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POLYSACCHARIDASE AND GLYCOSIDASE PRODUCTION OF AVICEL GROWN RUMEN FUNGUS *ORPINOMYCES* SP. GMLF5

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Extracellular and cell-associated enzyme preparations were obtained from ruminal anaerobic fungi *Orpinomyces* sp. GMLF5 grown in culture containing microcrystalline cellulose (avicel) as sole energy source and degradation capacities of the preparations towards several polysaccharides and glycosides were studied. Fungus showed substantial increases in xylanase, carboxymethyl cellulase (CMCase), lichenase, amylase, β-xylosidase, β-glucosidase and α-L-arabinofuranosidase activities between 72 and 168 hours. High amounts of cell associated β-xylosidase were noted in 4 and 5 days old cultures. Optimum temperature and pH of the polysaccharidases were found at 50 °C and 6.0-6.5, respectively. Xylanase was found to be virtually stable at 50 °C, CMCase and lichenase were stable at 40 °C for 200 min, however amylase was found more sensitive to heat treatment. The fibrolytic enzymes of the isolate GMLF5 were observed to be capable of hydrolyze the avicel.

Keywords: Orpinomyces – avicel – degradation – polysaccharidase – glycosidase

INTRODUCTION

Anaerobic fungi are indigenous to the rumen and actively degrade plant cell wall carbohydrates. Rumen fungi produce highly active cellulases and hemicellulases and especially xylanases and xylan debranching enzymes. Anaerobic fungi posses the unique capacity to penetrate the surface cuticle of plants, and the lignified tissues of cell walls. Furthermore, they digest recalcitrant substrates such as straw more extensively (37–50%) than do ruminal bacteria [13].

Anaerobic fungi are the initial colonizers of lignocellulose in herbivorous animals and they play vital role in biodegradation of the plant biomass ingested by herbivores. The colonization of lignocellulose by anaerobic fungi concludes the weakening and physical breakdown of plant tissues [10]. They degrade the plant cell walls by their broad range of polysaccharidase [22] and glycoside hydrolase enzymes [23] that could be localized on rhizoids [15] or secreted into the culture medium [19]. They

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also produce large scale of multienzyme cellulase and hemicellulase complexes, commonly known as cellulosomes [1].

Anaerobic fungi are grouped according to their monocentric or polycentric growth pattern [3] and both of them produce the enzymes capable of degrading plant cell wall polysaccharides [4]. The genus *Orpinomyces* [3] (type species for this genus, *O. joyonii*) accommodates the fungi with polyflagellated zoospores and they show polycentric reproduction [3]. The enzymes of *Orpinomyces* isolates were studied and similar enzyme profiles were observed compared with monocentric isolates [4]. Several studies focusing on enzyme activity of *Orpinomyces* isolates were reported and 17 enzyme encoding genes have been cloned and characterized from the isolate PC-2 grown on avicel or xylan containing media [reviewed in 14].

This study aims to investigate the time course production of several enzymes by the ruminal fungus *Orpinomyces* sp. GMLF5 which was grown in the medium containing avicel as sole carbon source. The effects of pH and temperature on the enzyme activity were also tested. In industrial processes thermal inactivation was the major problem in the utilization of enzymes; therefore investigation on the thermal inactivation of studied enzymes was carried out, too. Furthermore, avicel hydrolysis by using the crude enzyme extract of *Orpinomyces* sp. GMLF5 was conducted.

MATERIAL AND METHODS

Organism and culture condition

The anaerobic ruminal fungal isolate *Orpinomyces* sp. GMLF5 was obtained from the culture collection of Biotechnology and Gene Engineering Laboratory in Kahramanmaras Sutcu Imam University, Turkey. GMLF5 was isolated from frozen cattle feces and and classified as *Orpinomyces* according to its morphological characters such as polycentric development, polyflagellated zoospores [3] and whole ITS region sequence data (data not shown). Routine anaerobic techniques for the preparation of maintenance medium were reported earlier [9] and 10 ml of medium was dispensed into Hungate tubes under the CO_2 stream. For the maintenance media, ca. 100 mg wheat straw used for each Hungate tubes before the addition of liquid medium. Growth medium for enzyme production contained avicel as the sole energy source with a concentration of 0.5% (w/v). All incubations for the fungal isolate used in this study were performed at 39 °C without shaking.

Enzyme preparation

The cultures were centrifuged at 1250 *g* for 10 min for the separation of the culture medium and the fungal biomass. Cell-free supernatant was used to determine extracellular enzyme activity. Fungal biomass was washed twice with 50 mM sodium-phosphate buffer (pH 6.5) and then broken down by using ball-mill dismembranator

(Retsch, Germany) and resuspended in the same buffer. Cellular debris was subsequently removed by centrifugation and the clarified extract was used as cellular (cell-associated) activity. Extracellular and cellular fractions were stored at –20 °C until required.

Enzyme assay procedures

The ability of GMLF5 to synthesize a range of enzymes in avicel containing medium was investigated. The technique reported earlier [2] was used for determination of cellulase (CMCase), xylanase, lichenase and amylase activities by using carboxymethyl cellulose, oat spelts xylan, lichenan (from *Cetraria islandica*) and starch as substrates, respectively. The concentrations of substrate solutions in all assays were 0.5% (w/v) in 50 mM sodium phosphate buffer (pH 6.5). Reducing sugar, resulted from the enzymatic reaction, was determined by measuring the absorbance at 540 nm (Spectromax, UK) xylose for xylanase activity and glucose for cellulase, lichenase and amylase activities as standard. Glycoside hydrolase activity was determined by measuring the *p*-nitrophenyl (pNP) release from the appropriate *p*-nitrophenyl sugar derivatives (3 mM in 50 mM sodium phosphate buffer, pH 6.5) for incubation at 40 °C for 60 min. The reaction was halted by the addition of 2 M Na_2CO_3 solution. The amount of pNP released was determined by spectrophotometrically at 420 nm. The chromogenic substrates, which used in this study, were *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xylopyronoside, *p*-nitrophenyl-α-L-arabinofuranoside. Units of activity are defined as nmoles of product released per minute under assay conditions. Starch and avicel were obtained from Merck and the other substrates were purchased from Sigma. All assay procedures were conducted in triplicate, repeated twice and the mean values are used.

Determination of pH, temperature and thermal stability

The effects of pH on CMCase, xylanase, lichenase and amylase activity were assayed under different pH values ranging from 3.5 to 9.0 with 0.5 unit increments by preparing the appropriate substrates in 50 mM acetate buffer (for pH 3.5–5.5), phosphate buffer (for pH 6.0–7.5) and Tris-HCl buffer (for pH 8.0–9.0) solutions. Effect of temperature on enzyme activity was determined by assaying the activity at different reaction temperatures ranging between 30 and 80 °C with 10 °C increments in 50 mM sodium phosphate buffer (pH 6.5). Thermal stability determination was performed by preincubation of the enzyme without substrate at the temperatures of 40, 50, 60 and 70 °C for different times (5 to 200 min). Enzyme samples were immediately cooled on ice for 5 min after preincubation and the residual activities of each sample were then assayed with the addition of relevant substrates. All enzymatic measurements were performed by carrying out the standard assay procedures as described above.

Avicel hydrolysis

Hydrolysis of avicel was performed according to report of Dijkerman et al. [10]. Avicel was suspended in 50 mM sodium phosphate buffer (pH 6.0) with a final concentration of 2% (w/v). The substrate suspension was sterilized at 121 °C for 15 min. Extracellular enzyme preparation (0.25 ml) was added to the substrate solution (1.75 ml) and the mixture was incubated at 40 °C for 48 hours. Reactions were stopped for every 24 hours by heating the mixture in a boiling bath for 5 min. The samples were centrifuged and clarified supernatant were analyzed for reducing sugar as described above.

Protein estimation

Protein concentrations in the enzyme preparations were determined by using commercial protein assay kit (Favorgen Biotech. Coorp., Taiwan) with bovine serum albumin as standard.

RESULTS

Orpinomyces sp. GMLF5 was grown efficiently in the medium containing avicel as sole energy source. During the time course of cultivation of the isolate, protein content of fungal biomass was observed at peak level at the end of 4th day of incubation, thereafter shown relatively stable figure with slight decrease (Fig. 1).

Production of xylanase, CMCase, lichenase and amylase was studied at 39 °C for 7 days in anaerobic medium containing 0.5% avicel and the enzyme profiles of the *Orpinomyces* sp. GMLF5 culture was given in Table 1. Extracellular and cellular enzyme activities in the avicel culture produced time-dependent increases for xyla-

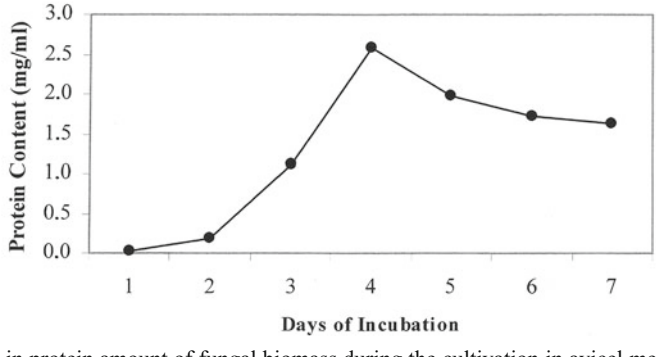

Fig 1. Variation in protein amount of fungal biomass during the cultivation in avicel medium. BSA standards were used to calculate the protein content with a correlation coefficient of 0.99. Error bars were negligible that is why they were not shown

Table 1
Extracellular and cell associated polysaccharide degrading enzyme production of *Orpinomyces* sp. GMLF5 grown in avicel containing culture

		Enzyme	*Orpinomyces* sp. GMLF5 incubation time course (day)						
			1	2	3	4	5	6	7
Enzyme activities (U/ml)	Extracellular	Xylanase	ND[a]	0.97 ± 0.12	4.17 ± 0.21	12.87 ± 0.34	16.64 ± 0.40	17.99 ± 1.29	19.44 ± 0.33
		CMCase	0.52 ± 0.08	0.53 ± 0.19	1.99 ± 0.51	4.63 ± 0.34	7.26 ± 0.46	6.52 ± 0.88	6.31 ± 0.25
		Lichenase	ND	0.46 ± 0.16	3.12 ± 0.19	9.22 ± 0.43	10.86 ± 0.42	11.81± 0.21	10.83 ± 0.44
		Amylase	1.02 ± 0.08	1.61 ± 0.28	1.83 ± 0.32	9.55 ± 0.15	11.80 ± 0.13	16.10 ± 0.18	17.45 ± 0.24
	Cell Associated	Xylanase	ND	1.67 ± 0.05	4.71 ± 0.61	13.81 ± 1.27	13.31 ± 1.65	17.10 ± 1.16	23.56 ± 1.80
		CMCase	1.54 ± 0.55	2.94 ± 0.14	3.74 ± 0.32	4.01 ± 0.50	3.75 ± 0.22	7.69 ± 0.95	6.55 ± 0.64
		Lichenase	ND	ND	1.26 ± 0.11	7.21 ± 0.36	8.77 ± 0.65	8.15 ± 0.31	8.20 ± 0.80
		Amylase	0.90 ± 0.18	0.44 ± 0.03	1.40 ± 0.38	2.56 ± 0.11	3.45 ± 0.16	5.15 ± 0.40	2.83 ± 0.67

[a]ND, not detected.

Table 2
Extracellular and cell associated glycoside hydrolase enzyme production of *Orpinomyces* sp. GMLF5 grown in avicel containing culture

		Enzyme	*Orpinomyces* sp. GMLF5 incubation time course (day)						
			1	2	3	4	5	6	7
Enzyme activities (U/ml)	Extracellular	pNP-X[a]	0.81 ± 0.08	0.49 ± 0.11	0.73 ± 0.07	0.61 ± 0.12	2.50 ± 0.45	3.89 ± 0.29	3.75 ± 0.50
		pNP-A[b]	0.08 ± 0.01	0.17 ± 0.03	0.15 ± 0.02	0.16 ± 0.03	0.17 ± 0.01	0.18 ± 0.01	0.21 ± 0.01
		pNP-G[c]	1.42 ± 0.12	3.61 ± 0.28	3.47 ± 0.23	10.72 ± 0.47	18.43 ± 1.32	22.98 ± 1.44	24.68 ± 0.52
		pNP-C[d]	ND	ND	ND	1.79 ± 0.23	1.39 ± 0.08	1.89 ± 0.07	2.12 ± 0.11
	Cell Associated	pNP-X	1.52 ± 0.14	2.48 ± 0.58	37.54 ± 0.96	160.91 ± 0.60	176.17 ± 3.33	75.76 ± 6.07	81.80 ± 7.66
		pNP-A	ND	0.40 ± 0.01	3.42 ± 0.06	12.80 ± 0.77	14.56 ± 0.15	7.94 ± 0.24	6.18 ± 0.14
		pNP-G	ND	2.10 ± 0.41	8.79 ± 1.84	14.31 ± 0.56	16.70 ± 1.60	19.44 ± 1.65	10.32 ± 1.62
		pNP-C	ND	ND	ND	ND	ND	ND	ND

[a]β-xylosidase; [b]α-L-arabinofuranosidase; [c]β-glucosidase; [d]β-cellobiosidase; ND, not detected.

nase, CMCase, lichenase, and amylase. CMCase and amylase were the pioneer enzymes that were detected at first day of incubation both in supernatant and cell associated fractions although they were observed relatively less amounts. Cellular and extracellular activities of xylanase, CMCase and lichenase were found proportional but amylase was mainly secreted into the culture medium.

β-xylosidase was seemed to be the major enzyme that produced by *Orpinomyces* sp. GMLF5 in avicel containing culture and the enzyme were accumulated mainly in cell. During the culturing period significant increase were noted and after the 4–5th day, marginal decrease were observed but still substantial β-Xylosidase activity were exist. β-glucosidase activity was found in both extracellular and cellular fraction and increased in time dependent manner. α-L-arabinofuranosidase activity was determined as to be associated with cellular debris and only a small proportion of the enzyme was released into the culture medium. Cell associated β-cellobiosidase activity was negligible whilst supernatant fraction of the culture at 4th day showed notable amount of activity which was maximized at 7th day of incubation. Glycoside hydrolase activities of the isolate GMLF5 were illustrated in Table 2.

Reducing sugar accumulation in the avicel containing culture medium of the isolate GMLF5 was analyzed and reducing sugar was started to accumulate after 4 days of incubation which is correlated with the high production of polysaccharidases and glycosidases in 4 to 7 days old culture (data not shown). Enzyme preparation of 7th day culture of GMLF5 was incubated with avicel (2%, w/v) and the enzymes exhibited high capacity to degrade microcrystalline cellulose. Reduced sugar was released from avicel in time dependent manner, 0.28 and 0.45 mM reduced sugar was liberated in 24 and 48 h, respectively.

Temperature and pH profiles of enzymes from *Orpinomyces* sp. GMLF5 was given in Fig. 2. All enzymes exhibited a similar profile and enzyme activities were increased by the temperature increments until the optimum of 50 °C. Enzyme activities declined over the temperature of 50 °C but amylase was differed from the other enzymes with the loss of 69% activity at 60 °C. Enzyme activities were remarkably affected by pH (Fig. 2). The favorable pH for xylanase, lichenase and amylase was found at 6.0; however, the optimum pH of CMCase was determined at the pH 6.5. All enzyme

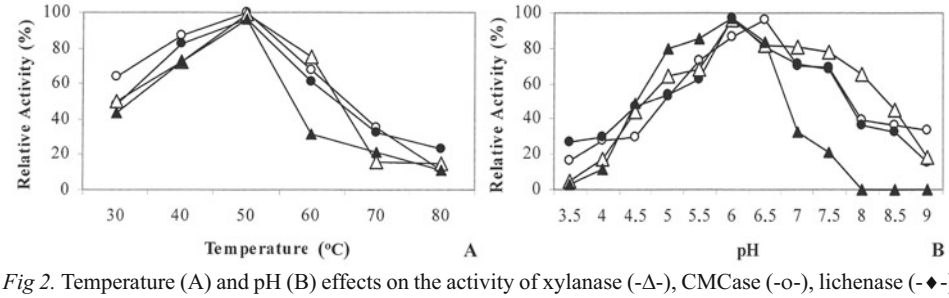

Fig 2. Temperature (A) and pH (B) effects on the activity of xylanase (-Δ-), CMCase (-o-), lichenase (-♦-) and amylase (-▲-) enzymes of *Orpinomyces* sp. GMLF5. Effect of the pH determined at 50 °C and effect of the temperature determined at pH 6.5. Relative activities are expressed as percentage of the maximum activity for each enzyme. Error bars were negligible that is why they were not shown

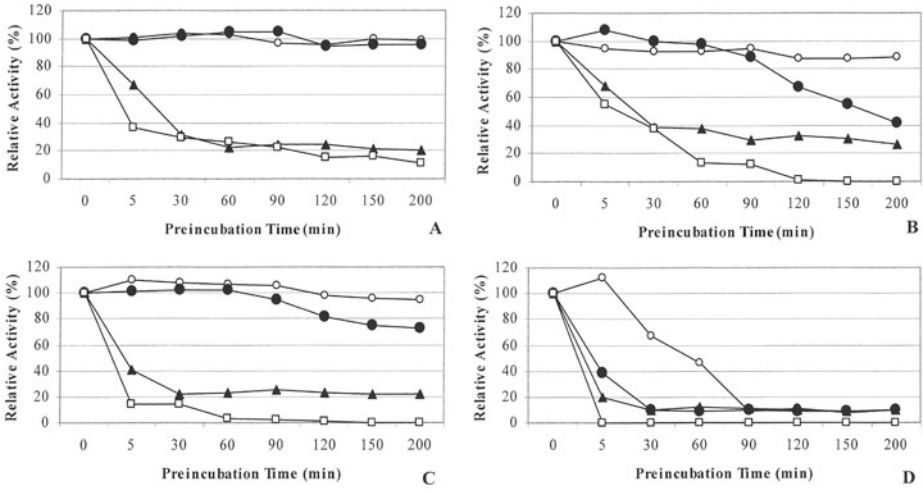

Fig 3. Thermal stabilities of xylanase (A), CMCase (B), lichenase (C) and amylase (D) produced by *Orpinomyces* sp. GMLF5. Relative activities are expressed as percentage of the original activity. Symbols: 40 °C (-o-), 50 °C (-•-), 60 °C (-▲-), 70 °C (-□-). Error bars were negligible that is why they were not shown

activities were observed less than 50% at outrange of pH 4.5–8.0. Alkaline pH was tending to have adverse effect mainly on amylase; on the other hand amylase was found to be more active at the pH ranges of 4.5–6.0. The pH profiles indicated that the enzyme complex from *Orpinomyces* sp. GMLF5 could be useful for any application at the pH ranges between 5.0 and 7.5 apart from amylase its activity decline rapidly during alkali pH values.

The results concerning the thermal inactivation of xylanase, CMCase, lichenase and amylase are shown in Fig. 3. Thermal stability studies were carried out by preincubating the enzymes up to 200 min in the range of 40–70 °C. Xylanase was reasonably stable at temperatures up to 50 °C, yet the remaining activities decreased very rapidly when denaturation takes place at 60–70 °C. The maximum thermal stability was obtained at 40 °C for CMCase and 88% of activity was still remained following incubation for at least 200 min. Under the same conditions, lichenase tend to be more stable than CMCase which preserved its 73% of activity at 50 °C after 200 min. Figure 2 has also shown that relatively short incubation periods at 40 and 50 °C resulted in substantial losses in amylase activity, and at the end of the 90 min amylase activity was reduced by 90%. Experiments were also carried out to estimate the enzyme stability concerning its storage at low temperatures (–20 °C and 4 °C). The crude enzyme preparation was preserved its full activity for 6 months when stored at –20 °C. The enzyme was stored for 100 hours at 4 °C and no activity loss was observed in xylanase, CMCase, lichenase and amylase activity.

DISCUSSION

Orpinomyces sp. GMLF5 produced the polysaccharidases of xylanase, CMCase, lichenase, amylase and glycosidases of β-xylosidase, β-glucosidase, α-L-arabinofuranosidase and minor amounts of β-cellobiosidase when they grown in the avicel containing culture. In similar culture conditions, *N. frontalis* produced enzymes capable of degrade cellulose, xylan and starch and the highest enzyme activities were reported in the extracellular fraction [20] which was parallel to the results of this study. Xylanase was known as the most active enzyme among the endo-acting polysaccharidases of rumen fungi [4, 19]. Xylanase activity level was found dependent on the carbon source used in the medium [19] and xylan was known as the best inducer for xylanase production [19] but avicel also enhanced the xylanase activity of *N. hurleyensis* [11], which was similar to our results, too. CMCase activity of *O. joyonii* A4 grown on avicel was reached the maximum level at 96 h in the extracellular fraction but cell associated activity was remained limited [12], however half of the activity was observed cell associated in the present study. Lichenase activity of *Orpinomyces* sp. GMLF5 was detected mostly in extracellular fraction but an important amount of activity was also found in cell associated. *Orpinomyces* sp. PC-2 secreted a lichenase into the culture medium [8] however *N. patriciarum* and *P. communis* exhibited their lichenase activities mostly on vegetative fractions [22].

It was observed that the amylase production of *Orpinomyces* sp. GMLF5 was enhanced by avicel when used as growth substrate. High amylolytic activities were measured in the vegetative preparations from *N. patriciarum*, *P. communis* and an isolate F grown on starch [22], whereas *N. frontalis* was released amylase mainly into the culture fluid, and starch and maltose was found to be best inducer for α-amylase production [18]. *O. joyonii* exhibited the great propensity to digest starch in wheat and barley grains more than *N. patriciarum* and *P. communis* due its extracellular amylase enzymes [16].

Cellular enzyme preparation of *Orpinomyces* sp. GMLF5 had substantial amounts of β-xylosidase, α-L-arabinofuranosidase and β-glucosidase activity. α-L-arabinofuranosidase activity of rumen fungi was observed in the vegetative cells or rhizoids by fluorescence microscopy [6] and high proportions of β-xylosidase activity located in the vegetative preparations was reported for rumen fungi [23] as in this study. On the other hand, β-xylosidase activities of monocentric isolates were observed relatively higher than *Orpinomyces* isolates [5]. β-Glucosidases of *Orpinomyces* sp. GMLF5 was showed similar distribution with the rumen fungi that reported by Williams and Orpin [23] whereas *Orpinomyces* sp. PC-2 were secreted their β-glucosidases into the culture medium [7] and β-glucosidase activity produced by *O. joyonii* A4 was mainly found in the cell wall fraction [12].

The optimum pH and temperature values of our study were in parallel to the findings of earlier reports focused on rumen fungal enzyme characteristics [7, 17, 18]. Thermal stability of the enzymes is a desirable property for particularly in industrial applications [24]. Xylanase of GMLF5 was found to be very stable at the temperature of 50 °C whereas CMCase and lichenase was stable at 40 °C for 200 min. Amylase

activity of the isolate was different from the other enzymes by rapid thermal inactivation even at lower temperatures. A xylanase from *Aspergillus foetidus* was found sensitive to 50 °C and 36% of activity was retained after 3 h [21]. *N. frontalis* amylase was found stable at 50 °C for 1 hour [18] whereas 30% of the xylanase activity obtained from the same fungus was lost after incubation at 50 °C in 1 hour [19]. Instability is reported for CMCases in the absence of substrate at the temperature of 50 °C [15, 17]. No activity loss was observed for purified lichenase enzyme from *Orpinomyces* sp. PC-2 at 40 °C for 24 h but inactivation was occurred at 55 °C after 1 hour incubation [8]. Another important aspect for industrial purposes is the storage of enzymes without an important activity loss. Enzymes obtained from the isolate GMLF5 were retained its 85–90% of activity at 4 °C for 100 h and its full activity at –20 °C for 6 months. Lowe et al. [15] observed negligible loss in CMCase activity of *Neocallimastix* at 4 °C, however 30% loss was observed in xylanase activity after 100 h incubation.

Effective degradation of avicel by the enzymes of anaerobic fungi is well established [11]. Enzymes from *Piromyces* sp. E2 and *Neocallimastix* N2 exhibited higher capacity to degrade the microcrystalline cellulose than the commercial enzymes [10]. This study demonstrated that the local isolate *Orpinomyces* sp. GMLF5 exhibited the capacity of xylanase, CMCase, lichenase, amylase, β-xylosidase, β-glucosidase, α-L-arabinofuranosidase and minor amounts of β-cellobiosidase production by utilizing avicel as the sole energy source. The enzyme preparation also degraded the avicel at a higher rate which encourage its use in processes performed at moderate temperatures and pH values.

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