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ORIGINAL PAPER

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Production of an endoglucanase by the shipworm bacterium, *Teredinobacter turnirae*

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Abstract The nutritional behavior of a cellulolytic nitrogen-fixing shipworm bacterium, *Teredinobacter turnirae*, is described, with respect to various carbon and nitrogen sources, in terms of endoglucanase production. Also, the effects of various surfactants on enzyme production are reported. Among the carbon sources, sucrose results in the maximum enzyme production, followed by cellulose. Ammonium phosphate proves to be the best nitrogen source for endoglucanase production. Various surfactants enhance the enzyme titers, with Triton X-100 yielding the best results. A combination of the above-mentioned components improves the enzyme production by 3.6-fold.

Keywords Carbon nutrition · Nitrogen nutrition · Endoglucanase · Shipworm bacterium · Marine bacterium · *Teredinobacter turnirae*

Introduction

Deshayes, in 1848, was the first to describe a gland in shipworms consisting of dark or brown irregular tissue. This gland, which was subsequently named after its discoverer, was found in the gills of the molluscan host; and ducts led from this special gland into the digestive tract inside the shipworm. Through the years, speculation abounded that the gland was associated with the shipworm's ability to grow in fixed-nitrogen-deficient environments [8]; and it remained unclear until recent times, when Waterbury and coworkers isolated a bacterium that existed as a pure culture in Deshayes gland tissue and showed that the bacterium was capable of both nitrogen fixation and cellulose digestion [28]. This isolate was unique in that it represented the only marine bacterium known to both digest cellulose and fix nitrogen. It was postulated that the bacterium enabled the host shipworm to grow on wood because it provided a ready supply of proteins and cellulolytic enzymes.

Later, the cellulolytic activity produced by the shipworm bacterium was studied extensively [8, 10, 12, 14]. Cell-free media from bacterial cultures extensively and visibly disrupted filter paper, yet little or no sugar was solubilized. At the same time, cell-free preparations of the cultures considerably increased the reducing sugar content of carboxymethyl cellulose (CMC). These observations indicated that the extracellular enzyme activity was endoglucanolytic in nature. Identification of the degradation products from cellodextrins (specifically radiolabeled in the terminal nonreducing glucose unit) and the kinetic analysis of product formation verified that the extracellular activity was primarily endoglucanolytic [12]. Indirect and admittedly scant evidence suggested that the exoglucanase activity remained cell-bound [12]. Zymographic analysis of CMCase activities secreted by the shipworm bacterium demonstrated the presence of multiple endoglucanases [3, 8]. Studies aimed at understanding the adhesive properties of the shipworm [13] and the extracellular shipworm bacterial CMCase activity [14] with respect to insoluble, cellulose-containing substrates were also carried out.

An endoglucanase was subsequently isolated from this symbiotic bacterium and characterized [10, 12]. This enzyme was of special interest because of its unusual characteristics. The enzyme showed half-maximal activity over the pH range 4.5–8.5 and exhibited salt

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tolerance up to 4 M [10]. Also, it was thermally stable up to 60 °C. It had a long half-life in solution and could be stored for years as a lyophilized powder. Another remarkable attribute of this enzyme was its resistance to various proteases like trypsin, chymotrypsin, and pronase. Table 1 compiles the salient features of the endoglucanase activity secreted by the shipworm bacterium and compares it to that secreted by the native strain of an important cellulase-producing fungal organism, *Trichoderma viride*.

It is important to note that the hydrolysis of crystalline cellulose is accomplished by actions of many enzymes. The first step in the process involves the enzymatic process that renders cellulose amorphous. The next step is comprised of the synergistic actions of endoglucanase and exoglucanase to create oligosaccharides. These oligosaccharides are then converted to cellobiose by the action of cellobiohydrolase. Finally, β-glucosidase converts cellobiose to the final product of the hydrolysis process, i.e. glucose [17]. This paper focuses on the production of endoglucanolytic activity by the shipworm bacterium, which may be the result of multiple endoglucanases. The work involved studying several growth conditions that could increase the enzyme titers. It was hoped that the knowledge acquired for the shipworm bacterium could be applied to many other marine microbes and be useful for the development of a high-cell-density fermentation process for endoglucanase production. With the aim of increasing the enzyme activity titer, numerous nutritional conditions were studied. First, various carbon sources, nitrogen sources, and surfactants were studied individually. Subsequently, various combinations of the best components were used. These studies led to a significant improvement in the enzyme activity titer of endoglucanase.

Materials and methods

Materials and chemicals

All chemicals used in this study, except casamino acids, were purchased from Sigma Chemical Co., St. Louis, Mo. Casamino acids were purchased from Difco Laboratories, Detroit, Mich. A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce, Rockford, Ill. Membrane tubing for dialysis was obtained from Spectrum Medical Industries, Los Angeles, Calif.

Microorganism

Cultures of *Teredinobacter turnirae* were generously supplied by Dr. Richard Greene (USDA, Peoria, Ill.). Stock cultures of this bacterium were prepared according to the instructions given elsewhere [1] and stored at −70 °C.

Media and growth conditions

The composition of the basal medium was the same as reported elsewhere [9]. The basal medium was autoclaved separately from carbon and nitrogen sources (and surfactant, when included in the

Table 1 Properties of extracellular endoglucanase activities from *Teredinobacter turnirae* and *Trichoderma viride*

Property	*Ter. turnirae*[a]	*Tri. viride*
Endoglucanase production		
Units per liter	200	100,000[b]
Number of enzymes	3–7	4–6[c,d,e]
Thermal sensitivity		
Maximum activity	60 °C	48–60 °C[c]
Inactivation	75 °C[f]	–
pH sensitivity		
Maximum activity	6.0–7.0[g]	4.0–4.5[e]
Stability (>50% maximum activity)	4.5–8.5	3.0–9.0[e]
Influence of salinity (NaCl)		
>50% maximum activity	0–4 M	–
Half-life		
Liquid, 25 °C	35 days	–
Liquid, 4 °C	63 days	–
Lyophilized powder	Indefinite	–
Protease susceptibility		
Trypsin	Resistant	–
Chymotrypsin	Resistant	–
Pronase	Resistant	–

[a] Cultures were grown aerobically in artificial seawater medium containing 1% microcrystalline cellulose and 0.1% ammonium chloride. Cultures were harvested in early stationary phase at a total protein concentration of about 1 g/l. Units of activity are micromoles of glucose reducing sugar equivalents formed from carboxymethyl cellulose (CMC) per minute at 25 °C and pH 7.0
[b] Cultures used Solka Floc (ground) as substrate. Units of activity are micromoles of glucose reducing sugar equivalents formed from CMC per minute at 50 °C and pH 4.8 [18]
[c] From reference [2]
[d] From reference [15]
[e] From reference [25]
[f] In the presence of substrate, residual activity was observed even after exposure to temperatures >100 °C
[g] From reference [10]

medium). After autoclaving, the two solutions were mixed aseptically. In all experiments, the final concentrations of carbon and nitrogen sources were 0.5% and 0.1%, respectively. Surfactant concentration in the growth media varied between 0% and 0.2% or was fixed at 0.1%, depending on the experiment. All experiments were conducted in duplicate with 50 ml of culture in 250-ml Erlenmeyer flasks shaken at 120 rpm and 30 °C in an incubator shaker. The control medium (SM) used in all experiments contained cellulose (0.5% Sigmacell 100) and ammonium chloride (0.1%) as carbon and nitrogen sources, respectively. All culture propagations prior to inoculations of experimental media were done in SM.

Inoculum

Each time an experiment was conducted, 500 µl of the stock culture was used to inoculate 50 ml of SM. The culture was incubated for 7 days (at which time the cells were in the late exponential growth phase) and subsequently transferred into the same medium with 10% inoculum. After 7 days of incubation, the culture was used to inoculate flasks containing different experimental media. A 10% inoculum was also used at this step.

Measurement of growth

Cell growth was monitored by measuring extracellular protein, which was previously shown to be proportional to cell protein [11], which in turn was used as a marker for growth of the shipworm bacterium [9]. Optical density could not be used to monitor cell growth due to the presence of cellulose in most of the experimental media. Moreover, it was reported that the bacterial cells tended to form clumps and adhere to the sides of the culture tubes [9], which prohibited the use of optical density to evaluate growth. However, for the growth medium containing sucrose, a relationship between extracellular protein and dry cell weight (dcw) was established, which equaled 1 g dcw/l per 1 g extracellular protein/l. This correlation could also be used to estimate cell growth by extracellular protein estimations for other growth media. The protein estimations were done by either BCA or Bradford assay. The latter assay was used to estimate the protein contents in media that interfered with the BCA assay. These included the media containing CMC, cellobiose, dextrin, galactose, glucose, lactose, maltose, starch, xylan, cysteine, tyrosine, and tryptophan. In the case of CMC and xylan, it was the formation of hydrolyzed products from carbon sources that interfered with the BCA assay. The BCA assay was performed according to the instructions provided by the manufacturer (Pierce), and the Bradford assay was performed as described elsewhere [3]. Blanks were prepared using the starting media. A set of protein standards of concentrations from 0.1 mg/ml to 1.0 mg/ml was made using bovine serum albumin. All assays were performed in duplicate.

Measurement of endoglucanase activity

Enzyme activity was monitored by an endoglucanase assay. The dinitrosalicylic acid reagent used in the assay was prepared as described elsewhere [19]. The CMC reagent was prepared and the endoglucanase assay performed according to the instructions given elsewhere [22], except that 20 mM sodium phosphate buffer (pH 7.0) and a wavelength of 620 nm were used. Blanks were prepared by the addition of 20 mM sodium phosphate buffer instead of CMC solution prior to incubation at 25 °C. The assay was standardized using known amounts of glucose. One unit of enzyme activity represented 1 µmol of glucose reducing equivalents released from CMC per minute at 25 °C and pH 7.0. All enzymatic assays were performed in duplicate. For the carbon sources that interfered with glucose estimation, i.e. cellobiose, dextrin, galactose, glucose, lactose, maltose, starch, and xylan, the samples were dialyzed before being assayed. Samples (2 ml) were placed in a dialysis bag (molecular weight cut-off = 3,500) and dialyzed overnight against sodium phosphate buffer at pH 7.0. After dialysis, the samples were assayed. Endoglucanase activity loss during the dialysis step was considered negligible.

Statistical analysis

Mean values for various parameters were calculated using the data from three independent runs. Standard errors of the means were calculated at 95% confidence interval.

Results

The bacterium grew slowly on most substrates and most of the experiments lasted for at least 10 days. The parameter chosen for medium-optimization studies for endoglucanase production was the maximum endoglucanase activity.

Effect of carbon source on endoglucanolytic activity production

Various carbon sources were studied in an attempt to increase the enzyme activity. The experiment was conducted with ammonium chloride as the nitrogen source and was carried out for 16 days. With most carbon sources, the maximum enzyme activity was obtained at 7–10 days. Sucrose gave the maximum endoglucanase activity, followed by cellulose. The other substrate that produced significant enzyme activity was CMC. All other carbon sources resulted in low or no enzyme activity; and they did not yield any significant growth of the bacterium. These results are summarized in Table 2. The extracellular protein estimated by the Bradford assay was maximum in the case of sucrose, followed by cellulose (Table 2). Not only did sucrose result in maximum growth and endoglucanase activity, it also reduced the lag phase. CMC yielded the highest specific activity.

Table 2 Effect of carbon source on endoglucanase activity. Maximum endoglucanolytic activity obtained in the control and the corresponding protein reading were 291 units/l and 0.17 g/l, respectively. Cellulose type was Sigmacell 100. *ND* Not detected

Carbon source	Maximum endoglucanase activity (% of control)	Extracellular protein (% of control)
Cellobiose, glucose, lactose, maltose	ND	≤ 5
Dextrin, galactose, starch, xylan	≤ 25	≤ 32
Cellulose (control)	100	100
Carboxymethylcellulose	74 ± 2	29 ± 5
Sucrose	108 ± 2	132 ± 10

Effect of nitrogen source on endoglucanase activity

An important consideration while studying bacterial metabolism, in addition to carbon source consumption, is the dissimilation of the nitrogen source. In this study, a nitrogen source is defined as the compound that serves as the sole source of nitrogen for the microorganism. Various nitrogen sources, including all 20 amino acids (L-forms), were studied to determine their effect on enzyme production. Arginine, histidine, and lysine were used in the hydrochloride forms. Glycine was used in the aminoacetic acid form. A combination of five amino acids, i.e. leucine, serine, methionine, aspartate, and lysine, was also used. This amino acid supplement was used since it was previously shown to enhance the protease activity produced by the bacterium [11]. The experiment was conducted with cellulose as the carbon source and was carried out for 10 days. Ammonium chloride as a nitrogen source was used as the control. Among the amino acids, arginine and asparagine resulted in the highest endoglucanase activity. The other amino acids that gave good enzyme activity (90–110% of the control) were glutamic acid, glutamine, and the amino acid combination described above. Interestingly, this combination resulted in higher endoglucanase activity than that achieved by any of the amino acids used alone.

In addition to amino acids, numerous inorganic and organic compounds were provided as nitrogen sources. A number of selected nitrogen sources were the compounds whose chemical structures were similar to those derived from marine plants and animals [21]. Of all the nitrogen sources tested, ammonium phosphate resulted in the maximum enzyme activity. Casein proved to be the second-best source of nitrogen for enzyme activity. Table 3 lists all nitrogen sources used, their maximum CMCase activities, and the corresponding extracellular protein values. As was the case with carbon sources, the maximum enzyme activity for most nitrogen sources was obtained at 7–10 days. Ammonium phosphate also yielded the maximum growth, as indicated by extracellular protein estimation. Among the sources that gave endoglucanolytic activity values greater than 90% of the control value, the amino acid combination yielded the highest specific activity (units per gram of protein), followed by ammonium phosphate. The protein estimation in this experiment was performed by the BCA assay, except for the media containing cysteine, tyrosine, and tryptophan, for which the Bradford assay was used.

Effect of surfactants on enzyme production

There are a number of reports in which the use of surfactants increased enzyme production [5, 23, 24]. An improvement in the enzyme titer as high as 120-fold has been reported [23]. Although the exact mechanism of their action is not understood, it is believed that the surfactants improve enzyme production by affecting cell permeability. In an attempt to increase enzyme production by the shipworm bacterium, various surfactants, i.e. sodium dodecyl sulfate, Tween 80, Triton X-100, and Antifoam 289, were incorporated in the medium at a concentration of 0.1%. Among these surfactants, Antifoam 289 and Triton X-100 yielded the best and equivalent endoglucanase activities (Table 4). However, since Triton X-100 gave the highest specific activity among all detergents (Table 4), it was selected for further experiments. As shown in Table 4, the presence of surfactants did not interfere with the assay results. The

Table 3 Effect of nitrogen source on endoglucanase activity. Maximum endoglucanase activity and extracellular protein obtained in the control were 292 units/l and 0.75 g/l, respectively. The following yielded less than 90% of the control value: acetamide, adenine, alanine, ammonium oxalate, aspartic acid, beef extract, betaine, casein (0.05%) with ammonium chloride (0.05%), corn steep liquor, creatine, cysteine, formic acid, glycine, guanidine, hippuric acid, histidine, isoleucine, leucine, lysine, methionine, nicotinic acid, phenylalanine, proline, pyridine, serine, threonine, tryptophan, tyrosine, urea, and valine. ND values indicate that accurate extracellular protein estimation was not possible due to the presence of casein

Nitrogen source	Maximum CMCase activity (% of control)	Extracellular protein (% of control)
Amino acids (leucine, serine, methionine, aspartate, lysine; at 0.01% each)	105 ± 9	65 ± 4
Ammonium chloride (control)	100	100
Ammonium phosphate	262 ± 4	143 ± 11
Ammonium sulfate	100 ± 7	106 ± 8
Aniline	97 ± 9	47 ± 2
Arginine	105 ± 8	88 ± 6
Asparagine	104 ± 6	101 ± 8
Casamino acids	97 ± 1	98 ± 5
Casein	133 ± 1	ND
Casein (0.05%) with 0.05% amino acids (leucine, serine, methionine, aspartate, lysine; at 0.01% each)	110 ± 4	ND
Glutamic acid	99 ± 10	87 ± 8
Glutamine	93 ± 3	69 ± 4
Tryptose broth	99 ± 7	89 ± 8

Table 4 Effect of surfactants on endoglucanase activity. Endoglucanase assay control included 0.1% of the surfactant in the assay mixture. *SDS* Sodium dodecyl sulfate

Surfactant	Endoglucanase (units/l)	Endoglucanase assay control (units/l)	Extracellular protein (g/l)	Specific activity (units/g)
Control	280 ± 10	276 ± 15	0.71 ± 0.05	395
SDS	180 ± 9	185 ± 10	0.22 ± 0.03	824
Tween 80	239 ± 11	240 ± 8	0.55 ± 0.02	439
Triton X-100	447 ± 16	456 ± 12	0.48 ± 0.05	928
Antifoam 289	450 ± 19	460 ± 11	0.65 ± 0.04	693

maximum endoglucanase activity was obtained at 7–10 days. Extracellular protein was estimated by the BCA assay. Note that surfactant concentration is a critical parameter and two surfactants may give the same result at different concentrations.

Effect of combinations of carbon source, nitrogen source, and surfactant on enzyme production

As already discussed, sucrose resulted in the maximum endoglucanase activity, with cellulose as the next-best substrate. For subsequent studies, both these carbon sources were used in conjunction with the best nitrogen source (ammonium phosphate), ammonium chloride, and the best surfactant (Triton X-100). Surfactant concentration was varied between 0.0% and 0.2%. The results for these combinations are summarized in Table 5. The optimum surfactant concentration was not the same for each combination. The maximum enzyme activities for various combinations were obtained at 7–10 days. Among all combinations in Table 5, the maximum enzyme activity was observed for the combination containing cellulose, ammonium phosphate, and 0.05% Triton X-100. Further experiments showed that the maximum endoglucanase activity was obtained when cellulose (0.5%), ammonium phosphate (0.1%), and 0.025% Triton X-100 were employed. A 3.6-fold increase in enzyme activity, concomitant with a 2.5-fold increase in specific activity, was observed in this case, when compared with the control. The protein estimations in all these experiments were done by the BCA assay. Fig. 1 illustrates the improvement in endoglucanase activity achieved with the best combination.

The final pH of each solution was also measured. In all media, the pH typically decreased from 8.0 to 6.5 as growth occurred (data not shown). Such a pH drop with bacterial growth on cellulosic substrates has been

Table 5 Effect of substrate combinations on endoglucanase activity. All values are given as units of activity per liter. *AC* Ammonium chloride (0.1%), *AP* ammonium phosphate (0.1%)

[a] Control combination

Combination	Triton X-100			
	0%	0.05%	0.10%	0.20%
Sucrose + AC	319 ± 1	285 ± 14	274 ± 14	306 ± 2
Sucrose + AP	528 ± 22	481 ± 15	462 ± 20	409 ± 8
Cellulose + AC	295 ± 12[a]	355 ± 9	437 ± 1	495 ± 15
Cellulose + AP	772 ± 19	1051 ± 4	710 ± 34	178 ± 4

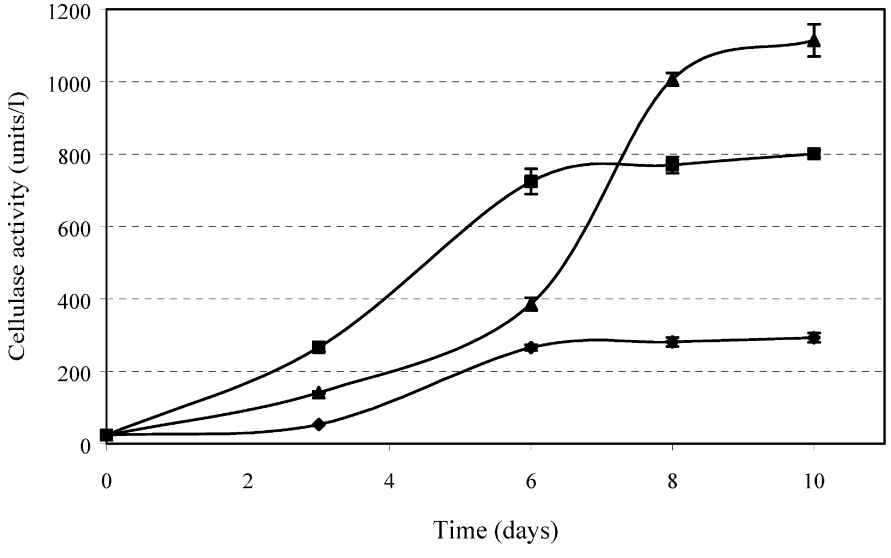

Fig. 1 Variation in endoglucanase activity with time, using Sigmacell 100 as the cellulose type. *Diamonds* control, i.e. cellulose with ammonium chloride, *squares* cellulose with ammonium phosphate, *triangles* cellulose with ammonium phosphate and 0.025% Triton X-100. *Error bars* indicate standard errors of the means at 95% confidence interval ($n = 3$)

attributed to the secretion of organic acids, such as succinic, acetic, and formic acids in the fermentation broth [9].

Discussion

Interest is growing in marine bacteria and fungi as sources of unusual chemicals that cannot be produced by terrestrial organisms. In addition to developing the salt-resistant reactors that are needed to grow these marine bacteria, numerous technical advances in bioprocess engineering are required before the potential of marine organisms as sources of valuable compounds can be realized [27]. From a bioprocess point of view, equally important is the understanding of the behaviors of such organisms with respect to the different chemical environments that typically exist when they are grown in a laboratory. This work is an attempt in that direction for the shipworm bacterium.

By optimizing the conditions, we were able to significantly enhance the enzyme activity for endoglucanase. A 3.6-fold improvement in enzyme activity, concomitant with a 2.5-fold improvement in specific activity, was achievable by providing appropriate quantities of the optimal carbon source, nitrogen source, and surface-active agent. The use of sucrose as a carbon source resulted in the maximum endoglucanase activity. This is an interesting result, as endoglucanase activity is not required for hydrolyzing sucrose per se. Another advantage associated with sucrose was the reduction in the lag phase. This points to the general superiority of soluble over crystalline substrates for the production of a metabolite. However, the use of soluble substrates, e.g. sucrose, may not result in the production of the whole enzyme (cellulase) complex, for which a crystalline substrate (cellulose) may be required.

Not only does casein improve endoglucanase activity, it also increases the proteolytic activity secreted by the bacterium [11]. Ammonium phosphate proved to be the best nitrogen source for both growth and endoglucanase activity and the second-best source for specific activity. Since phosphorus is a constituent of nucleic acids, phospholipids, and coenzymes [26], ammonium phosphate may trigger a response leading to the overproduction of biomass and endoglucanase activity.

The results obtained with surfactants point toward the general usefulness of surface-active agents in improving enzyme titers. The mechanism for the action of surface-active agents however, remains largely conjectural. It is believed that surfactants act on the cell membranes to facilitate enzyme release. The presence of extracellular enzymes seems to be a function of their release from the cells. It is as though, at a certain level of enzyme, the enzyme synthesis is turned off by product inhibition. And so, if the critical level of the enzyme inside the cells is never reached, since the enzymes are constantly escaping from them, the synthesis of the enzymes proceeds on a continuous basis. Hence, any factor that favors enzyme release will lead to an increased enzyme yield [23]. Other possible modes for the actions of surfactants have been put forth. Reese and Maguire [23] noted that, while using cellobiose as the carbon source for cellulase production, the surfactants that increased cellulase production also increased the production of cellulase inducers. The surfactant may be favoring access of cellobiose to a membrane-bound enzyme, such as a transferase. And since cellobiose may be responsible for the production of inducing compounds, the presence of surfactants may lead to enhanced cellulase yield [23].

The incorporation of Triton X-100 in the medium resulted in an increased enzyme titer in the presence of cellulose, but not sucrose (Table 5). This observation is in conflict with the above discussion, as one would expect the same surfactant effect irrespective of the carbon source. It may be that the concentrations of Triton X-100 used in the experiment were too high and lower concentrations of Triton X-100 may yield the expected behavior. The concentration dependence of the surfactant effect on medium components is clear from the results in Table 5, where changing the nitrogen source from ammonium chloride to ammonium phosphate with cellulose as the carbon source shifted the optimal value toward a lower surfactant concentration.

This work attempted the use of a bioprocess approach to enhance the CMCase activity produced by the shipworm bacterium. Although the approach was successful in increasing the enzyme yield significantly, it did not have a dramatic effect on the process. Hence, we suggest the use of genetic engineering to enhance the production of this enzyme, whose applications for industrial enzymes [7], including endoglucanase cloning [4, 16], are increasingly being reported. An alternate route is random mutagenesis, which historically has been proven to substantially enhance cellulase production by *Trichoderma* species [20].

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