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Optimal conditions for *Aspergilus fumigatus* to biosynthesize endo-ß-1,4-glucanase and its physico-chemical characteristics

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Abstract
Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to smaller sugar components like glucose units. These enzymes are produced by fungi and bacteria. The aim of this research was to identify a *Aspergillus fumigatus* with over production of endo-β-1,4-glucanase. Properties of endo-β-1,4-glucanase from a culture filtrate of the *Aspergillus fumigatus* was also studied. Our results show that *Aspergillus fumigatus* can biosythesized endoglucanase utmost after 96 hours of cultivation in MT1 medium, pH 6.5, shaking 200 rpm at 30^{o} C, substrate CMC 2%, carbon source from soybean waste (3%) and ammonium acetate (0.3%). This endoglucanase enzyme presents some major properties: optimal reaction at temperature 50 ^{o}C, pH 5.0; stability at 30-50 ^{o}C, pH 4.5-5.5. At 5, 10 and 15 mM endoglucanase activity is increased by Fe^{2+}, Cu^{2+} and EDTA. Meanwhile Ag^{+}, K^{+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, CO^{2+} can decreased endoglucanase activity; especially Ag^{+} can decreased endoglucanase dramatically to 43% at concentratio 15 mM after 2 hours of incubation at 37^{o}C. Several solvents such as acetone, ethanol, methanol, iso propanol and n-butanol are able to reduce endoglucanase activity. Partically, 30% methanol decreases endoglucanase activity to 52% after 2 hours of incubation at 37 ^{o}C. Detergents are demonstrated in lowering endoglucanase activity; especially SDS can reduce this enzyme activity to 20% after incubation with 2% SDS in 2 hours at 37 ^{o}C.

Keywords: *Aspergillus* fumigatus, biosynthesis, endo-β-1,4-glucanase.

1. 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). Cellulose has enormous potential as a renewable source of energy (Coral *et al*., 2002) and several microorganisms use it as a carbon source. It has also attracted the interest of biotechnologists, who wish to use it as a renewable source of fuels and chemicals (Beguin, 1990). Cellulolytic enzymes degrade cellulose by cleaving the glycosidic bonds (Han *et al*., 1995). A number of fungi and bacteria are capable of producing multiple groups of enzymes, which are collectively known as cellulases, that act in a synergistic manner to hydrolyze the β-1,4-D-glycosidic bonds within the cellulose molecules (Akiba *et al*.1995). Cellulases can be classified into three types: endoglucanases (endo-β-1,4glucanase) and exoglucanases (exo-β-1,4-glucanase) and and β-glucosidases (β-D-glucoside glucohydrolase).
Endo-ß-1,4-glucanase can be synthesized from different sources such as bacteria, actinobacteria, fungi and yeast. There are several species of fungi able to biosynthesize endo-ß-1,4-glucanase such as *Aspergillus niger* (Coral G *et al*., 2002; Dahot MU *et al*., 1996), *A. flavus*, *A. fumigatus* (Das M *et al*., 1997), *A. terreus* (Gao J *et al*., 2008), T*richoderma reesei* [53], *Penicillium persicinum, P. brasilianum* (Henning J *et al*., 2005), *Penicillium* sp., *Phanerochaete chrysosporium* (Henriksson G *et al*., 1999). Beside fungi, bacteria are also able to
biosynthesize endo-ß-1,4-glucanase such as *Acidothemus cellulobuticus* (Bergquist PL *et al*., 1999), *Bacillus pumilis* (Gordon R *et al*., 1973), *Cellulomonas flavigena, C. udai* (Bagnara C *et al*., 1985, 1987), *Pseudomonas fluoressens (*Kim BH, 1987*), Clostridium* (Sharma VK, Hagen JC, 1995). Some actinobacteria are also able to biosynthesize endo-ß-1,4-glucanase such as *Actinomyces griseus, Streptomyces reticuli* (Wachinger G *et al*.,1989). Moreover endo-ß-1,4-glucanase is also produced from plant *Arabidopsis* (Campillo ED, 1999), protozoa and molusk (Watanabe H, Tokuda G, 1997; *Mytilus edulis* (Xu B *et al*., 2001).
Purpose of our research is to investigate optimal conditions for *Aspergilus fumigatus* to biosynthesize endo-ß-1,4-glucanase and its physico-chemical characteristics.

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2. Material & Method

2.1 Material

Aspergilus fumigatus strain is supplied from Pasteur Institute, HCM City, Vietnam

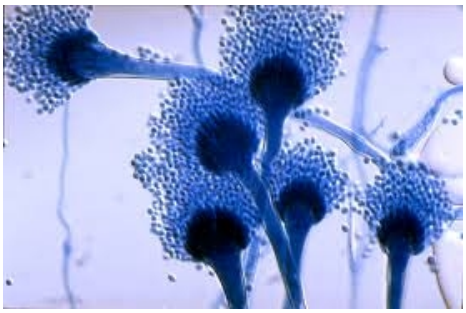

Fig 1: *Aspergilus fumigatus*

2.2 Research method

2.2.1 Fungi cultivation

Aspergilus fumigatus strains are activated in Czapek medium at 30 ^{o}C, pH 6.5, and shaked 200 rpm in 72 hours. After being activated they are cultured in MT1 medium with 0.5% CMC as substrate at 30 °C, shaked at 200 rpm. After 96 hours of cultivation, we collect broth, centrifigue to get crude enzyme.

2.2.2 Determination of endoglucanase activity

2.2.2.1 Qualification of endoglucanase

Qualification of endoglucanase is executed by diffusion of enzyme on agar dishes. These dishes contain 0.5% CMC with 100 mM potassium phosphate pH 6.5, thickness 0.5 cm with diameter of holes 0.5 cm. Take 50 µl of enzyme into holes, incubate 12 hours at 4 ^{o}C, and continue incubating 8 hours at 37 ^{o}C. Drop 1% lugol and measure the decomposition circle.

2.2.2.2 Determination of endoglucanase activity

Endoglucanase activity is determined by the reduced sugar formation under the spectrophotometry.

2.2.3 Optimal condition to endoglucanase biosythesis

2.2.3.1 Cultivation time

Aspergilus fumigatus is cultivated under submergence in MT1 medium to biosythesize endoglucanase. We examine different cultivation times from 24-240 hours to determine endoglucanase activity.

2.2.3.2 Effect of cultivation temperature

Aspergilus fumigatus is cultivated under submergence in MT1 medium to biosythesize endoglucanase. We examine different cultivation temperature 25 ^{o}C, 28 ^{o}C, 30 ^{o}C, 32 ^{o}C, 37 ^{o}C at pH 7.0 after 96 hours to determine endoglucanase activity.

2.2.3.3 Effect of substrate concentration

Aspergilus fumigatus is cultivated under submergence in MT1 medium to biosythesize endoglucanase. We examine different CMC substrate concentration 0.2-4.0% at pH 7.0, 200 rpm shaking, and 30 ^{o}C. After 96 hours of cultivation, we determine endoglucanase activity.

2.2.3.4 Effect of carbon source and its concentration

Aspergilus fumigatus is cultivated under submergence in MT1 medium with 2% CMC, 30 ^{o}C, pH 7.0, 200 rpm shaking. We examine different carbon souces and different carbon concentration (0-5% w/v). After 96 hours of cultivation, we determine endoglucanase activity.

2.2.3.5 Effect of nitrogen sources

Aspergilus fumigatus is cultivated under submergence in MT1 medium with 2% CMC, 30 ^{o}C, pH 7.0, 200 rpm shaking. We examine different nitrogen souces (ammonium sulphate, urea, and peptone) in the same concentration. After 96 hours of cultivation, we determine endoglucanase activity.

2.2.3.6 Effect of pH in cultivation

Aspergilus fumigatus is cultivated under submergence in MT1 medium with 2% CMC, 30 ^{o}C, 200 rpm shaking, 3% soybean waste, 0.3% ammonium acetate. We examine different pH values 3.0-8.0. After 96 hours of cultivation, we determine endoglucanase activity.

2.2.4 Enzyme purification

Enzyme filtrate is purified by Sephadex G-100

2.2.5 Physio-chemical characteristics of endoglucanase

2.2.5.1 Effect of substrate concentration

In order to find the appropriate substrate concentration for endoglucanase activity, we examine different CMC concentration 0.2-2.0% in 100 mM potassium phosphate, pH 6.5. Enyme activity is defined after 20 minutes at 50 ^{o}C.

2.2.5.2 Optimal temperature and thermal stability

A mixture of enzyme (0.002 mg protein) with 1.2% (w/v) CMC in potassium phosphate pH 6.5 is incubated at different temperatures 30-70 ^{o}C, in 20 minutes to verify the optimal temperature.

Enzyme (0.002 mg protein) is incubated at different times: 6, 12, 24, 36, 48, 60 and 72 hours at 30-70 ^{o}C, in 20 minutes to verify the thermal stability.

2.2.5.3 Optimal pH and pH stability

In order to determine the optimal pH, we use enzyme (0.002 mg protein) incubated with CMC in 100 mM sodium acetate, pH 3.5-5.5 and 100 mM potassium phosphate, pH 6.0-8.0. The mixture is incubated at 50 ^{o}C in 20 minutes.

In order to determine the pH stability, endoglucanase (0.002 mg protein) is incubated in various pH 3.5-8.0, in 2 hours at 37 ^{o}C. After that, we choose the pH to examine enzyme stability by times 6, 12, 24, 36, 48, 60, 72 hours.

2.2.5.4 Effect of solvent

In order to evaluate the effect of solvent to endoglucanase activity, enzyme is incubated with 10-30% solvents: acetone, ethanol, isopropanol, methanol and n-butanol at 37-C. After 2 hours of incubation, we analyse the remaining enzyme activity.

2.2.5.5 Effect of detergents

Enzyme (0.002 mg protein) is incubated with 0.5-2.0% detergents: tween 20, tween 80, SDS and triton X-100 at temperature 37 ^{o}C. After 2 hours of incubation, we analyse the remaining enzyme activity.

2.2.5.6 Effect of metal ion

In order to evaluate the effect of metal ion to endoglucanase activity, enzyme (0.002 mg protein) is incubated with Ag^{+},

Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Ni^{2+}, Zn^{2+} and EDTA at concentration 5-15 mM at 37 °C. After 2 hours of incubation, we analyse the remaining enzyme activity.

2.2.6 Determination of total protein
Total protein is determined by Bradford method.

2.3 Statistical analysis
All data are processeed by Excel 2003.

3. Result & Discussion
3.1 Optimal conditions for endoglucanase biosynthesis
3.1.1 Endoglucanase biosynthesis by time
Endoglucanase biosynthesis from *Aspergilus fumigatus* increases from 24-96 hours. At 96 hours of incubation, endoglucanase is maximun 0.55 IU/ml.

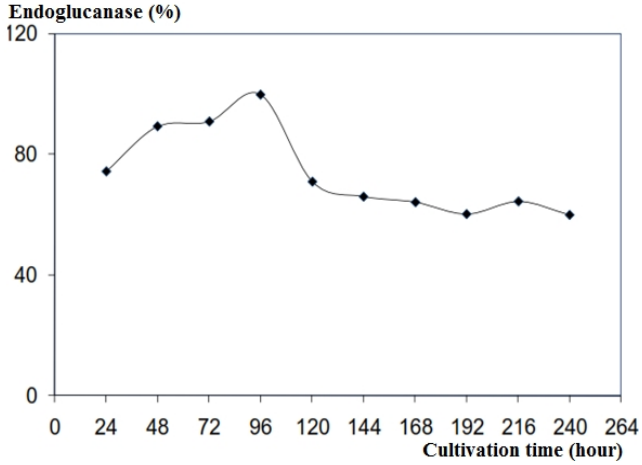

Fig 2: Endoglucanase biosynthesis from Aspergilus fumigatus by cultivation time

3.1.2 Cultivation temperature
Endoglucanase activity increases from 25 °C to 30 °C. At 30 °C, its activity reaches 0.56 IU/ml. At higher temperature, endoglucanase activity decreases gradually. At 37 °C, enzyme activity remains 0.44 IU/ml (78% compared to maximum value).

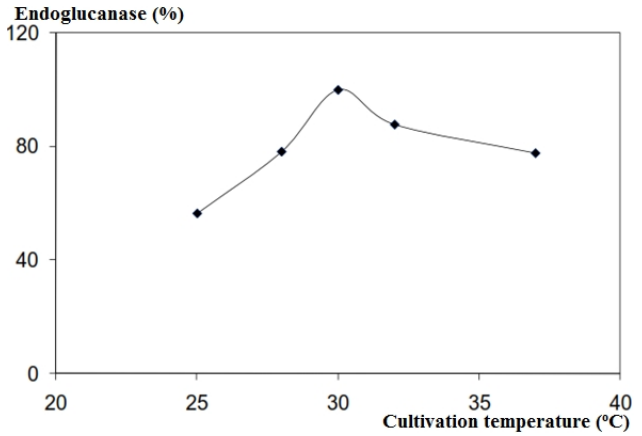

Fig 3: Endoglucanase biosynthesis from *Aspergilus fumigatus* by cultivation temperature

3.1.3 Effect of substrate concentration
Endoglucanase activity increases from 0-2% CMC; optimal at 2% (0.81 IU/ml).

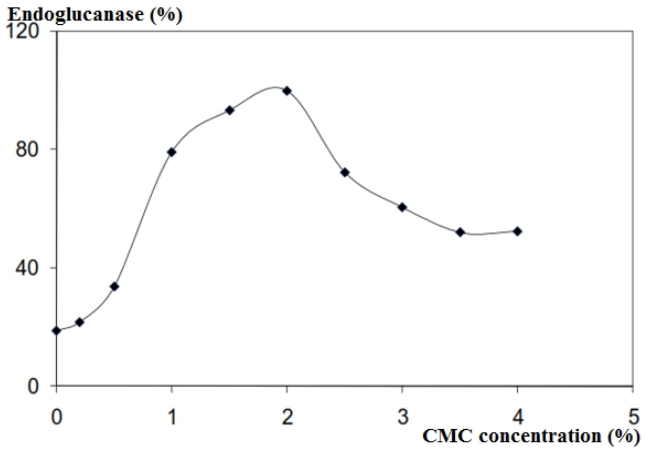

Fig 4: Endoglucanase biosynthesis from *Aspergilus fumigatus* by CMC concentration

3.1.4 Effect of carbon source and carbon concentration
Enzyme activity is maximum with soyben waste (0.87 IU/ml), following sugarcane waste (0.75 IU/ml) and glucose (0.70 IU/ml)

Table 1: Effect of carbon source

Carbon source	Endoglucanase activity	
	IU/ml	%
Sugarcane waste	0.75±0.146	86
Yeast extract	0.65±0.166	74
Soybean waste	0.87±0.158	100
Glucose	0.70±0.134	80

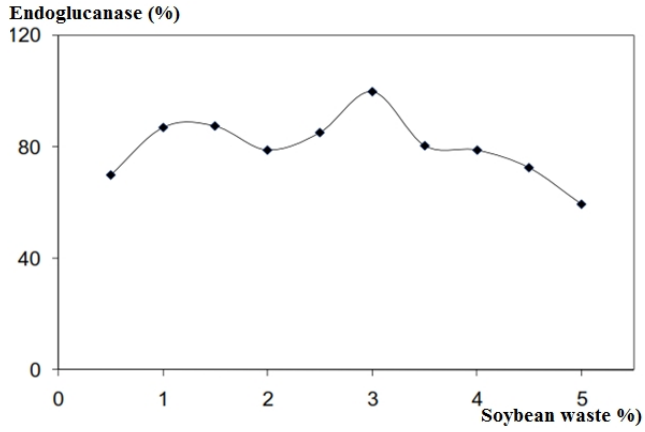

Fig 5: Endoglucanase biosynthesis from *Aspergilus fumigatus* by soybean waste concentration

3.1.5 Effect of nitrogen source and nitrogen concentration
Among different nitrogen sources, ammonium acetate is defined the appropriate source for *Aspergilus fumigatus* biosynthesize endoglucanase (4.88 IU/ml).

Table 2: Effect of nitrogen source

Nitrogen source	Endoglucanase activity	
	IU/ml	%
Ammonium acetate	4.88±0.129	100
Ammonium nitrate	3.91±0.104	80
Ammonium sulphate	4.19±0.124	86
Fish powder	4.10±0.118	84
Soybean powder	3.51±0.115	72
Peptone	3.67±0.105	75

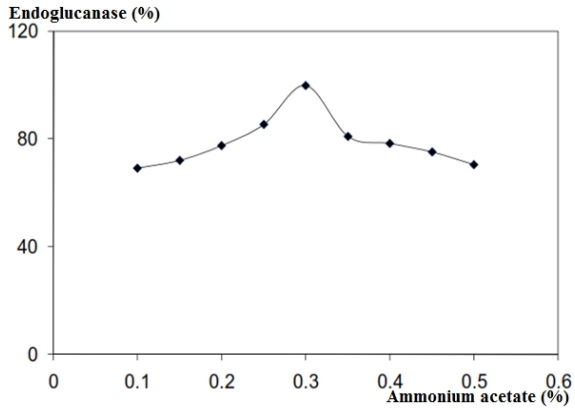

Fig 6: Endoglucanase biosynthesis from *Aspergilus fumigatus* by ammonium acetate

Enzyme activity increases in ammonium acetate range 0.1-0.25%, maximum at 0.3% amonium acetate (4.97 IU/ml).

3.1.6 Effect of cultivation pH

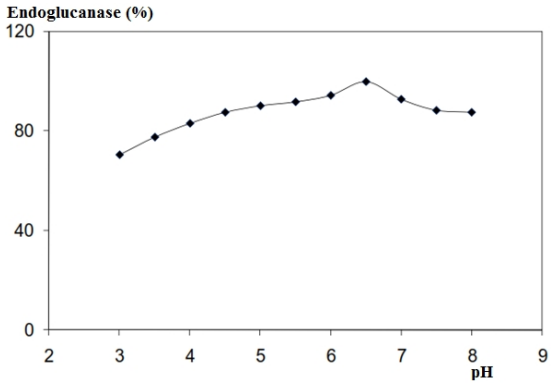

Fig 7: Endoglucanase biosynthesis from *Aspergilus fumigatus* by pH cultivation

Endoglucanase activity increases in pH range 3.0-6.5, maximum at pH 6.5 (5.22 IU/ml).

3.2 Physiochemical characteristics of endoglucanase
3.2.1 Effect of substrate concentration

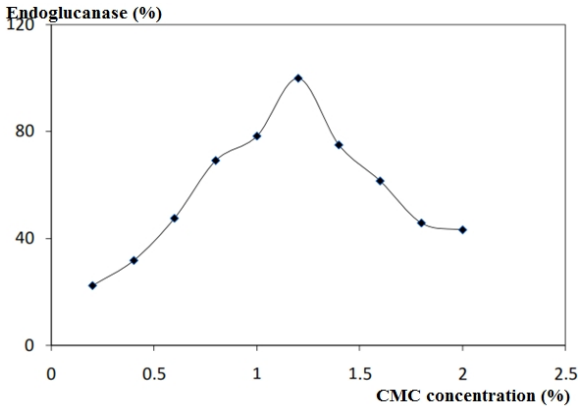

Fig 8: Effect os substrate concentration to endoglucanase activity

Endoglucanase activity increases in range 0.2-1.2% CMC, maximum at 1.2% CMC.

3.2.2 Optimal temperature

When increasing temperature 30 to 50 ºC, endoglucanase activity increases 42% to 100%. If continue increasing temperature over 50 ºC endoglucanase activity decreases.

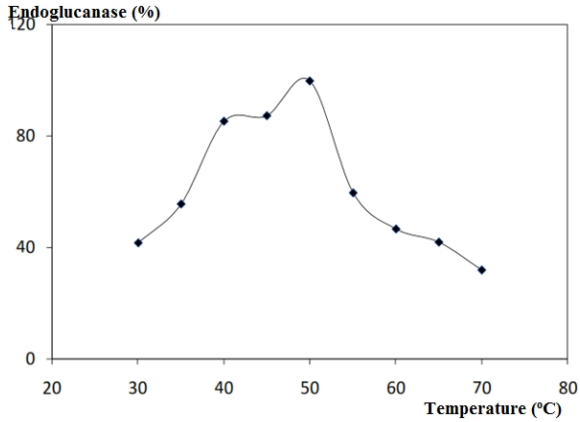

Fig 9: Effect of temperature to endoglucanase activity

3.2.3 Optimal pH

Reaction between enzyme and substrate is optimal at temperature 50 ºC, substrate 1.2% CMC in buffer sodium acetate and potassium phosphate having pH range 4.0-8.0

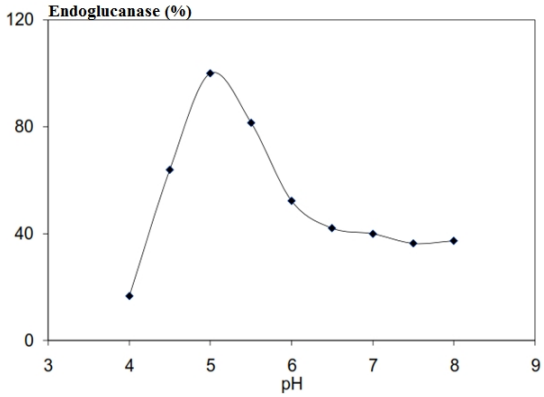

Fig 10: Effect of pH to endoglucanase activity

Endoglucanase activity increases in pH 4.0-5.0, maxium at pH 5.0 (4.08 IU/ml).

3.2.4 Thermal stability

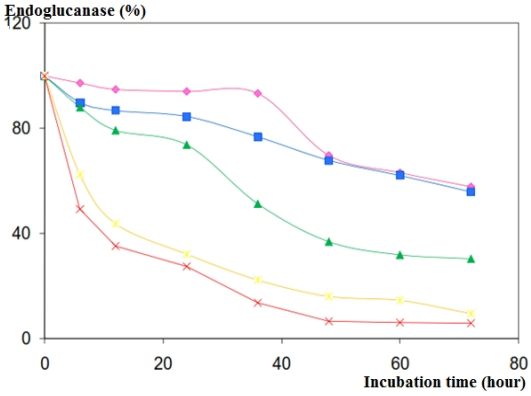

Fig 11: Effect os temperature to endoglucanase stability (◆): 30 °C; (■): 40 °C; (▸): 50 °C; (*): 60 °C; (✕): 70 °C

After 24 hours of incubations at 30ºC and 40ºC, enzyme activity decreases slightly to 94% and 85%. At 50ºC enzyme activity remains 79% after 24 hours of incubation. So temperature 30-50ºC is suitable for enzyme stability.

3.2.5 Effect of pH to enzyme stability

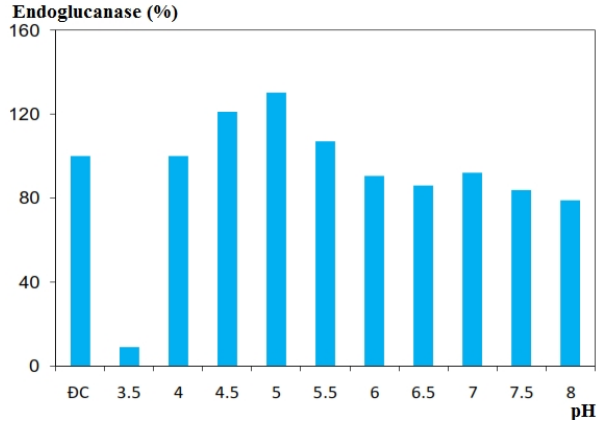

Fig 12: Effect of pH to endoglucanase stability

After 2 hours of incubation, endoglucanase is stable at sodium acetate pH 4.5-5.5.

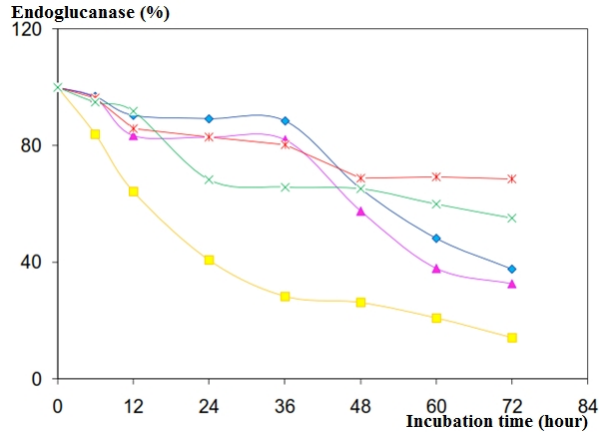

Fig 13: Effect of pH to endoglucanase stability (■): pH 4.0; (▲): pH 4.5; (◆): pH 5.0; (*): pH 5.5; (×): pH 6.0

3.2.6 Effect of organic solvent

After 2 hours of incubation with 30% (v/v) ethanol at 37°C, endoglucanase activity remains 53% compared to control. Ethanol is the strongest inactivation solvent and acetone is the weakest inactivation solvent.

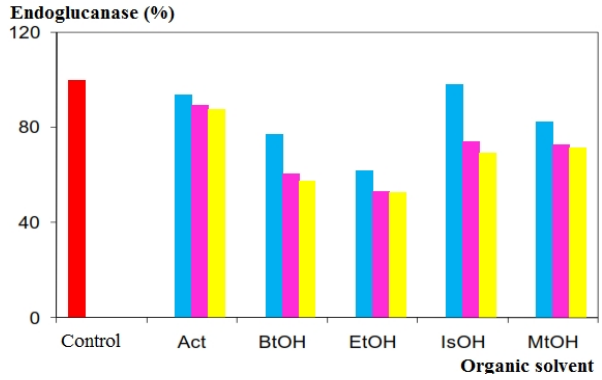

Fig 14: Effect of organic solvent to endoglucanase (■): 0%; (■): 10%; (■): 20%; (■): 30%; Act: Acetone; BtOH: n-Butanol; EtOH: Ethanol; IsoOH: Isopropanol; MtOH: Methamol.

3.2.7 Effect of metal ion

Table 3: Effect of metal ion

Metal ion concentration (mM)	Endogluacanse activity after 2 hours of incubation (%)		
	5	10	15
Ag^{+}	74	70	43
Ca^{2+}	97	95	90
Co^{2+}	99	96	79
Cu^{2+}	126	137	148
EDTA	146	153	155
Fe^{2+}	129	120	107
K^{+}	92	86	87
Mn^{2+}	70	63	54
Ni^{2+}	96	93	74
Zn^{2+}	94	79	78

Several ions such as Fe^{2+}, Cu^{2+} and EDTA increase enzyme activity. Meanwhile, some ions Ag^{+}, Ca^{2+}, Co^{2+}, K^{+}, Zn^{2+} decrease enzyme activity.

3.2.8 Effect of detergent

We examine different detergents Tween 20, Tween 80, SDS, Triton X-100 at different concentrations 0.5; 1.0; 1.5; 2.0%. After 2 hours of incubation, tween 20 at concentration 0.5-1.0% slightly affects to endoglucanase activity.

Triton X-100 decreases enzyme activity from 82% to 59% at concentration 0.5-2.0%. Regarding to SDS, endoglucanase is strongly inactivated.

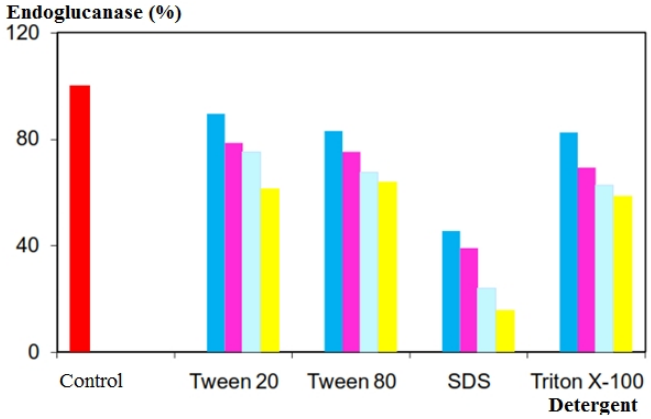

Fig 15: Effect of detergent to endoglucanase stability (■): 0%; (■): 0,5%; (■): 1%; (■): 1,5%; (■): 2%.

4. Conclusion

Endo-β-1,4-glucanases have wide applications in textile, paper and food industries. Most neutral cellulase producers are bacteria or anaerobic fungi. In this work, we have successfully investigated optimal conditions for *Aspergilus fumigatus* to biosynthesize endo-ß-1,4-glucanase and its physico-chemical characteristics.

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