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PRODUCTION OF CELLULASES FROM *ALTERNARIA* SP. MS28 AND THEIR PARTIAL CHARACTERIZATION

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Abstract

Cellulase production was studied after cultivation of *Alternaria* sp. MS28 in cellulose containing media. This fungus produces different component activities of cellulases, a property influenced by growth temperature. The data indicates that the production of cellulases is a genetically regulated process in the *Alternaria* sp. MS28, where endoglucanase production was triggered in the presence of CMC; β-glucosidase production by salicin; however, the presence of glucose suppressed the cellulase production. The growth and cellulase production kinetics revealed that the production of endoglucanase and β-glucosidase started in early log-phase and attained its peak in stationary phase. The higher rates of biomass production and shorter generation time were noted in SDB. The rate of endoglucanase and β-glucosidase production was higher in CMC and salicin containing medium, respectively. Partial characterization of cellulases in cell-free culture supernatant showed that endoglucanase has its optimum activity at 50°C and pH 6.0; while β-glucosidase at 45°C and pH 4.5. The melting temperature for endoglucanase and β-glucosidase were 57° and 59°C, respectively. Activity of both the enzymes was slightly affected by the presence of EDTA indicating that these enzymes may not require divalent metallic ions for their activity.

Introduction

Cellulases can convert world's most abundant biopolymer, 'cellulose' into reducing sugars and used in many biotechnological applications like biofuel production etc., (Bhat, 2000). Fungi are the main natural agents of cellulose degradation; are widely distributed in nature and used for commercial production of cellulases. Most of the fungi elaborate one or more cellulolytic enzymes including endoglucanase, exoglucanase and β-glucosidase (Bhat, 2000). Endoglucanase randomly hydrolyses the polymer to generate oligomers which in turn are acted upon by exoglucanase to release cellobiose and some other oligosaccharide residues. Finally, β-glucosidase can hydrolyze cellobiose or short chain oligosaccharides into glucose (Bhatia *et al.,* 2002). The synergistic action of these enzymes, therefore, is responsible to degrade cellulosic biomass into its completion (Davies & Coleman, 1984).

Amongst fungi, species of *Trichoderma* and *Aspergillus* are well known for cellulolytic potential (Lynd *et al.,* 2002). The cellulase preparation of *Trichoderma reesei* has a major limitation of being devoid of β-glucosidase activity (Rosgaard *et al.,* 2006). Therefore, either recombinant strains of *Trichoderma* are used commercially, or other microbial strains are being screened (Gusakov, 2011) and mutagenized (Javed *et al.,* 2011) that can express all of the component cellulolytic enzymes in appropriate proportions.

Alternaria species has long been reported for their cellulolytic potential (Logan & Siehr, 1966) and some strains had previously been mutagenized for improved productivity (Macris, 1984). It has also been proved that some of the species of *Alternaria* induce plant invasion by elaborating the cellulolytic enzymes (Eshel *et al.,* 2000); and genes encoding endoglucanases from this organism have been characterized (Eshel *et al.,* 2002). Previously, the hydrolytic potential of an indigenous strain MS28 of *Alternaria* has been reported (Sohail *et al.,* 2009a); in the present work nonetheless the production and partial characterization of cellulases are described.

Materials and Methods

Growth and enzyme production: *Alternaria* sp. MS28 was isolated from soil; identified and maintained as described earlier (Sohail *et al.,* 2009a). Growth and enzyme production studies were conducted in SDB and mineral salt medium (MSM; Mandels *et al.,* 1969) containing 1% (w/v) suitable carbon source at 35°C. Growth and enzyme production kinetics were as described earlier (Sohail *et al.,* 2009b).

Enzyme assays: Endoglucanase and β-glucosidase assays performed by incubating 0.5ml of crude enzyme preparation in 0.5ml Sodium citrate buffer (50mM; pH 4.5) containing 0.5% (w/v) CMC or salicin, at 50°C for 30 min. A filter paper strip (6x1cm) was coiled in 1 ml of reaction mixture containing 0.5 ml crude enzyme preparation in Sodium citrate buffer (50mM; pH4.5) and incubated at 50°C for one hr, for determining filter paperase activity. Cotton-cellulase activity was measured by loading 2 ml of enzyme preparation on 0.1g of cotton soaked in 100mM Sodium citrate buffer (pH 4.5), and incubating for 24 hrs at 50°C. Reducing sugars released were measured by DNS method (Miller, 1959) using glucose as standard. One international unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of glucose equivalents in 1 minute under the standard assay conditions.

Optimum temperature and pH and thermal stability: Optimum temperature for the enzyme activity was determined by performing enzyme assays at variable temperatures (40-80°C). The pH optima were determined by following the enzyme assays with an exception of using different buffers i.e., 50mM HCl-KCl buffer (for pH 1-2), 50mM Glycine-HCl buffer (for pH 2.5-3.5), 50mM sodium acetate buffer (for pH 4.0-5.5), 50mM citrate-phosphate buffer (for pH 6.0-7.0), 50mM TRIS-HCl buffer (for pH 7.5-9.0) and 50mM Glycine-NaOH buffer (for pH 9.5-10.0). Melting temperature (T_m) for all the enzymes were investigated by incubating a known amount of crude enzyme preparation at temperatures ranged between 50-80ºC for 15 minutes followed by determination of residual enzyme activity under the standard assay conditions.

Effect of metallic ions on enzyme activity: Enzyme preparation was dialyzed against 50mM Sodium citrate buffer, pH 5.0, and metallic ions or EDTA (at final concentration of 20 or 50mM) were added and assayed for endoglucanase or β-glucosidase activity.

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Results and Discussion

MS28 strain of *Alternaria* sp., was able to grow and elaborate cellulolytic activity in presence of various cellulosic substrates (Table 1). The highest IFPU and endoglucanase titers were obtained in the presence of 1% (w/v) CMC. Aslam *et al*., (2010) has also mentioned about an increase in endoglucanases titer when *Trichoderma* sp., was grown in the presence of CMC indicating that it is the substrate of choice for endoglucanase production. The highest β-glucosidase titers were obtained when MS28 strain was cultivated in mineral salt medium with 1% (w/v) salicin, as sole carbon source. The levels of endoglucanase were, in general, 4-8 times higher than either filter paperase or β-glucosidase irrespective of the cellulosic substrates. It was noted that the production of cellulolytic enzymes were repressed when *Alternaria* sp., MS28 was grown in MSM with glucose or in SDB, indicating that cellulase production was under catabolite-repression as has been reported for *Alternaria solani* earlier by Sands & Lukens (1974), where a decreased production of cellulases was observed in cellulose-deficient medium.

When *Alternaria* MS28 was cultivated in CMC containing medium and incubated at different temperatures it did not exhibit each component enzyme activity at all the temperatures (Table 2). It produced higher titers of endoglucanase and filter paperase at 35°C than at other temperatures but no detectable cotton-cellulase activity at 35°C and only endoglucanase activity at 40°C.

Table 1. Selection of substrate for cellulases production by *Alternaria* MS28 under submerged conditions.

Media	Enzyme titers (IU/ml)			
	IFPU	Endoglucanase	β-glucosidase	Cotton-cellulase
MSM + 1% (w/v) CMC	0.18	0.75	0.02	0
MSM + 1% (w/v) BMC	0.12	0.56	0.03	0
MSM + 1% (w/v) PASC	0.10	0.22	0.05	0
MSM + 1% (w/v) Filter paper	0.10	0.48	0.05	0
MSM + 1% (w/v) Cellulose acetate	0.05	0.34	0.03	0
MSM + 1% (w/v) Sigma cell	0.06	0.30	0.04	0
MSM + 1% (w/v) Salicin	0.15	0.58	0.11	0
MSM + 2% (w/v) Glucose	0	0	0	0
SDB	0	0	0	0

Table 2. Effect of growth temperature on the production of cellulases.

Enzyme	Enzyme activity (IU/ml) at different temperatures (°C)		
	30	35	40
IFPU	0.028	0.174	0
Endoglucanase	0	0.74	0.167
β-glucosidase	0.036	0.016	0
Cotton-cellulase	0.19	0	0

The growth and enzyme production kinetic studies show that the production of both endoglucanase and β-glucosidase was initiated during early log-phase; attained peak in stationary phase, when grown in CMC and salicin containing medium (Fig. 1) indicating the growth-associated regulation of enzyme production. In a recent study with *Aspergillus niger* MS82, it was revealed that the volumetric productivity of cellulases was higher at 25°C than at 40°C (Sohail *et al.,* 2009b); which may be attributed to the slow growth-rate of fungus at higher temperatures. The volumetric rate and specific productivity of the endoglucanase were higher in the presence of CMC than in the presence of salicin, consequently leading to higher volumetric and specific productivity of β-glucosidase (Table 3). The results of the present study support the findings of Jang & Chang (2005) on *Streptomyces* sp., where the organism remained in lag-phase for a day followed by a 4-days long log-phase in CMC supplemented Mendel's medium; furthermore a production of endoglucanase and β-glucosidase was observed during the log-phase of growth. A similar observation was made with *Curvularia inaequalis* by Gomes *et al*., (2001). It is noteworthy that the enzyme productivity rate obtained from *Alternaria* MS28 was much lower than reported by Rajoka *et al*., (2004) where *Cellulomonas biazotea* gave 5.2 to 12.5 $IU.L^{-1}.h^{-1}$ of volumetric productivity and 12 to 36 IU g^{-1} of specific productivity of β-glucosidase in CMC supplemented MSM. The difference in these values explain the differences in the metabolic and growth characteristics of bacteria and fungi (Papagianni, 2004).

It can be further interpreted that SDB and glucose supplemented mineral salt medium favor the growth of the fungus with a shorter generation time 'g', leading to a higher production of biomass but with no detectable enzyme activity. The strain, MS28 showed relatively longer generation time and less biomass production in CMC or salicin containing media. Walter & Schrempf (1996) also described the variability in the duration of the growth phases of *Streptomyces reticuli* on glucose and other substrates.

Table 3. Effect of media on generation time (g), volumetric rate of biomass production (Q_x), volumetric rate of cellulase production (Q_p) and specific productivity ($Y_{p/x}$).

Medium	g (h)	Q_x ($mg.h^{-1}$)	Q_p ($IU.L^{-1}.h^{-1}$)		$Y_{p/x}$ ($IU.mg^{-1}$ of cells)	
			Endoglucanase	β-glucosidase	Endoglucanase	β-glucosidase
MSM + CMC	8.215	0.135	0.808	0.02	1.02	0.0314
MSM + Salicin	7.117	0.141	0.47	0.106	0.776	0.175
MSM + Glucose	6.298	0.158	0	0	0	0
SDB	4.401	0.227	0	0	0	0

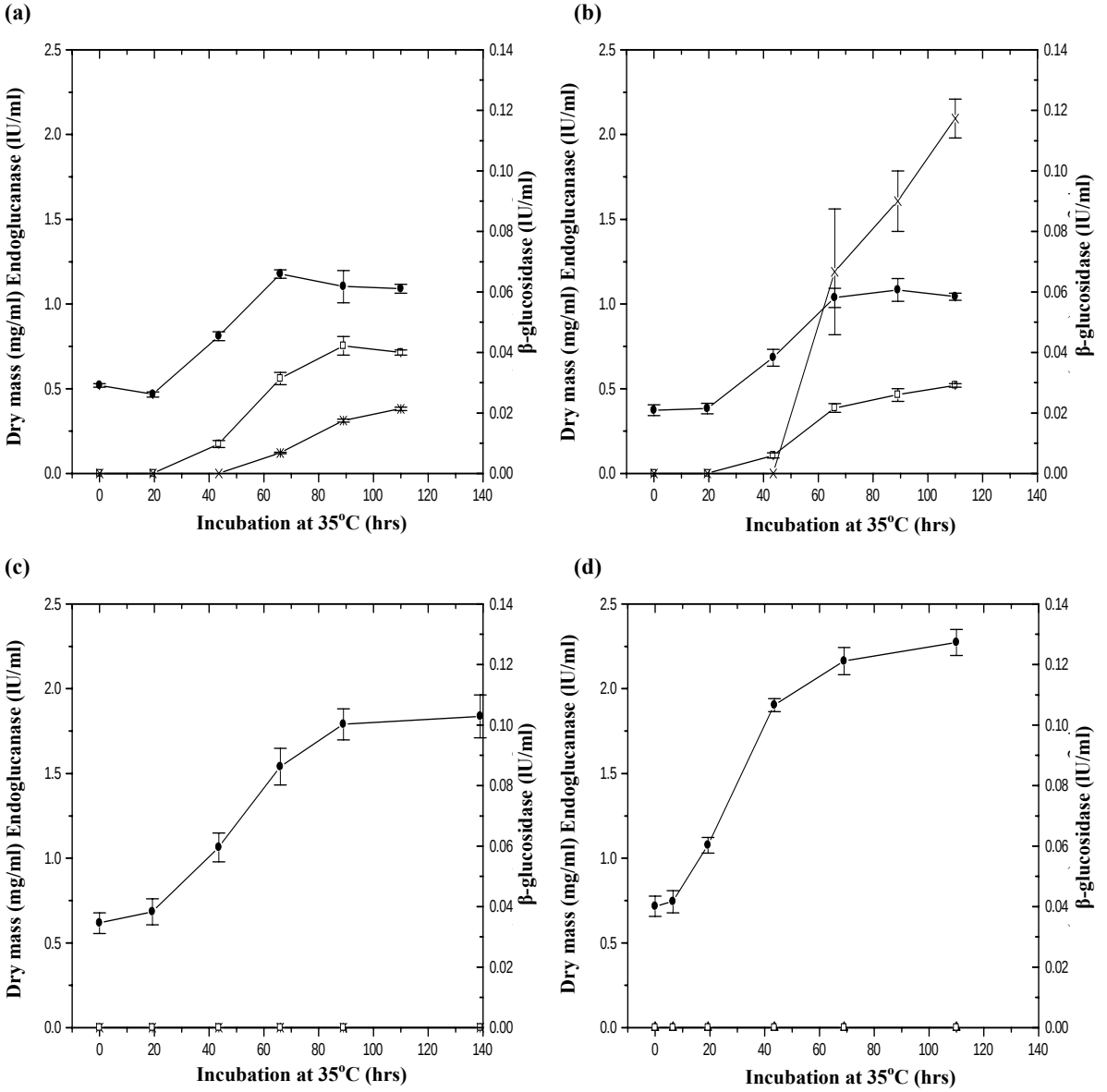

Fig. 1. Kinetics of Biomass (-■-),endoglucanase (-□-) and β-glucosidase (-x-) production in mineral salt medium containing (a) CMC (b) Salicin and (c) Glucose and in (d) SDB.

The endoglucanase and β-glucosidase activity had temperature optima of 50° and 45°C, respectively (Table 4). The activity of both the enzymes was inhibited if the reaction temperature was increased (Fig. 2). The optimum pH for endoglucanase and β-glucosidase was 6.0 and 4.5, respectively (Fig. 3). More or less similar values of optimum temperature (≥45°C) and pH (towards acidic side) were reported for cellulases from various other fungal strains (Grigorevski-Lima *et al.,* 2009; Singh *et al.,* 1990; Yun *et al.,* 2001). It is believed that cellulases showing optimum enzyme activity towards acidic side can be regarded as potential candidates for use in food industry (Bhat, 2000).

Melting temperature, in general, indicates the thermal stability of enzymes and the data suggest that it was 57°C for endoglucanase and 59°C for β-glucosidase activity (Fig. 4). Some earlier reports signify that T_m of cellulases were lower than noted in strain MS28 for instance, a T_m of 52°C for β-glucosidase (Rajoka *et al.,* 2004) and endoglucanase (Rajoka *et al.,* 2003) of *Cellulomonas biazotea*. It means that the endoglucanase and β-glucosidases of *Alternaria* sp., MS28 show relatively more heat stability and hence may find an application in food and syrup manufacturing industries, because heat stable enzymes can withstand pasteurization temperatures (Jang & Chang, 2005).

Table 4. Optimum temperature and pH for endoglucanase and β-glucosidase activity with their melting temperatures.

Enzyme	Optimum temperature (°C)	Optimum pH	Melting temperature; T_m (°C)
Endoglucanase	50	6.0	57
β-glucosidase	45	4.5	59

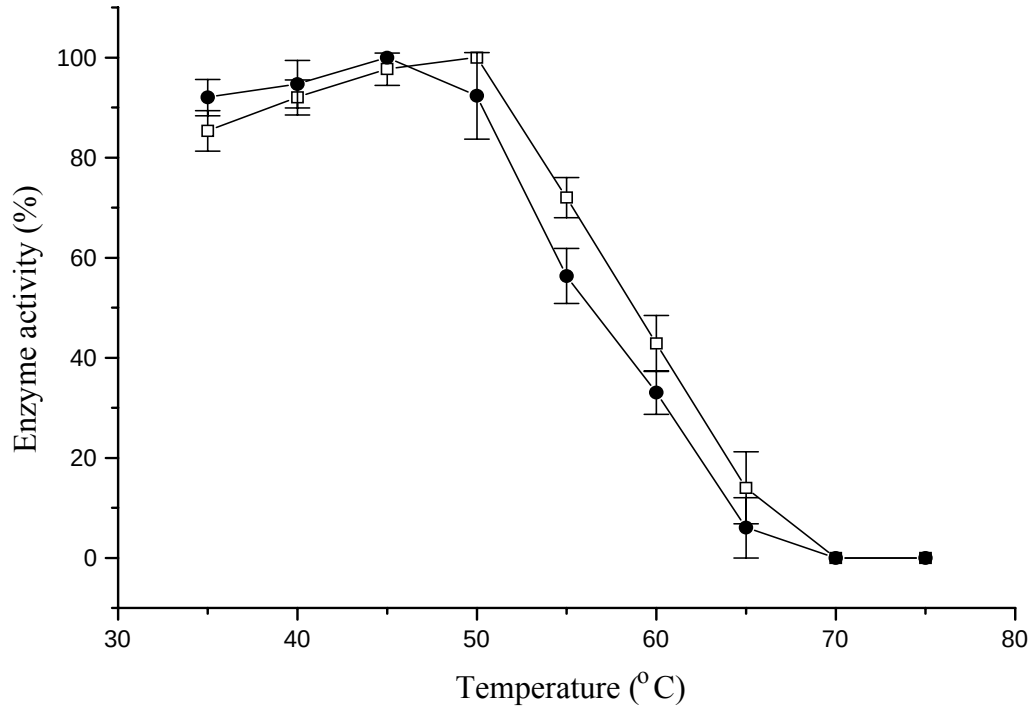

Fig. 2. Effect of temperature on endoglucanase (-□-) and β-glucosidase (-●-) activity.

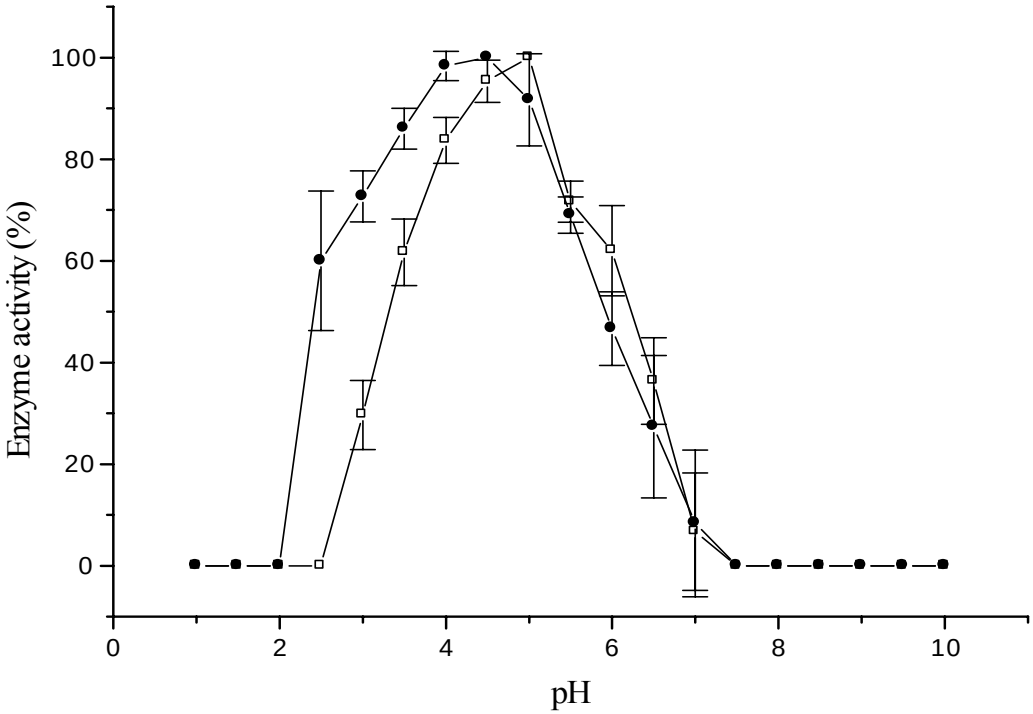

Fig. 3. Effect of pH on endoglucanase (-□-) and β-glucosidase (-●-) activity.

Metallic ions can alter the enzyme activity either by forming complexes with the substrate by making them inaccessible for enzymes (Blair, 1968) or by altering their activity through binding at specific site of enzyme. Preparations of cellulases and xylanases are generally applied on crude lignocellulosic preparations which may contain metallic-ions, in residual concentrations, thereby affecting the rate of these enzyme activities. The data revealed that activity of *Alternaria* MS28 endoglucanase and β-glucosidase remained unaltered in the presence of EDTA (Fig. 4) showing that these enzymes perhaps do not require divalent metallic ion, for activities. There are reports which also support the finding that the presence of EDTA in the reaction mixture does not modify the β-glucosidase activity of *Trichoderma harzianum* type C-4 (Yun *et al*., 2001) and *Candida peltata* (Saha & Bothast, 1996).

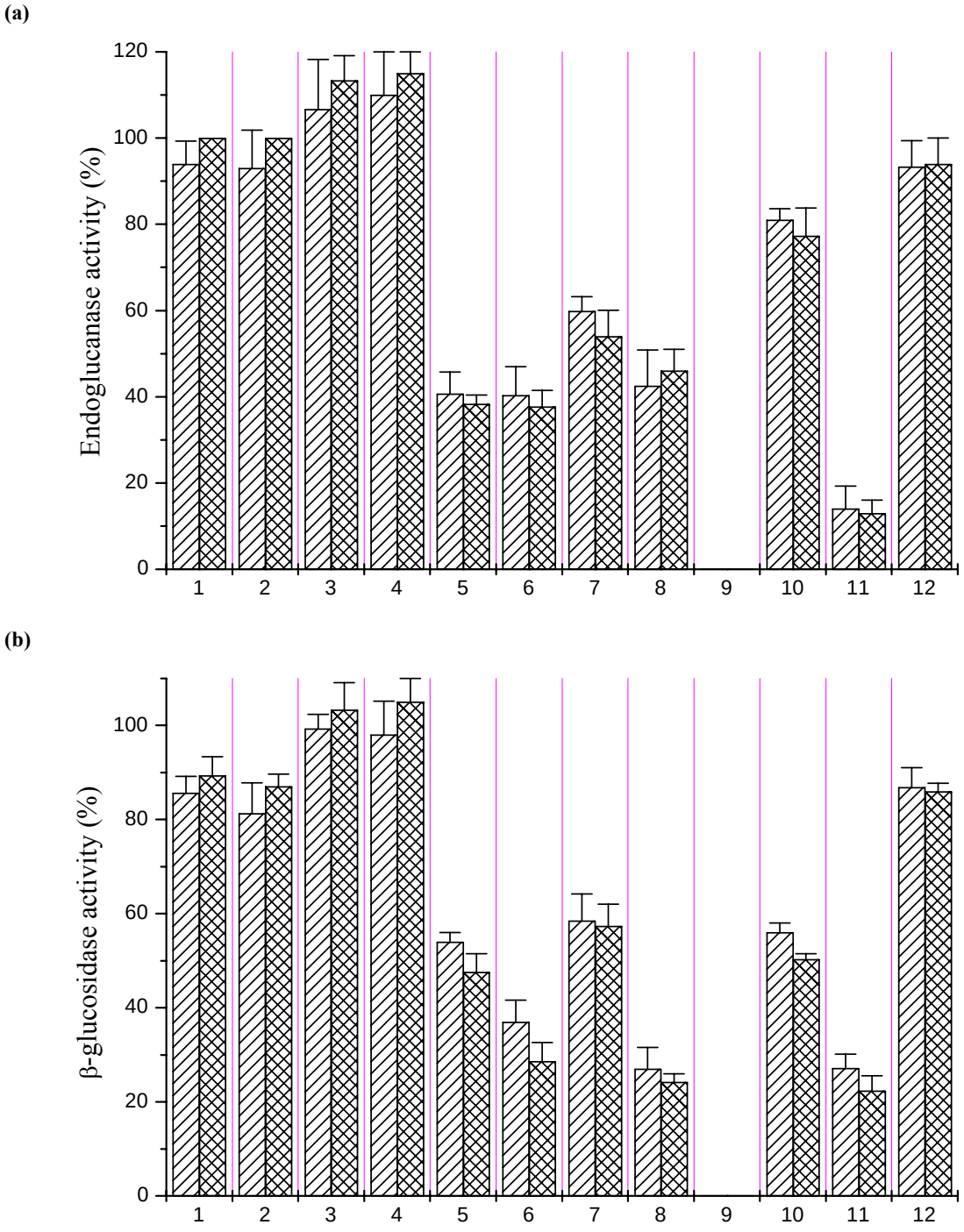

Fig. 4. Effect of metallic ions and EDTA on enzymes activity.
Metallic ions and EDTA were added at 20mM (//) and 50mM (#) to dialyzed cell-free culture supernatant followed by (a) endoglucanase and (b) β-glucosidase assays.

(1 is Na^+, 2 = K^+, 3 = Ca^{2+}, 4 = Mg^{2+}, 5 = Cu^{2+}, 6 = Co^{2+}, 7 = Ni^{2+}, 8 = Hg^{2+}, 9 = Fe^{3+}, 10 = Ba^{2+}, 11 = Ag^+ , 12 = EDTA)

Furthermore, the activities of *Alternaria* MS28 cellulytic enzymes remained unchanged in the presence of Na^+ or K^+, however, Ca^{2+} and Mg^{2+} slightly improved the enzyme activities. The presence of Fe^{3+} ion completely inhibited both the enzyme activities while other ions moderately lowered down the enzyme activity. The presence of Ag^{2+} strongly inhibited the enzyme activity which was in line with the study of Singh *et al*., (1990) where complete inhibition of *A. niger* endoglucanase was reported.

An increment in the activity of cellulases of *Pseudomonas fluorescens* was also noted when the concentration of Mg^{2+} was increased from 10 to 30 mM in the assay mixture (Bakare *et al*., 2005). Similarly, Kim *et al*., (2005) reported that the activity of cellulose, from an

alkalophilic *Bacillus* sp., was significantly affected in the presence of 1mM Fe^{3+} or Hg^{2+}. An earlier report suggests the inhibitory action of Hg^{2+} on endoglucanase activity from *Rhodotorula glutini* (Oikawa *et al.,* 1998). The data show that cellulases of *Alternaria* sp., MS28 is relatively better and may find some biotechnological applications.

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