased rapidly between 25-48 h to lead to the formation of 61.0 and 20.7 IU of CMCase and FPA per g SDM, respectively. The enzyme levels in the medium decreased slowly with further continuation of the fermentation. The ratio of these enzymic activities was about 10 up to 20 h fermentation (Fig. 2). The production of FPA fraction was, however, much rapid beyond 30 h fermentation as compared to that of CMCase and, therefore, resulted in the ratios which ranged between 2.6-3.3 during 24-64 h.

The data indicate that the cellulase production on wheat bran alone is much lower than that on other substrates evaluated. This probably is due to the preferential utilization of starch present in the substrate by the culture for biomass formation. The glucose formed during the utilization of the starch also repressed the biosynthesis of cellulolytic enzymes. It is of interest to note that the protein production during the fermentation is quite high and the

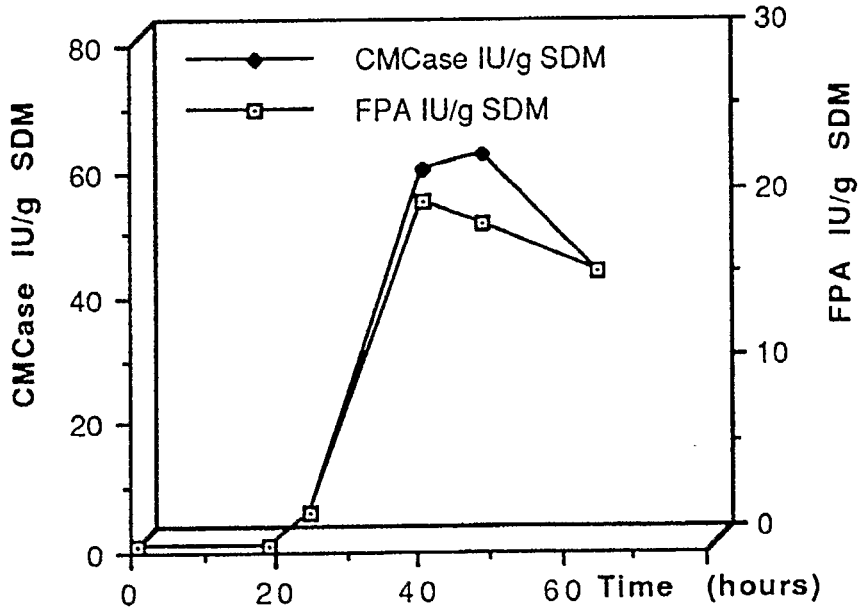

Fig. 2. Kinetics of carboxy-methyl cellulase (CMCase) and filter paper activity (FPA) of *Trichoderma harzianum* on wheat bran alone as carbon substrate.

level of protein in the medium at 50 h was 0.33 g/1 g SDM. The increase in protein is about 48% over that present initially in wheat bran. These results show the potential of using *T. harzianum* for efficient protein enrichment of wheat bran. The utility of the culture for cellulases production, however, is poor unless cellulases with 3:1 ratio of CMCase and FPA are required for the hydrolysis of specific lignocellulosic residues/wastes.

Sugar beet pulp alone as substrate

The sugar beets are used in France to produce sucrose, in contrast to the use of sugar cane in tropical countries. Large quantity of the byproducts and wastes are generated in North France, the region where sugar beet processing industries are located. Molasses, one of the byproducts, is used for production of yeasts and other microbial metabolites. Sugar beet pulp contains about 20% cellulose, 15% hemicellulose, 30% simple carbohydrates, 10% protein, 10% pectin, 8% lipids and 7% other matters (Roussos, 1987). However, it does not contain any lignin. It can form a good substrate for growth of filamentous fungi and also for production of various fungal metabolites, such as cellulase, in SSF system. The sugar beet pulp, as it exits from the sugar beet processing industry, contains about 80% moisture (Roussos, 1987).

In the present studies, the sugar beet pulp was found to be a good substrate for production of cellulolytic enzymes (Table 1). The production of FPA fraction in the column fermenter was initiated by about 3 h, while the CMCase activity was detectable at about 18 h. The production of both of these enzymes was at extremely low rate up to about 30 h, probably because of the preferential utilization of simple carbohydrates present in the substrate. The enzyme titres, however, increased at a faster rate between 30-64 h to reach a peak of 220 and 19 IU of CMCase and FPA fractions per g SDM at 64 h, respectively. The results on the ratios of CMCase and FPA fractions showed interesting trends. The ratio varied between 10 and 6.4 between 30-47 h, in contrast to those of 11.1-11.4 in the subsequent fermentation period.

The protein content of the medium at 0 h was about 0.21 g/1 g SDM and it increased to about 0.30 g/1 g SDM by 64 h fermentation. *T. harzianum* seems to preferentially utilize simple carbohydrates over the proteins as carbon source, as the sugar concentration decreased by about 50% during the entire course of fermentation and the protein concentration increased by about 9%. The data indicate the excellent potential of *T. harzianum* for protein enrich-

Table 1. Carboxy-methyl-cellulase (CMCase) and filter paper activity (FPA) production on sugar beet pulp alone in solid state fermentation system with *Trichoderma harzianum*.

Time (hours)	pH	Protein (g/g SDM)	Cellulolytic enzymes	
			CMCase	FPA
			IU/g SDM	
0	5.6	20.4	0	0
18	5.6	24.0	0	0
24	5.8	26.0	0	0
42	5.0	27.5	83.0	12.9
48	4.5	28.0	196.0	17.2
65	4.6	29.0	215.0	19.4

SDM= Substrate dry matter.
IU= International units.

ment of sugar beet pulp in SSF system. The kinetics of the changes in the pH of the medium showed interesting results. The pH was nearly stable at about 5.4 in the initial 18 h fermentation period, probably due to preferential utilization of urea over that of $(NH_4)_2SO_4$. The pH increased slightly between 18-27 h probably due to the accumulation of H^+ ions resulting from the assimilation of NH_4^+ ions during the utilization of $(NH_4)_2SO_4$. The sugar beet pulp also seems to have strong buffering action. It is interesting to note that the pH of the medium decreased rapidly during the growth of *T. harzianum* (strain CCM F-470) on microcrystalline cellulose in SmF process (Roussos and Raimbault, 1982).

Sugar cane bagasse alone as substrate

The manufacture sucrose from sugar cane 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 and fibres so obtained are used in the manufacture of paper. The sugar cane 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 where in the range of 5.9-6.3 during first 52 h fermentation. This is the fermentation period which led to maximum cellulase titres. Further continuation of fermentation beyond 52 h resulted in increasing the pH of the medium to 6.9 at 58 h and 64 h fermentation.

The data on the production of CMCase and FPA fractions indicated that no

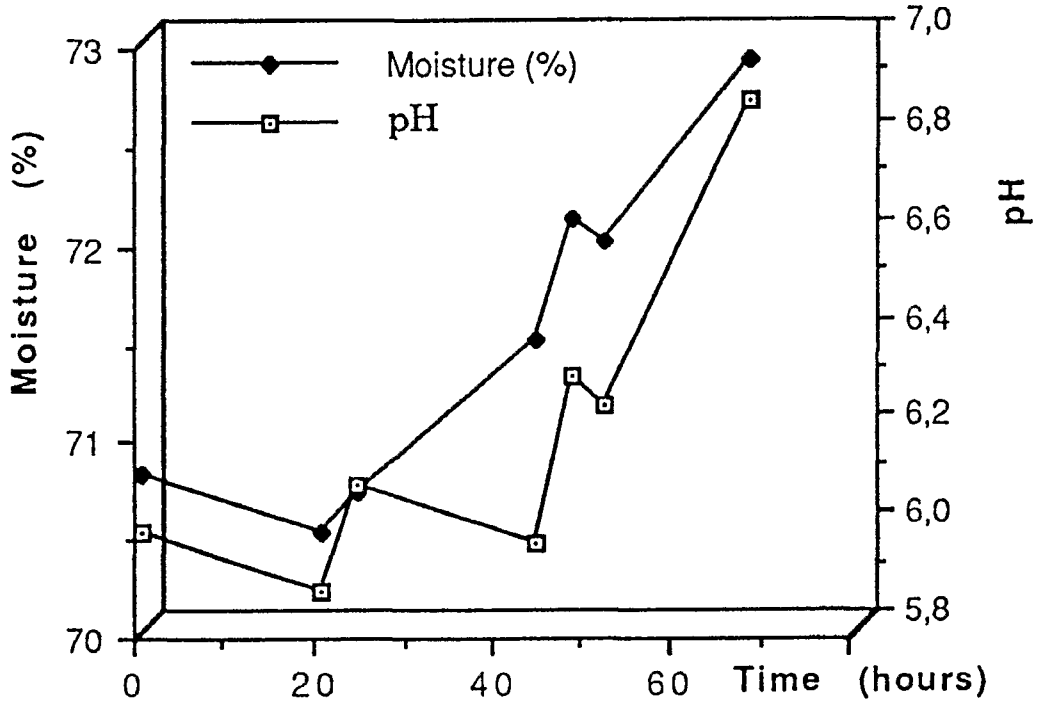

Fig. 3. Changes in moisture and pH levels of the medium based on sugar cane bagasse alone as carbon substrate.

CMCase was produced up to 24 h, in contrast to the production of FPA at slower rate right from the start of fermentation. 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 at 52 h for both the enzymes and their levels decreased if 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 wheat straw and wheat bran as substrate

The growth characteristics of *T. harzianum* in the medium containing wheat straw and wheat bran at the ratio of 80:20 in the column fermenter at 28±1°C revealed that the germination of the conidiospores was 100% by 17 h fermentation. The active mycelial growth of the culture was observed up to 45 h by which time the substrate was fully colonized by the culture. The conidiospores and phialides formation were initiated at about 48 h, thereby indicating either the exhaustion or non-accessibility of the nutrients to the culture. This can be, perhaps, prevented by adding more nutrients to

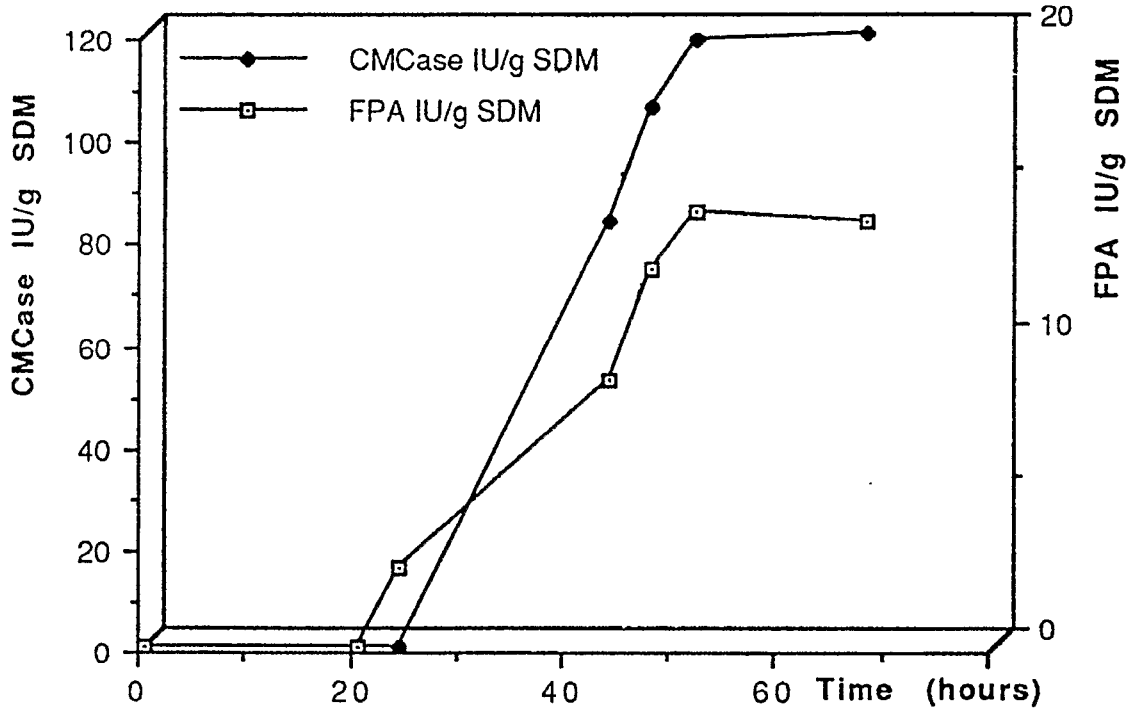

Fig. 4. Kinetics of cellulases production in *Trichoderma harzianum* culture on sugar cane bagasse alone as carbon substrate.

the medium or by giving more efficient pretreatment to the substrate with a view to provide the nutrients in more accessible form (Roussos and Sedha, 1983). The culture, in the present case, formed conidiospores from 60 h fermentation.

The fermentation of 80:20 ratio of wheat straw and wheat bran with 74% moisture content and initial pH of 5.8 in the column fermenter showed that the maximum cellulolytic activities at 60 h fermentation were 18.10 and 198 IU of FPA and CMCase fractions/g SDM, respectively (Fig. 5). The production of FPA and CMCase fractions was found to be initiated at about 4 and 10 h fermentation, respectively, and the rate of enzyme production was faster between 18-48 h. The ratios of CMCase and FPA fractions during 20-64 h fermentation were nearly the same and ranged between 10.5-12.0.

Combination of sugar cane bagasse and wheat bran as substrate
The use of sugar cane bagasse and wheat bran at the ratio of 80:20 in the column fermenter and under the fermentation parameters such as 20 g

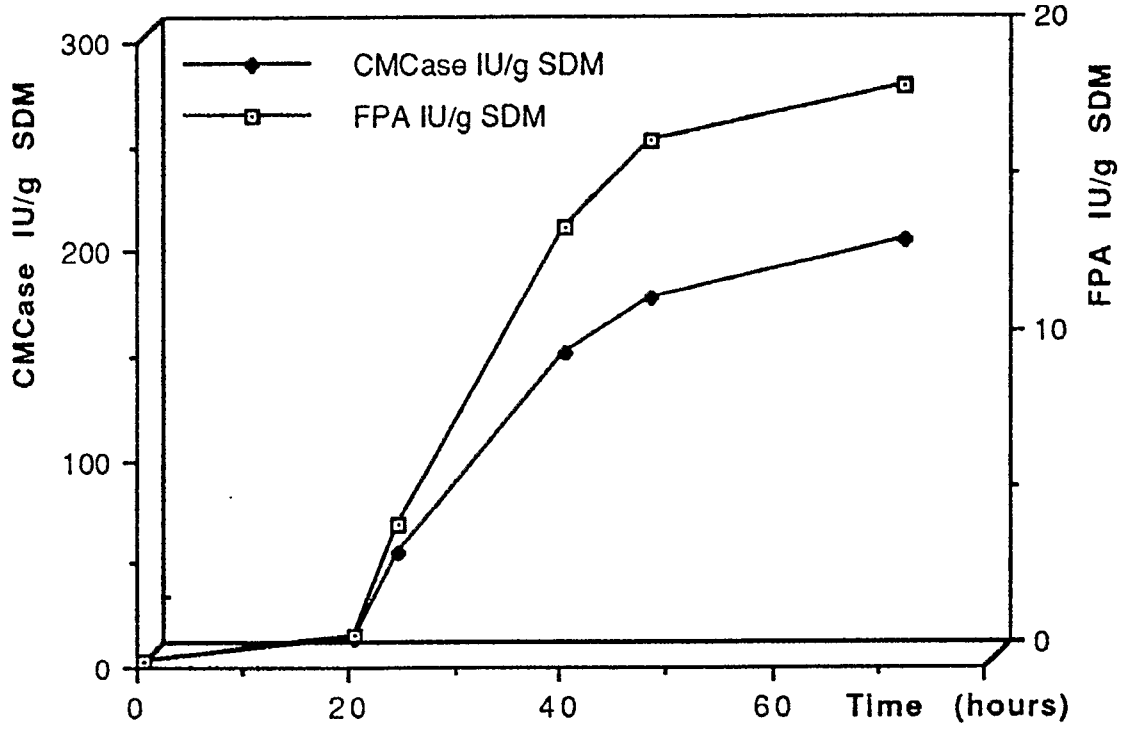

Fig. 5. Fermentation data of the medium consisting of wheat straw and wheat bran at 80:20 ratio as carbon sources.

medium in the column, 72% initial moisture content, 28±1°C incubation temperature and aeration at 5 L air $h^{-1}column^{-1}$ 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 up to 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 up to 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

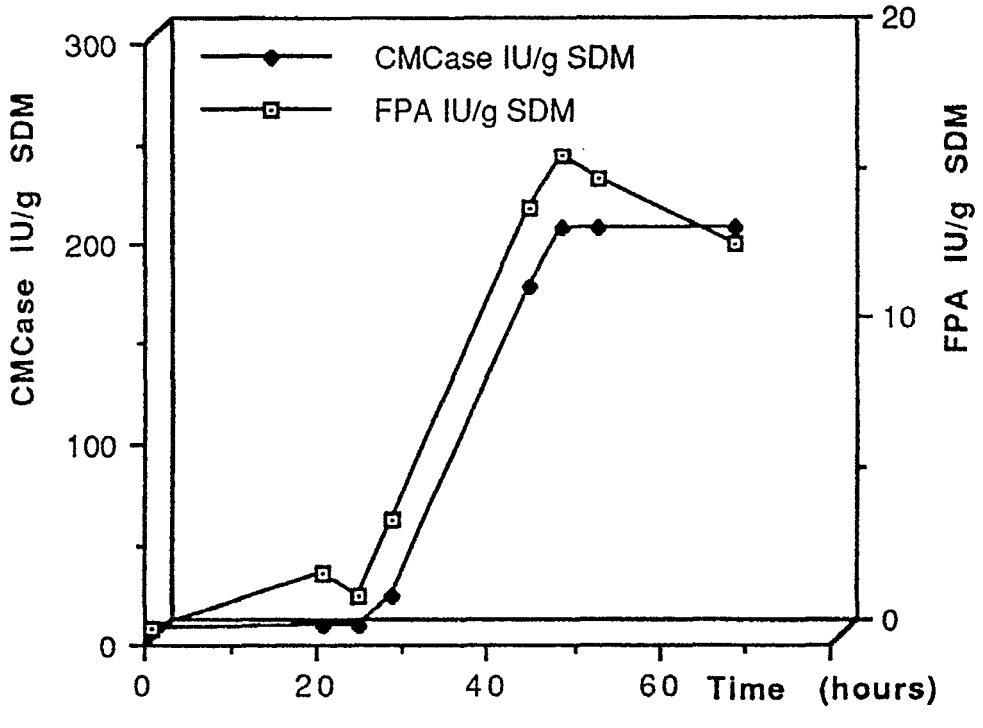

Fig. 6. Kinetics of the production of cellulases by *Trichoderma harzianum* in the medium containing sugar cane bagasse and wheat bran at a ratio of 80:20.

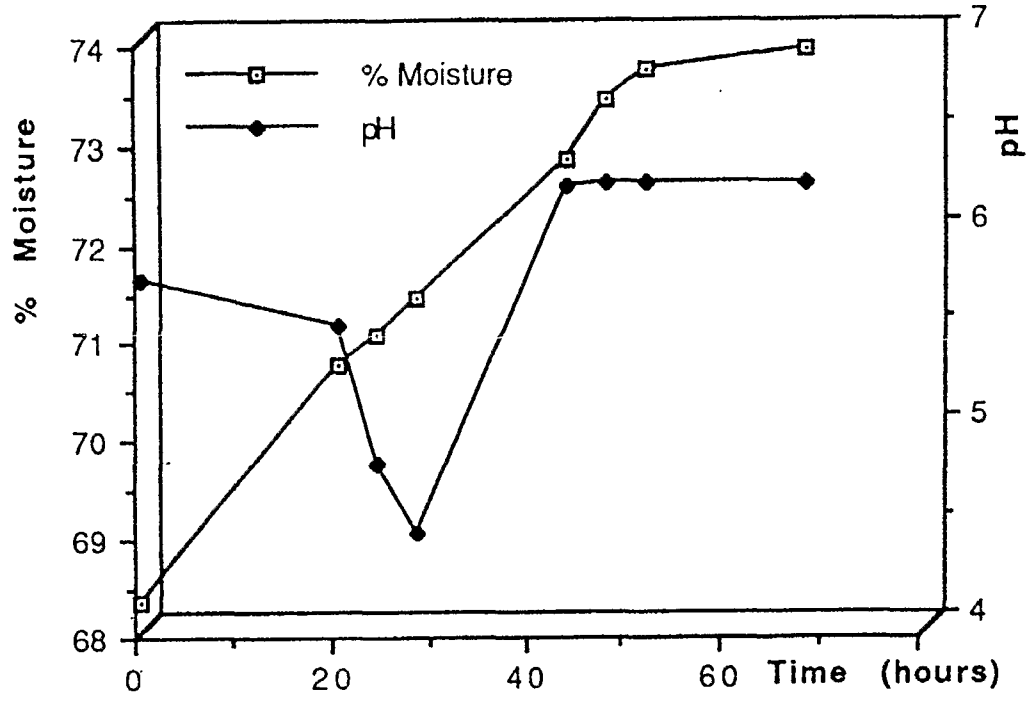

Fig. 7. Changes in moisture and pH levels of the medium consisting of sugar cane bagasse and wheat bran at a ratio of 80:20.

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.

Over-views

One of the interesting patterns that has emerged in this study is the relationship between pH changes in the medium and the growth patterns as well as cellulolytic enzyme production. The pH of the medium remains more or less unaltered during the conidiospore germination stage and then starts declining during active mycelial growth of the culture. The lowest value usually corresponds to the initiation of the biosynthesis as well as liberation of the cellulolytic enzymes. The pH, in the subsequent period of fermentation, starts rising with the increase in the accumulation of the enzyme in the medium. The kinetics of these pH changes and the shape of the curve of pH

change vs. time, perhaps can form a simple tool to monitor the progress of fermentation and also to determine the extent of enzyme production. It is interesting to note that these changes in pH of the medium are confined to a narrow range due to a good buffering action of the solid substrate evaluated. It, therefore, does not seem to be necessary to control the pH during the course of fermentation.

The growth pattern and metabolism of *T. harzianum* on different substrates in SSF system indicate that sugar beet pulp and wheat bran are not very good substrates for production of cellulolytic enzymes, probably due to the presence of easily fermentable carbohydrates in these substrates. The ability of these two substrates to support good growth of *T. harzianum* and higher accumulation of protein (30% in 45-50 h fermentation) in the fermented solids can be best exploited for protein enrichment of these substrates to obtain better quality animal feed. The preferential utilization of simple carbohydrates as carbon source over that of protein by *T. harzianum* is noteworthy in this context.

The lignocellulosic substrates, when used alone, allow slower growth of *T. harzianum* probably due to the non-accessibility of the nutrients to the culture. The nutrient accessibility can be improved by giving drastic pretreatment to the substrate but it will be prohibitively expensive. A simple and economic strategy to overcome these limitations has emerged from the present studies and involves the use of the combination of the lignocellulosic and starchy substrates. The data show that the combination of wheat straw and wheat bran or sugar cane bagasse and wheat bran at 80:20 ratio is efficient.

The lignocellulosic byproducts and wastes contain both crystalline and amorphous regions and their proportion varies depending on the type of the plant of origin (Acebal *et al.*, 1986). During enzyme attack on these celluloses, the amorphous portion is hydrolyzed rapidly but the rate of hydrolysis of the crystalline portion is very slow (Mandels *et al.*, 1976). Such slower rate of crystalline cellulose hydrolysis is quite understandable as it is this fraction which gives protection to the plant tissue against microbial and chemical attack (Fan *et al.*, 1981). It is also for this reason that a chemical pretreatment is given to the lignocellulosic materials to make them amorphous by reducing the crystallinity of the cellulose and also to solubilize it to some extent (Tanaka and Matsuno, 1985; Acebal *et al.*, 1986).

In the mechanism of the action of cellulases on cellulose, the FPA portion of the enzyme was shown to first attack the cellulose to form a susceptible water-insoluble cellulose, which is then hydrolyzed randomly by CMCase as per the model proposed by Reese *et al.* (1950). On the other hand, the action of FPA and CMCase on water-insoluble cellulose is stated to be completely inverse in the order of enzyme action in the most accepted concept of sinergistic action of the enzymes (Tanaka and Matsuno, 1985). It has been also reported that the hydrolysis of crystalline, highly hydrogen-bonded, ordered component of native cellulose requires the concerted action of CMCase and FPA fractions (Roux and Odier, 1986; Ooshima *et al.*, 1983). However, the amorphous, easily hydrated areas of cellulose can be hydrolyzed by either of these enzymes acting independently (Hoffman and Wood, 1985).

The kinetics of the enzymic hydrolysis of cellulose is also known to be dependent on three major factors, i.e., adsorption of cellulases on the cellulosic substrate, the structure of cellulose and the type of the cellulolytic enzymes (Tanaka and Matsuno, 1985). In fact, the adsorption of cellulases to cellulose is a prerequisite for the initiation of the cellulase reaction. It is emphasized that the adsorption behaviour is different for various components of cellulases (Castañon and Wilke, 1980). For example, the K_{es} , the half-saturation constant for adsorption of the enzyme, is independent of the type of the straw in case of FPA, in contrast to the preference of the CMCase activity for the α-cellulose-rich straw (Estrada *et al.*, 1988). Consequently, the FPA may get preferentially adsorbed to the non-cellulosic component of the straw. This assumes critical importance as most of the lignocellulosic residues and wastes contain 40-60% cellulose, the rest being hemicellulose, lignin and other materials (Acebal *et al.*, 1986). Moreover the ratios between E_{max} , the enzyme units adsorbed/g straw for FPA and CMCase fractions, were shown to be higher for the milled and sieved wheat straw due to higher specific adsorption (Estrada *et al.*, 1988). The ratios of CMCase and FPA fractions in the enzyme used for hydrolysis of different cellulosic substrates, thus, are of significant importance in the overall efficiency and economics of the process.

It has been stressed that the biosynthesis of CMCase and FPA fractions of cellulases is not coordinately controlled in fungi (Montenecourt and Eveleigh, 1977). Therefore, it becomes critically important to find out a simple and practicable technique to obtain appropriate and desired ratio of CMCase and FPA in the enzyme product. It appears that three different possibilities

can be employed to achieve the desired ratios. The selection of the specific microbial culture which gives the desired proportion of the enzymes in one such possibility. For example, the ratio of CMCase and FPA produced by *Trichoderma reesei*, strain QM 9414, on wheat straw was reported to be 8:1.2 (Acebal *et al.*, 1988), in contrast to that of 2.5:0.58 by *Pestalotiopsis versicolor* on bagasse medium (Rao *et al.*, 1982). Different ratios were also reported when six species belonging to different genera were evaluated for comparative production of cellulases (Grajek, 1987). However, this approach may not prove practicable as all the cultures are not potent enzyme producers. The second possibility is to use SmF or SSF techniques as the ratio differs widely when the same culture is grown in a particular type of fermentation technique, as it is reported for six species of different genera of the thermophilic fungi (Grajek, 1987). In this case, the utility of the method is of doubful nature as the desired fermentation technique may not be highly productive.

The data reported in the present communication provide the third possibility for obtaining the tailor-made ratios of CMCase and FPA fractions and consists of using specific lignocellulosic substrate and the fermentation time. Together with other advantages in production of cellulases by SSF technique, as indicated earlier, and also for its ability to produce many other metabolites or products of economic importance (Roussos *et al.*, 1991c), *Trichoderma harzianum* may prove to be the super fungi for commercial exploitation.

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