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Full Length Research Paper

Optimizing culture conditions for the production of endo-β-1,4-glucanase by *Aspergillus awamori* strain Vietnam Type Culture Collection (VTCC)-F099

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In the present study, twenty six strains of *Aspergillus awamori* from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) were used for the endoglucanase production by growing at 37°C in the growth medium. Result showed that *A. awamori* strain VTCC-F099 produced the highest level of endo β-1,4-glucanase in the growth medium, pH 6.5, at 30°C for 96 h, agitated at 200 rpm. The optimal concentration of the inducer CMC (carboxymethyl cellulose) for the endoglucanase production by *A. awamori* VTCC-F099 was 2%. Among tested carbon sources (coconut fiber, coffee shell, corncob, dried tangerine skin, peanut shell, rice bran, saw dust, sugar-cane bagasse as organic wasters and glucose, lactose sucrose as pure carbon sources), corncob showed the highest endoglucanase production by *A. awamori* VTCC-F099 at the concentration of 3%. Ammonium acetate was the best among nitrogen source (casein, peptone, fish powder, soybean powder as organic sources and CH_3COONH_4, NH_4NO_3, $(NH_4)_2SO_4$, urea as inorganic sources) for the endoglucanase production by *A. awamori* VTCC-F099 at the concentration of 0.3%.

Key words: *Aspergillus awamori,* carboxymethyl cellulose, endoglucanase production, optimization of culture conditions.

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Murai et al., 1998; Hong et al., 2001). Biological degradation of cellulose involves the synergistic action of three enzymes: Endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1,4-glucanase, E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (exo β-1,4-glucanase, E.C. 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) (Gielkens et al., 1999; Kang et al., 1999). Endo- β -1, 4-glucanase randomly hydrolyzes internal β-1,4-D-glycosidic bonds in cellulose producing oligos and reducing polymer length, while exo- β-1,4-glucanase cleave cellobiosyl residues from the non-reducing end of cellulose chain. Then, cellobiose is hydrolyzed by β-glucosidase to yield two glucose units (Coughlan et al., 1985).

Cellulases have a broad variety of applications in food, animal feed (Ramamurthy et al., 1987), brewing, paper pulp, and detergent (Bhat and Bhat, 1997), textile (Belghiht et al., 2001; Anish et al., 2006), fuel and chemical industries as well as waste management and pollution treatment (Mandels 1985; Ole et al., 2002). Among theses enzymes, endoglucanases have been well studied and are produced by various microbes (bacteria, yeast, and fungi), plant, and protozoans. Especially, the filamentous fungi *Aspergillus* spp. (*awamori*, *fumigatus*, *niger*, *terreus*) are preeminent in endoglucanase produc-tion (Onsori et al., 2005; Gao et al., 2008; Grigorevski-Lima, 2009). The purpose of this present work was to produce the endo-

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Abbreviations: CMC, carboxymethyl cellulose; **CMCase,** carboxymethyl cellulose.

glucanase from easily available carbon and nitrogen sources by using *A. awamori* VTCC-F099.

MATERIALS AND METHODS

Fungal strains and culture conditions

Twenty six strains of *A. awamori* from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) were used for the endoglucanase production by growing at 37°C in the growth medium (Mandels et al., 1976) containing (w/v): 0.03% urea, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% $CaCl_2$, 0.03% $MgSO_4.7H_2O$, 0.1% peptone, 1% yeast extract, 0.1% Tween 80, 1% CMC. Trace elements were also added, using a 1% (v/v) solution of salts: 18 mM FeSO4.7H2O; 6.6 mM $MnSO_4$; 4.8 mM $ZnSO_4$, 15 mM $CoCl_2$. The initial pH of the production media was adjusted to 6.5-7.0 before sterilization.

Chemicals

Peptone and Tween 80 was purchased from Bio Basic Inc. (USA), yeast extract from Difco (USA), 3,5-dinitrosalicylic acid (DNS) from Fluka (Germany), carboxymethyl cellulose (CMC) from Prolabo (France), urea from Merck (Germany).

Endoglucanase assay

Endoglucanase activity was examined relatively by measurement of the halo diameter of enzyme diffusion on agar plates containing substrate: After 96 h of growth in the growth medium containing 0.5% (w/v) CMC, 50 µl of the culture supernatant were dropped into well on agar plates containing 0.5 % (w/v) CMC and incubated for 24 h at 4°C to diffuse enzyme. After that, the agar plates were incubated for further 24 h at 37°C (Incubator, Sanyo, Japan) and stained with 1% (w/v) lugol dye.

Endoglucanase activity was determined by Mandels et al. (1976) with 0.5% (w/v) CMC in 0.1 M potassium phosphate buffer pH 6.5. The amount of reducing sugars released in the reagent solution at 50°C for 20 min was read at the wavelength of 540 nm (spectrophotometer UV-2500, Hewlett Package, USA). Glucose was used as the standard for the estimation of reducing sugars. One unit of the endoglucanase activity was defined as the amount of enzyme required to release 1 µmol glucose per minute under experimental conditions.

Endo β-1,4-glucanase production

A. awamori VTCC-F099 was grown in 100 ml shaking flask containing 20 ml growth medium with initial pH 6.5 at 30°C, agitated at 200 rpm (Certomat HK, Sartorius, Germany). After every 24 h of cultivation, 1 ml of culture was obtained, centrifuged (MIKRO22, Hettich, Germany) and the supernatant was used to determine the endoglucanase activity as mentioned above.

Optimization of culture temperature

In order to determine the effect of culture temperature on the endo-glucanase production, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium for 96 h at different temperatures varied from 25, 28, 30, 32, 37°C and agitated at 200 rpm.

Optimization of pH

To optimize the initial pH, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing growth medium with an initial pH range of 3 to 8.

Optimization of inducer concentration

To investigate the effect of CMC as an inducer on the endoglucanase production, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium, pH 6.5 added with CMC at various concentrations ranging from 0.2 to 4% (w/v).

Optimization of carbon source and its concentration

To optimize the carbon source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium except yeast extract, which was substituted by another carbon source (coconut fiber, coffee shell, corncob, dried tangerine skin, peanut shell, rice bran, saw dust, sugar-cane bagasse, glucose, lactose, sucrose, yeast extract). After determination of the carbon source for the maximal endoglucanase production, *A. awamori* strain VTCC-F099 was grown in the growth medium containing corncob as the optimum carbon source at different concentrations ranging from 0.5 to 5% (w/v).

Optimization of nitrogen source and its concentration

To optimize the nitrogen source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml with carbon source optimized growth medium and one of various nitrogen sources including ammonium acetate, ammonium sulfate, casein, fish powder, peptone, soybean powder, and urea. After determination of the optimal nitrogen source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml optimal growth medium with an initial pH 6.5, with 3% corncob, and ammonium acetate at concentrations ranging from 0.1 to 0.55% (w/v).

RESULTS AND DISCUSSION

Screening *A. awamori* strains producing endoglucanase

The endoglucanase production by 26 filamentous *A. awamori* strains from Vietnam Type Culture Collection was examined; all of the strains showed endoglucanase activity on the agar plates containing CMC as the substrate. Among them, four strains showed the largest helo of clearing zone on CMC containing plates including *A. awamori* VTCC-F099 (29 mm), VTCC-F245 (28 mm), VTCC-F261 (27 mm), VTCC-F350 (26 mm) (Table 1). Among these fours strains, *A. awamori* VTCC-F099, VTCC-F245, and VTCC-F350 showed the highest levels of endoglucanase production in submerged fermentation, with an endoglucanase activity of 0.513, 0.489, and 0.484 U/ml, respectively. *A. awamori* strain VTCC-F099 was selected for optimization of the endoglucanse production (Figure 1).

Table 1. Endoglucanase production by 26 *A. awamori* strains. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

A. awamori strain	Diameter of clearing zone (mm)	CMCase activity (U/ml)	*A. awamori* strain	Diameter of clearing zone (mm)	CMCase activity (U/ml)
VTCC-F-014	12.5 ± 0.35	0.42 ± 0.020	VTCC-F-261	27 ± 0.30	0.33 ± 0.027
VTCC-F-020	12.5 ± 0.42	0.38 ± 0.011	VTCC-F-262	19 ± 0.34	0.33 ± 0.017
VTCC-F-061	15 ± 0.21	0.45 ± 0.035	VTCC-F-269	15.5 ± 0.45	0.23 ± 0.010
VTCC-F-062	13.5 ± 0.23	0.46 ± 0.065	VTCC-F-270	9.5 ± 0.48	0.38 ± 0.020
VTCC-F-063	15 ± 0.32	0.38 ± 0.025	VTCC-F-296	4 ± 0.50	0.34 ± 0.018
VTCC-F-064	18 ± 0.41	0.42 ± 0.042	VTCC-F-311	13 ± 0.56	0.40 ± 0.021
VTCC-F099	29 ± 0.35	0.51 ± 0.059	VTCC-F-312	14 ± 0.48	0.39 ± 0.033
VTCC-F-100	17.5 ± 0.12	0.45 ± 0.040	VTCC-F-317	15.5 ± 0.10	0.41 ± 0.018
VTCC-F-135	15.5 ± 0.05	0.43 ± 0.124	VTCC-F-350	26 ± 0.46	0.48 ± 0.007
VTCC-F-207	15 ± 0.25	0.33 ± .0120	VTCC-F-353	19 ± 0.70	0.36 ± 0.023
VTCC-F-229	18.5 ± 0.16	0.47 ± 0.021	VTCC-F-356	15 ± 0.59	0.39 ± 0.040
VTCC-F-245	28 ± 0.57	0.49 ± 0.069	VTCC-F-401	16 ±0.32	0.46 ± 0.057
VTCC-F-259	14 ± 0.43	0.44 ± 0.020	VTCC-F-406	14.5 ± 0.21	0.46 ± 0.028

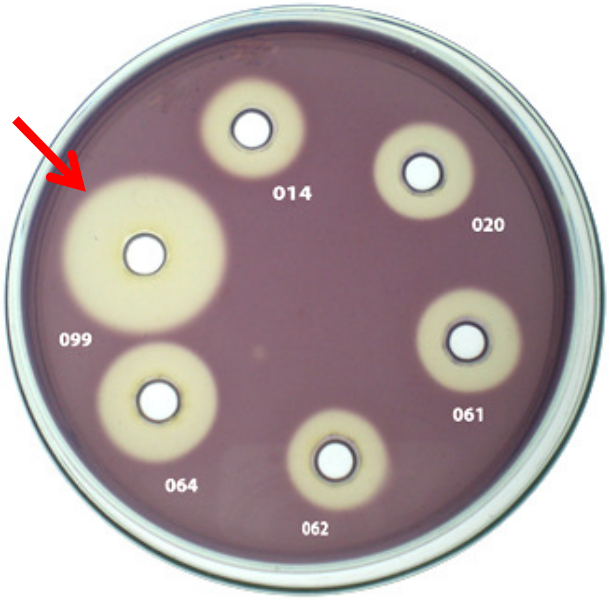

Figure 1. Endoglucanase activity on agar plates containing 0.5% (w/v) CMC and staining with lugol. 014, 020, 061, 062, 064, 099, are abbreviated for *A. awamori* VTCC-F014, *A. awamori* VTCC-F020, *A. awamori* VTCC-F061, *A. awamori* VTCC-F062, *A. awamori* VTCC-F064, and *A. awamori* VTCC-F099.

Endo β-1,4-glucanase production course

The endoglucanase production by *A. awamori* VTCC- F099 increased gradually from 0.41 U/ml (74%) at 24 h of cultivation to the maximum of 0.55 U/ml (100%) at 96 h of cultivation (Figure 2). Then the endoglucanase production decreased strongly to 0.39 U/ml (71%) at 120 h of cultivation and remained constant 0.39 - 0.33 U/ml (71 to 60%) in a long time interval from 120 to 240 h of cultivation (Figure 2).

The highest levels of endoglucanase production by *A. fumigatus* corresponded to 0.365 U/ml and was obtained using sugarcane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation within 6 days of cultivation (Grigorevski-Lima, 2009).

Optimization of cultivation temperature

The endoglucanase production by *A. awamori* VTCC-F099 was maximum (0.551 U/ml) at 30°C (Figure 3). The optimum temperature for endoglucanase production by *A. fumigatus* (Grigorevski-Lima, 2009) was also 30°C.

Optimization of initial medium pH

A. awamori VTCC-F099 produced the highest levels of endoglucanase (5.22 U/ml) at the initial medium pH 6.5 (Figure 4). At the lower (pH 3) or higher (pH 8) initial medium pH, the endoglucanase production was also very high 4.59 U/ml (88%) and 3.65 U/ml (70%) in comparison to the maximum at the initial pH of 6.5, respectively.

Optimization of inducer concentration

The CMC is a substrate for endoglucanases and showed an induction effect on the endoglucanase production. The addition of CMC at the concentration from 0.2 to 2% to the growth medium increased the endoglucanase production gradually from 0.17 U/ml (21%) to the maximum 0.81 U/ml. The addition of more CMC (at the concentration of 2.5 - 4%) to the culture medium decreased the endoglucanase production gradually to

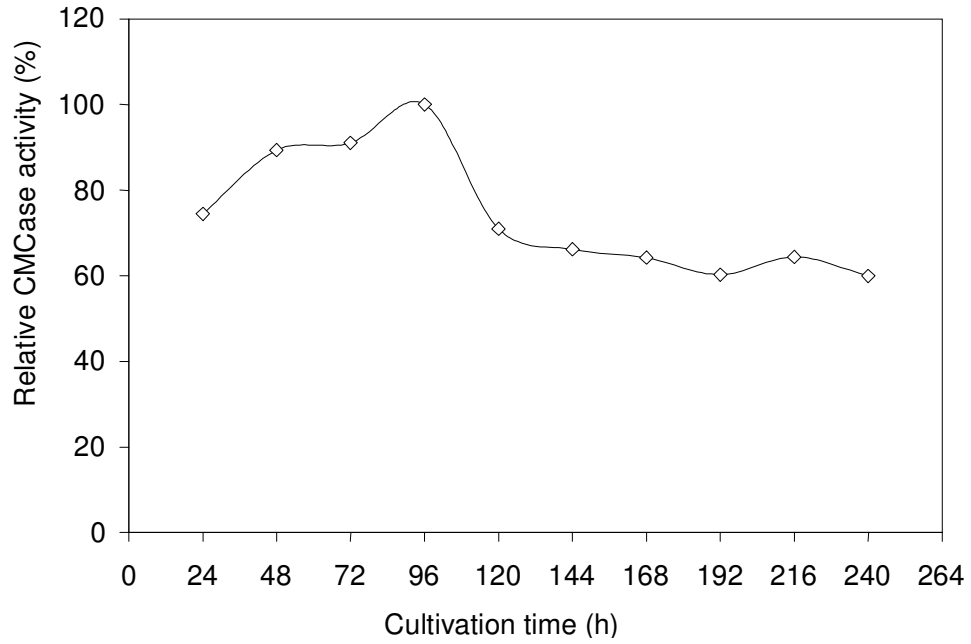

Figure 2. Time course of the endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

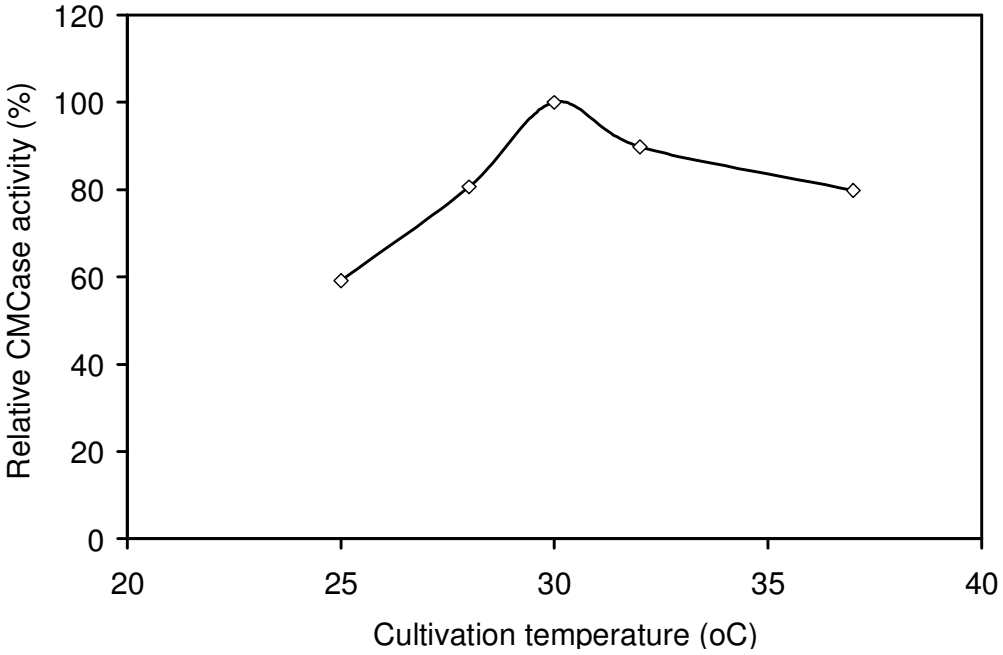

Figure 3. Effect of cultivation temperature on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

0.42 U/ml (52%). No addition of CMC showed a similar effect on endoglucanase production as the addition of 0.2% CMC, just 0.15 U/ml (18%) in comparison to the maximum production (Figure 5).

Optimization of carbon source and its concentration

Among the investigated carbon sources, corncob was the most appropriate carbon source for the endoglucanase

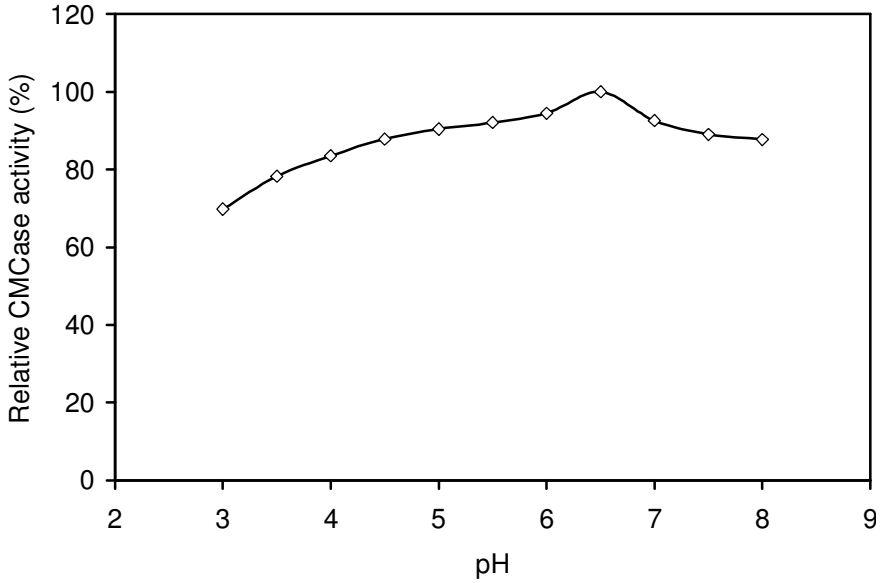

Figure 4. Effect of initial pH of culture medium on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

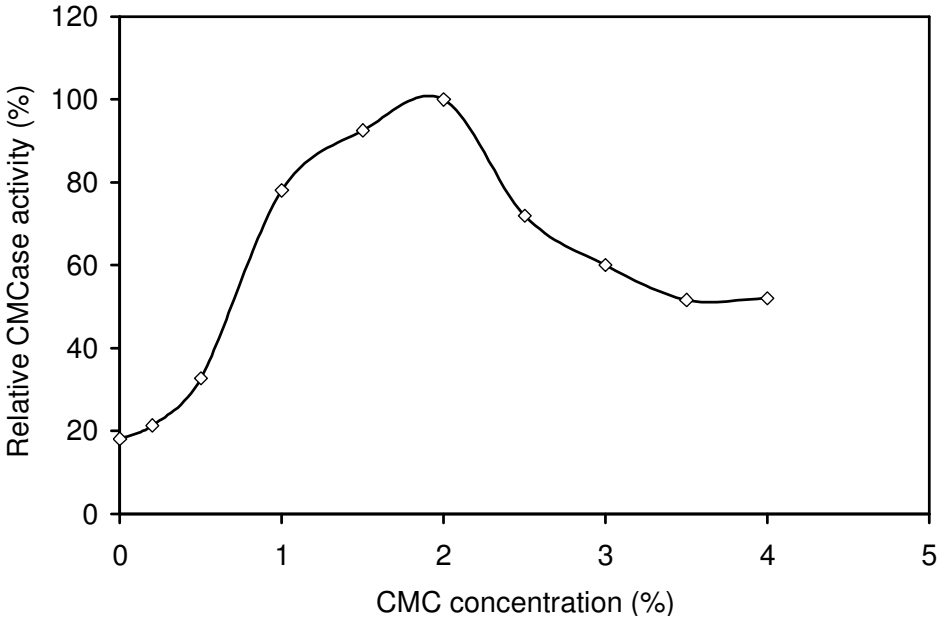

Figure 5. Effect of CMC concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

production (0.868 U/ml, 100%) by *A. niger* VTCC-F099 (Table 2). Other carbon sources produced the enzyme at the level of 34-85% in comparison to corncob as carbon source (Table 2). Sugar-cane bagasse was a suitable carbon source for the endoglucanase production by *A. fumigatus* strain (Grigorevski-Lima, 2009).

The endoglucanase production by *A. awamori* VTCC-F099 increased from 0.64 U/ml (71%) in the growth medium containing 0.5% corncob to the maximum 0.90 U/ml in the growth medium containing 3% corncob (Figure 6). The endoglucanase production decreased gradually to 0.53 U/ml (59%) in the growth medium containing 5% corncob. The highest level of endoglucanase by *A. fumigatus* was obtained using 1% sugarcane

Table 2. Effect of carbon source on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

Carbon source	Endoglucanase activity	
	U/ml	%
Coconut fiber	0.521 ± 0.046	53
Coffee shell	0.643 ± 0.125	74
Corncob	0.868 ± 0.233	100
Dried mandarin skin	0.511 ± 0.055	59
Glucose	0.686 ± 0.146	79
Lactose	0.295 ± 0.087	34
Peanut shell	0.553 ± 0.117	64
Rice bran	0.554 ± 0.048	64
Sucrose	0.607 ± 0.038	70
Saw dust	0.411 ± 0.089	42
Sugarcane bagasse	0.734 ± 0.196	85
Yeast extract	0.652 ± 0.212	75

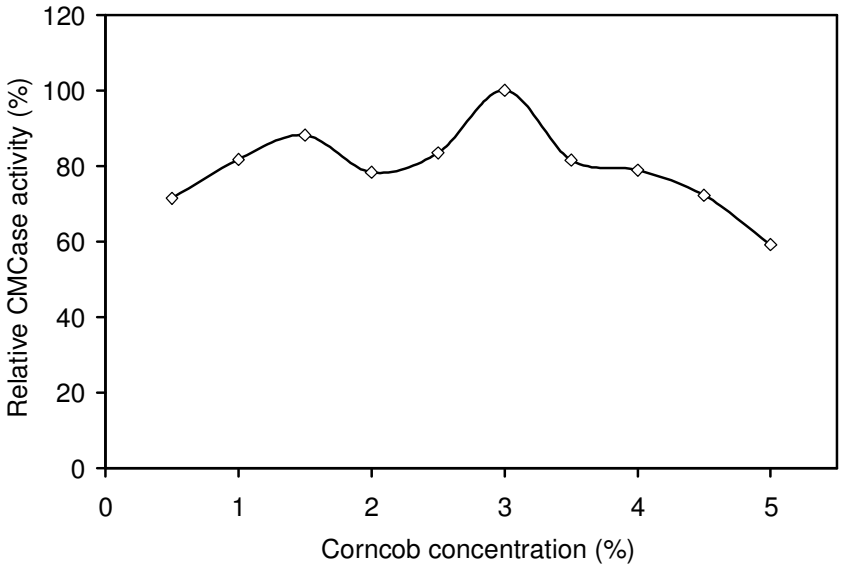

Figure 6. Effect of corncob concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

ugarcane bagasse (Grigorevski-Lima, 2009).

Optimization of nitrogen source and its concentration

Among the examined nitrogen sources (Table 3), ammonium acetate was the best nitrogen source for the endoglucanase production by *A. awamori* VTCC-F099 (4.85 U/ml). The enzyme production in growth medium containing other nitrogen sources was obtained only (70 - 81%) in comparison to ammonium acetate (Table 3). *A. awamori* VTCC-F099 produced the highest levels of endoglucanase amount (4.98 U/ml) when ammonium acetate was used with the amount of 0.3% (Figure 7). The lowest (0.1%) and highest (0.55%) amount of ammonium acetate in the growth medium reduced the endoglucanase production to 71% (3.53 U/ml) and 68% (3.39 U/ml) in comparison to the optimal amount of ammonium acetate 0.3%.

Conclusion

In conclusion, *A. awamori* VTCC-F099 is capable of

Table 3. Effect of nitrogen source on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

Nitrogen source	Endoglucanase activity	
	U/ml	%
Ammonium acetate	4.847 ± 0.189	100
Ammonium sulfate	4.187 ± 0.124	87
Casein	3.440 ± 0.128	71
Fish powder	4.101 ± 0.118	85
Peptone	4.101 ± 0.150	75
Soybean powder	3.508 ± 0.115	72
Urea	3.866 ± 0.077	80

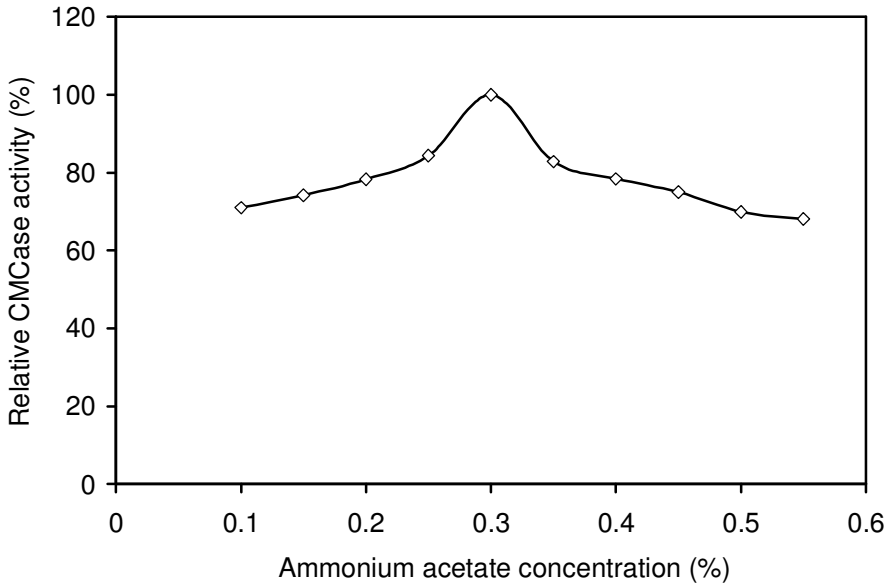

Figure 7. Effect of ammonium acetate concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

producing endoglucanase from corncob and ammonium acetate. The endoglucanase production from corncob and ammonium acetate was highest at 96 h of production, with an initial pH 6.5 and at 30°C.

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