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EFFECTS OF CULTURE CONDITIONS ON THE PRODUCTION OF ENDO-β-1,4-GLUCANASE BY *ASPERGILLUS AWAMORI* VTCC-F-099

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SUMMARY

Cellulases are a group of hydrolytic enzymes and are capable of degrading lignocellulosic materials. These enzymes are produced by several microorganisms including bacteria and fungi. Endo β-1,4-glucanase is one of three types cellulase, which has a wide range of applications such as in food, animal feed, pulp industry, waste management, *etc. Aspergillus awamori* VTCC-F-099 (*A. awamori* VTCC-F-099) strain produced the highest amount of endo β-1,4-glucanase (CMCase) among 26 *A. awamori* strains were screened. Some culture conditions for *A. awamori* VTCC-F-099 strain producing CMCase were examined to assess their effect on enzyme production. CMCase production of the strain *A. awamori* VTCC-F-099 was the highest after 96 hours of fermentation in MT1 medium with 2% CMC. The optimum temperature and pH for CMCase production from the strain *A. awamori* VTCC-F-099 were 30°C and 6.5, respectively. The strain *A. awamori* VTCC-F-099 produced CMCase highest in case the corncob used as carbon source (3.0%) among tested carbon sources (lactose, glucose, saccharose, sugar-cane bagasse, coffee grounds, rice brand, corncob, peanut shell, dried mandarin shell, saw dust, coconut fiber). Ammonium acetate (0.3%) was the best nitrogen source for the strain *A. awamori* VTCC-F-099 producing highest CMCase among tested nitrogen sources (peptone, urea, fish powder, soybean powder, casein, CH_3COONH_4, NH_4NO_3, $(NH_4)_2SO_4$).

Keywords: *Aspergillus awamori VTCC-F-099, CMCase, culttivation conditions, endoglucanase, optimization*

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Hong *et al.*, 2001; Murai *et al.*, 1998). It is an unbranched glucose polymer composed of an β-1,4 glucose units linked by a β-1,4-D-glycosidic bond (Gielkens *et al.*, 1999). Cellulose is commonly degraded by an enzyme complex called cellulase. Cellulases can be classified into three types: endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1,4-glucanase, EC 3.2.1.4), exoglucanase or cellobiohydrolase (exo β-1,4-glucanase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) (Gielkens *et al.*, 1999; Kang *et al.*, 1999). Endo β-1,4-glucanase randomly hydrolyze internal β-1,4-D-glycosidic bonds in cellulose. Cellulases have a wide range of applications. Potential applications are in food, animal feed (Ramamurthy *et al.*, 1987), brewing, paper pulp, and detergent industries (Bhat and Bhat, 1997), textile industry (Anish *et al.*, 2006; Belghiht *et al.*, 2001), fuel, chemical industries, waste management and pollution treatment (Mandels, 1985; Ole *et al.*, 2002; Wu, Lee, 1997). Cellulases are produced by different microorganisms such as fungi, bacteria, yeast, plant, protozoa, etc. Especially, the fungus *Aspergillus spp. (fumigatus, oryzae, niger, kawachi, terreus)* and *Trichodderma spp.* are pre-eminent in cellulases production (Acharya *et al.*, 2008; Gao *et al.*, 2008; Immanuel *et al.*, 2006; Mangelli, Forchiassin, 1999; Ojumu *et al.*, 2003; Onsori *et al.*, 2005; Shin *et al.*, 2000; Solomon *et al.*, 1999). Numerous studies on producing cellulase by fungal have been done and some optimal conditions for producing of cellulase from *A. flavus* Linn Isolate NSPR 101 (Ojumu *et al.*, 2003), *Aspergillus sp.* (R4) (Onsori *et al.*, 2005), *A. terrus* (Ali *et al.*, 1991; Pothiraj *et al.*, 2006), *A. niger* (Acharya *et al.*, 2008) have been studied. The recent thrust in bioconversion of agricultural and industrial wastes to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by fungi and bacteria (Baig *et al.*, 2004; Solomon *et al.*, 1999). In this study, *A. awamori* VTCC-F-099 was selected among 26 surveyed *A. awamori* for optimization of cultivation conditions for producing of endo β-1,4-glucanase.

MATERIALS AND METHODS

Fungal strains and media

26 strains of *A. awamori* were isolated from soil samples from various locations in Viet Nam. All strains were used as the enzyme source. They were maintained on Czapek-Dox agar slant at 4°C.

Medium composition described by Mandles (MT1 medium) was used for CMCase production. The basic MT1 medium consist of (g/l): urea, 0.3; $(NH4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$, 0.3, $MgSO_4.7H_2O$, 0.3; peptone, 1; yeast extract, 10; Tween 80, 1; CMC, 10; Trace elements were also added, using 1% (v/v) solution of salts: $FeSO_4.7H_2O$ 18 mM; $MnSO_4$ 6.6 mM; $ZnSO_4$ 4.8 mM, $CoCl_2$ 15 mM. The pH was adjusted to 7.0 before sterilization (Mandels *et al.*, 1976).

Chemicals

Chemicals used in experiment were purchased from various companies such as peptone (Bio Basic Inc), yeast extract (Difco), 3,5-dinitrosalicylic acid (DNS) (Fluka), carboxymethyl cellulose (CMC) (Prolabo), tween-80 (Bio Basic Inc), urea (Merck) etc.

CMCase assay

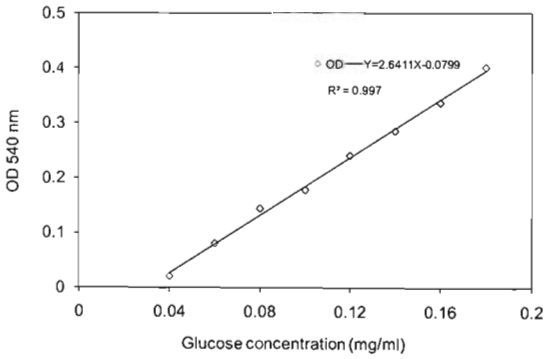

Figure 1. The standard curve of glucose-OD 540 nm.

CMCase activity was determined by Miller's spectrometric method (1959) with 0.5% CMC (w/v) in 0.1 M potassium phosphate buffer at pH 6.5. The content of reducing sugars released in the reagent solution at the temperature of 50°C for 20 minutes was determined by spectrometry at the wavelength of 540 nm (Figure 1). The absorption was compared with the standard curve of glucose to calculate the equivalent content of reducing sugars. One unit of CMCase activity is defined as the amount of hydrolyzed catalytic enzyme required to release 1 µmol glucose per minute under experiment conditions.

Endo β-1,4-glucanase production

Cultivation of *A. awamori* VTCC-F-099 was carried out in 500 ml flask containing 200 ml MT1 at 30°C, initial pH 7.0 on rotary shaker with 200 rotations per minute (rpm). After every 24 hours of fermentation, 1 ml of cultivation broth was taken to determine the CMCase activity.

Selection of temperature

In order to determine the effect of culture temperature on CMCase production, *A. awamori* VTCC-F-099 strain was cultivated in shake flasks for 96 hours at 200 rpm at different temperatures of 25, 28, 30, 32, 37°C in MT1 medium, initial pH 7.0.

Selection of inducer concentration

In order to determine the inducer concentration, *A. awamori* VTCC-F-099 strain was grown in the MT1 medium, initial pH 7.0, CMC concentration ranging from 0.2 to 4.0% (w/v).

Selection of carbon source and its concentration

A. awamori VTCC-F-099 strain was cultured in MT1 medium, initial pH 7.0 for 96 hours in which yeast extract was replaced with another carbon source (lactose, glucose, saccharose, sugar-cane bagasse, coffee grounds, rice straw, corncob, peanut shell, dried mandarin shell, saw dust, coconut fiber) at the same concentration. After determination of the best carbon source, *A. awamori* VTCC-F-099 strain was grown in the MT1 medium with the best carbon source of different concentration ranging from 0.5 to 5% (w/v).

Selection of nitrogen source and its concentration

The source of nitrogen in the basic MT1 medium was the peptone used to culture *A. awamori* VTCC-F-099 strain. This is a very good source of nitrogen. However, it is not suitable for production of later enzyme products due to its high cost. In order to enable the use of available materials, peptone was replaced with other nitrogen sources such as urea, ammonium sulfate, ammonium nitrate, ammonium acetate, casein, soybean powder, fish powder at the

same concentration. After determination the best nitrogen source, *A. awamori* VTCC-F-099 strain was shaken flask for 96 hours at 200 rpm in MT1 medium with 3% corncob at 30°C, initial pH 7.0 and ammonium acetate at concentrations ranging from 0.1 to 0.55% (w/v).

Selection of pH

The *A. awamori* VTCC-F-099 strain was shaken flask for 96 hours at 200 rpm in MT1 medium with 2% CMC, 3% corncob, 0.3% ammonium acetate, at 30°C and initial pH ranges from 3.0 to 8.0 to determine the optimum pH medium.

RESULT

Selection of the strain over producing CMCase

The selection of over producing CMCase from *A. awamori* strains was based on the diameter of clearing zone on CMC containing agar plates (Figure 2) and CMCase specific activity. The size of clearing zone diameter and CMCase specific activity for each isolate are shown in Table 1. Among 26 *A. awamori* strains were screened, the *A. awamori* VTCC-F-099 strain produced CMCase as the highest (0.51 IU/ml).

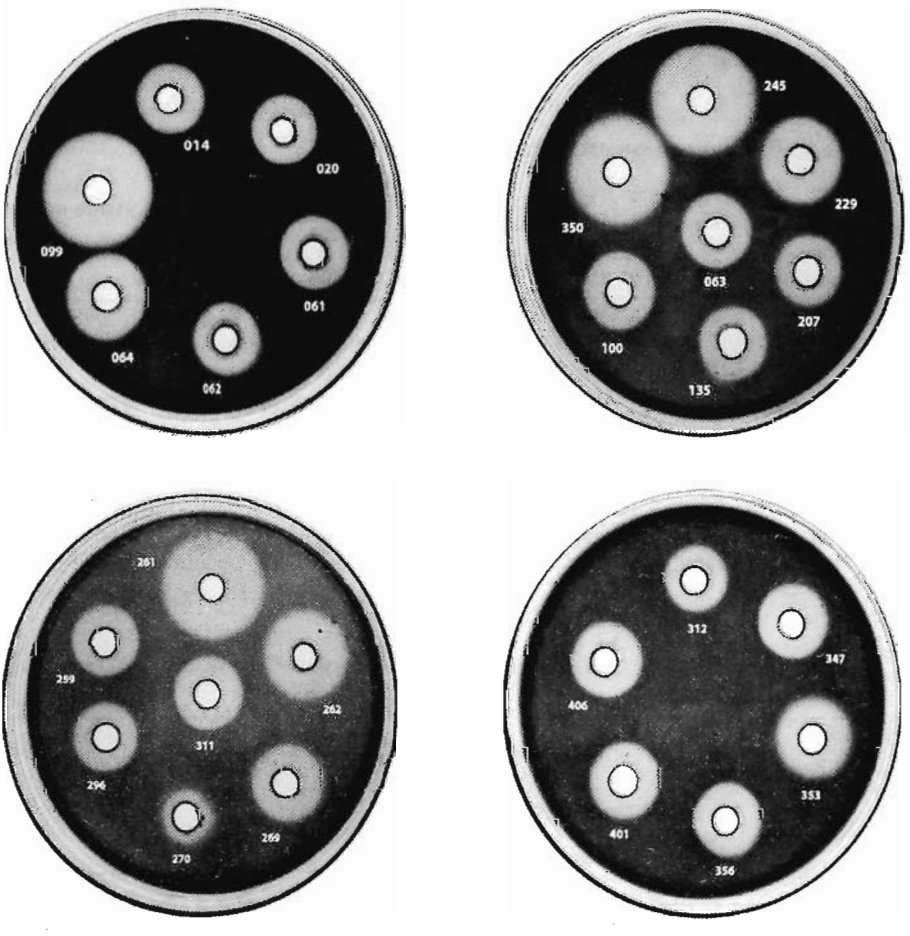

Figure 2. Determination of CMCase activity on agar plates.

Table 1. CMCase activity of 26 *A. awamori* strains.

Strain	Diameter of Clearing zone (mm)	CMCase activity (U/ml)	Strain	Diameter of Clearing zone (mm)	CMCase activity (U/ml)
VTCC-F-014	12.5	0.42	VTCC-F-261	27.0	0.33
VTCC-F-020	12.5	0.38	VTCC-F-262	19.0	0.33
VTCC-F-061	15.0	0.45	VTCC-F-269	15.5	0.23
VTCC-F-062	13.5	0.46	VTCC-F-270	9.5	0.38
VTCC-F-063	15.0	0.38	VTCC-F-296	4.0	0.34
VTCC-F-064	18.0	0.42	VTCC-F-311	13.0	0.40
VTCC-F-099	**29.0**	**0.51**	VTCC-F-312	14.0	0.40
VTCC-F-100	17.5	0.45	VTCC-F-317	15.5	0.41
VTCC-F-135	15.5	0.43	VTCC-F-350	26.0	0.48
VTCC-F-207	15.0	0.33	VTCC-F-353	19.0	0.36
VTCC-F-229	18.5	0.47	VTCC-F-356	15.0	0.40
VTCC-F-245	28.0	0.49	VTCC-F-401	16.0	0.46
VTCC-F-259	14.0	0.44	VTCC-F-406	14.5	0.46

Endo β-1,4-glucanase production

The CMCase production of the *A. awamori* VTCC-F-099 strain increased after 24 hours and reached the highest level of 0.55 IU/ml after 96 hours culture. Then the CMCase activity reduced (Figure 3).

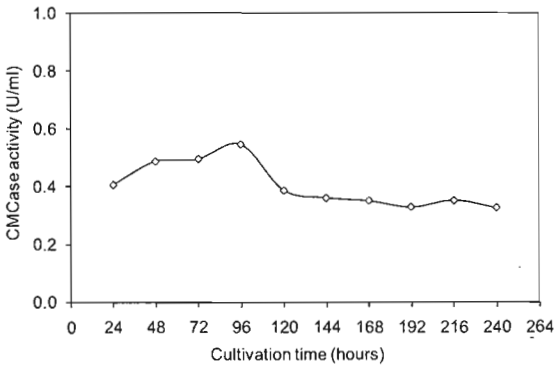

Figure 3. Time course of CMCase activity of the strain *A. awamori* VTCC-F-099.

Effect of temperature

At the temperature of 30°C, *A. awamori* VTCC-F-099 strain obtained the highest CMCase production (0.56 IU/ml) (Figure 4).

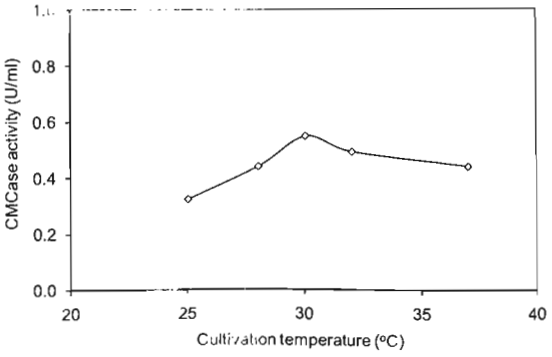

Figure 4. The effect of culture temperature on CMCase production by *A. awamori* VTCC-F-099.

Effect of inducer concentration

CMC was used in culture medium at concentrations ranging from 0.2 to 4.0% (w/v). The strain *A. awamori* VTCC-F-099 produced CMCase highest (0.81 IU/ml) at the concentration of CMC 3.0% (w/v) (Figure 5).

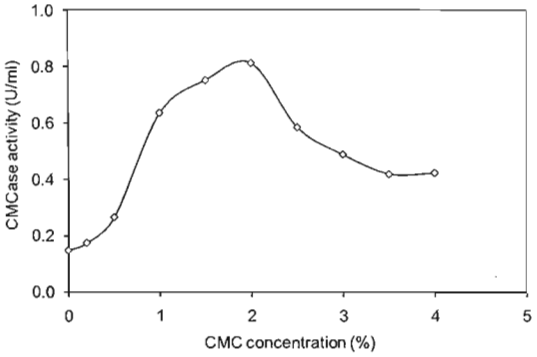

Figure 5. The effect of CMC concentrations on CMCase production by *A. awamori* VTCC-F-099.

Effect of carbon source and its concentration

Among the surveyed carbon sources (lactose, glucose, saccharose, sugar-cane bagasse, coffee grounds, rice brand, corncob, peanut shell, dried mandarin shell, saw dust, coconut fiber), the corncob was the best carbon source to CMCase production which were 0.87 IU/ml by *A. awamori* VTCC-F-099 (Table 2).

After the corncob was determined the best carbon source, it was added to the medium with concentrations from 0.5 to 5.0%. The strain *A. awamori* VTCC-F-099 produced CMCase highest (0.90 IU/ml) at the corncob concentration of 3.0% (Figure 6).

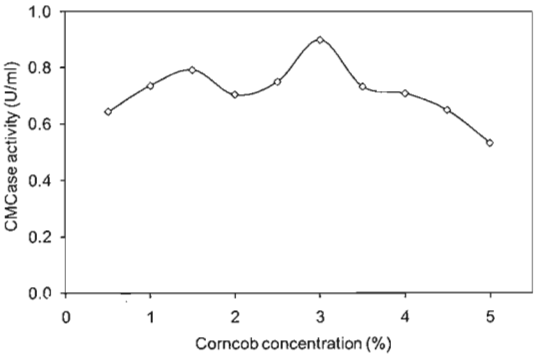

Figure 6. The effect of corncob concentrations on CMCase production by *A. awamori* VTCC-F-099.

Effect of nitrogen source and its concentration

Among the surveyed nitrogen sources, ammonium acetate was the best nitrogen source for CMCase production by *A. awamori* VTCC-F-099 (4.88 IU/ml). However, ammonium sulfate, as an inorganic salt, and fish powder, as a cheap and available source of nitrogen, were also suitable for the production of CMCase (Table 3).

After ammonium acetate was determined as the best nitrogen source, it was added to the medium at the different concentrations from 0.1 to 0.55%. The CMCase production by *A. awamori* VTCC-F-099 achieved the highest level (4.97 IU/ml) at 0.3% (w/v) ammonium acetate concentration (Figure 7).

Table 2. The effect of carbon source on CMCase production by *A. awamori* VTCC-F-099.

Carbon source	CMCase activity		Carbon source	CMCase activity	
	IU/ml	%		IU/ml	%
Yeast extract	0.65	74	Rice brand	0.57	65
Glucose	0.70	80	**Corncob**	**0.87**	**100%**
Lactose	0.28	32	Peanut shell	0.54	62
Sucrose	0.60	69	Dried mandarin shell	0.51	59
Sugar-cane bagasse	0.75	86	Saw dust	0.39	45
Coffee grounds	0.64	74	Coconut fiber	0.52	60

Table 3. The effect of nitrogen source on CMCase production by *A. awamori* VTCC-F-099.

Nitrogen source	CMCase activity		Nitrogen source	CMCase activity	
	IU/ml	%		IU/ml	%
Peptone	3.67	75	Casein	3.44	71
Fish powder	4.10	84	Urea	3.87	79
Ammonium sulfate	4.19	86	Soybean powder	3.51	72
Ammonium acetate	**4.88**	**100**			

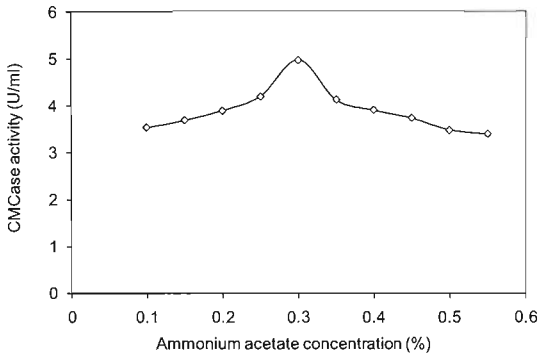

Figure 7. The effect of ammonium acetate concentration on CMCase production by *A. awamori* VTCC-F-099.

Effect of pH

The effect of pH on CMCase production was determined at initial pH from 3.0 to 8.0 and the highest CMCase activity (5.22 IU/ml) was obtained at pH 6.5 (Figure 8).

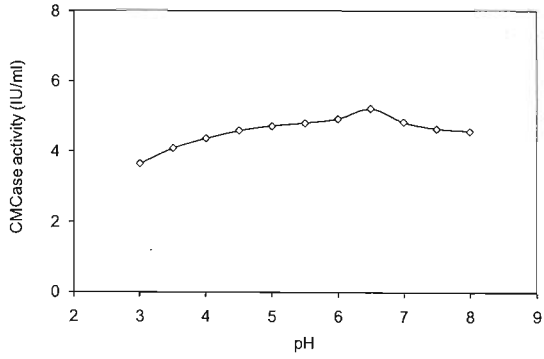

Figure 8. The effect of initial medium pH on CMCase production of the strain *A. awamori* VTCC-F-099.

DISSCUSION

A. awamori is known to produce a variety of cellulolytic enzymes including endo β-1,4-glucanase (CMCase). In this study, we identified the *A. awamori* VTCC-F-099 strain produced CMCase with the highest yield among the 26 *A. awamori* strains. Some culture medium conditions were optimized. The *A. awamori* VTCC-F-099 strain produced CMCase with the highest yield (0.545 IU/ml) after 96 hours of culture. Our result also matched with other studies. *Trichoderma harzianam, Trichoderma spp, Phanerochaete chrysosporium* and *Aspergillus niger* strains produced the highest CMCase level of 1.88, 1.53, 2.40 and 0.096 IU/ml of CMCase activity was achieved after 96 hours fermentation, respectively (Acharya *et al.*, 2008; Khan *et al.*, 2007). The highest level of cellulase activity from *A. flavus* was obtained at the 12 hours of fermentation (Ojumu *et al.*, 2003). The optimum temperature and pH for CMCase production from microbes were various. Akiba *et al.* (1995) observed that the optimum pH for CMCase producing from *A. niger* was found to be between 6.0 and 7.0 (Akiba *et al.*, 1995). In another report the optimum pH and temperature for cellulase production from *A. niger* were between 4.0 - 4.5 and 28°C, respectively (Acharya *et al.*, 2008). The strain *A. awamori* VTCC-F-099 produced CMCase highest at the temperature of 30°C and pH 6.5.

CMC was shown as an inducer for CMCase production. In our study, CMC was used in culture medium at various concentrations from 0.2 to 4.0% (w/v) and CMCase production of *A. awamori* VTCC-F-099 strain was highest at CMC concentration of 2% (w/v).

The carbon and nitrogen sources effected on the growth and enzyme production by microbes. In this study, we used various carbon and nitrogen

sources for examination. Among the surveyed carbon sources, the corncob (3%) was the best for CMCase production by *A. awamori* VTCC-F-099 (0.87 IU/ml). Ojumu et al (2003) reported the highest level of cellulase activity when 3% pretreated saw dust substrate was used (0.0743 IU/ml) (Ojumu *et al.*, 2003). The activity of CMCase obtained from *A. niger* was maximum (0.1813 IU/ml) when 9.6% saw dust was used as the carbon source (Acharya *et al.*, 2008). Ammonium acetate (0.3%) was the best among various nitrogen sources (urea, ammonium sulfate, ammonium nitrate, ammonium acetate, casein, peptone, soybean powder, fish powder) used for CMCase production by *A. awamori* VTCC-F-099 (4.98 IU/ml). According to studies of Narasimha et al (2006), at 0.03% urea, peptone and $NaNO_3$ used as nitrogen source, the cellulase activity obtained were 0.824, 0.421 and 0.401 IU/ml, respectively (Narasimha *et al.*, 2006).

CONCLUSION

We identified the *A. awamori* VTCC-F-099 strain produced CMCase with the highest yield among the 26 *A. awamori* strains. Some cultivation conditions were optimized. The *A. awamori* VTCC-F-099 strain produced CMCase with the highest yield (0.545 U/ml) after 96 hours of cultivation, at 30°C and pH 6.5. CMC (2%) was shown as the best inducer for CMCase production by *A. awamori* VTCC-F-099. Among the examined carbon sources, the corncob (3%) was the best for CMCase production by *A. awamori* VTCC-F-099 (0.90 U/ml). Ammonium acetate (0.3%) was the best nitrogen source among various nitrogen sources (urea, ammonium sulfate, ammonium nitrate, ammonium acetate, casein, peptone, soybean powder, fish powder) used for CMCase production by *A. awamori* VTCC-F-099 (4.97 U/ml).

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ẢNH HƯỞNG MỘT SỐ ĐIỀU KIỆN NUÔI CẤY CHỦNG *ASPERGILLUS AWAMORI* VTCC-F-099 SINH TỔNG HỢP ENDO-β-1,4 GLUCANASE

Nguyễn Văn Tuân, Quyền Đình Thi*

Viện Công nghệ sinh học

TÓM TẮT

Endo-β-1,4 glucanase là một trong ba nhóm enzyme thuộc hệ cellulase, tham gia thủy phân các liên kết β-1,4 glucoside ở bên trong các phân tử cellulose và một số loại polysaccharide tương tự khác. Enzyme này chủ yếu có nguồn gốc từ vi sinh vật, trong đó có các loài nấm mốc. Mục đích của nghiên cứu này là tuyển chọn chủng *Aspergillus awamori* tự nhiên sinh tổng hợp endoglucanase cao và tìm môi trường tối ưu cho khả năng sinh tổng hợp endoglucanase của chủng nấm mốc này. Từ 26 chủng *A. awamori* đã tuyển chọn được một chủng *A. awamori* VTCC-F-099 có khả năng sinh tổng hợp endoglucanase cao nhất. Một số điều kiện thích hợp cho khả năng sinh tổng hợp endoglucanase của chủng nấm mốc trên đã được khảo sát. Khả năng sinh tổng hợp endoglucanase của chủng này mạnh nhất sau 96 h nuôi cấy ở nhiệt độ 30°C và pH 6,5. Cơ chất cảm ứng được sử dụng trong môi trường nuôi cấy là CMC (Cacboxylmethylcellulose) với nồng độ tối ưu là 2% (w/v). Nguồn carbon thích hợp nhất cho khả năng sinh tổng hợp endoglucanase của chủng nấm mốc trên là lõi ngô (3%), với hoạt tính đạt 0,90 IU/ml. Amonium acetate với nồng độ 0,3% là nguồn nitrogen tốt nhất để chủng *A. awamori* 099 sinh tổng hợp endoglucanase (hoạt tính đạt 4,97 IU/ml).

Từ khóa: *Aspergillus awamori VTCC-F-099, CMCase, điều kiện nuôi cấy, endoglucanase, tối ưu*

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