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Full Length Research Paper

Enzymatic hydrolysis of pseudoplastic paint thickener (hydroxyethyl cellulose) by a local isolate of *Aspergillus niger*

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A pseudoplastic paint manufactured by the Protal Paint Company in Alexandria, Egypt was examined for its quality to resist microbial degradation. The paint was hydrolyzed by extracellular endoglucanase of a local fungal isolate. The main hydrolysed component was the hydroxylethyl cellulose (HEC) thickener. The fungal isolate was identified as *Aspergillus niger* which showed a DNA monomorphism of 86% to *A. niger* ATCC 16404 by RAPD. A Plackett–Burman design was performed to determine the factors influencing endoglucanase activity. The analysis revealed the significance of negative effect of glucose concentration on endoglucanase specific activity.

Key words: Hydroxylethyl cellulose, *Aspergillus niger*, endoglucanase, Plackett-Burman.

INTRODUCTION

Paints contain a wide range of organic and inorganic constituents and provide different ecological niches that may be exploited by a large variety of microbial species (Cifferi, 1999). Characterization of the microbial flora present on paints has been limited to select groups of microorganisms. Surveys have often been limited to fungi (Bianchi et al., 1980; Tiano and Gargani, 1981; Agrawal et al., 1988; Guglielminetti et al., 1994), bacteria (Lazar, 1971; Giacobini et al., 1988; Altenburger et al., 1996; Rolleke et al., 1998), or cyanobacteria and eukaryotic algae (Ortega et al., 1993; Arino et al., 1996).

Hydroxyethyl cellulose (HEC) is a nonionic, water-soluble polymer that can thicken, suspend, bind, emulsify, form films, stabilize, disperse, retain water, and provide protective colloid action. It is readily soluble in hot or cold water and can be used to prepare solutions with wide range of viscosities and has long been recognized as an outstanding thickener for both interior and exterior latex paints. Paints thickened with HEC are pseudoplastic (Flick, 1991).

HEC undergoes decomposition by the action of cellulases (Petrov et al., 2007). Cellulase preparations are able to decompose natural cellulose as well as modified celluloses such as carboxymethyl cellulose (CMC) or HEC (Buchholz et al., 1983). One of these cellulases is β-1,4-endoglucanase (1,4-β-D-glucan 4-glucanohydrolase) which cleaves internal β-1,4-glycosidic bonds (Kaur et al., 2007). Endoglucanases degrading HEC were described in several fungi (Mansfield et al., 1998). The fungus *Aspergillus niger* is the main source of industrial cellulases. β-1,4 endoglucanase is the major component of *A. niger* cellulase (Khademi et al., 2002). Strains as *A. niger* 16404, *A. niger* 9172, *A. niger* 9029, and others produce endoglucanases (Onsori et al., 2005) which have been assayed using HEC (Bailey, 1988) or CMC as a substrate (Miller, 1959).

The aim of this work is to isolate and identify a local fungal isolate capable of hydrolyzing HEC in the pseudoplastic paint. It is also aimed to determine whether the enzyme is expressed extracellularly or intracellularly and study the factors affecting enzyme activity.

MATERIALS AND METHODS

Fungal strain

A. niger A.E was locally isolated from an infected residential kitchen ceiling. It was identified by the Fungal Center of Assuit University, Assuit, Egypt.

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Paint

The whole paint and its components (HEC thickener and polyvinyl alanine polymer), were manufactured and kindly provided by Protal Paint Company of Amria, Alexandria, Egypt.

Fungal isolation and cultivation

Fungal colonies grown on a kitchen ceiling were removed by scraping, and grinding the infected area, then spreading on a solid minimal medium MM (0.3% Glucose, 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.05% sodium citrate, 0.01% $MgSO_4.7H_2O$, 0.1% NH_4SO_4) (Jones and Ballou, 1969). The plates were incubated at 30°C for 4 days.

The isolated colonies were purified and maintained on glucose peptone media (2% glucose, 0.2% yeast extract (Difco), and 0.5% peptone (Bacto Peptone [Difco]). All media were sterilized by autoclaving at 121°C for 15 min.

Qualitative hydrolysis tests

The hydrolysis of 0.5% of paint, HEC or Polyvinyl alanine was investigated by growing the isolated fungus on solid MM plates supplemented with each of the tested substrates. The degradation of paint or its components was detected by the appearance of clear zones around the fungal growth.

RAPD analysis

After morphological identification of the experimental fungus, RAPD analysis was performed using randomly selected primers, for comparison between the identified local strain and a control strain of the same species purchased from ATCC (*A. niger* ATCC 16404). DNA was isolated from both strains using Fungal DNA kit (Omega, Comp. USA). Analysis was performed using RAPD primers (1- GCGACATTGC; 2- CCTAAGTCCT and 3- CTAAGTACTA, provided by Amersham/Pharmacia Biotech Company). PCR reaction conditions consisted of an initial denaturation for 60 s at 90°C and for 90 s at 95°C, then subjected to 45 cycles by using the following temperature profile: 95°C for 30 s, 36°C for 1 min and 72°C for 2 min. Samples were then incubated at 72°C for 10 min (Pafetti et al., 1996). PCR amplified DNA fragments were separated by electrophoresis in agarose gels (1.5%) and stained with ethidium bromide for visualization.

Measurement of enzyme activity

The fungal strain was allowed to grow in 100 ml MM amended with 0.5% HEC and incubated for 4 days at 30°C under static conditions. Cells were harvested by centrifugation and supernatant was used for determination of extracellular enzyme activity. Cell pellets were suspended in 2 ml of treatment buffer (0.125 M Tris HCL pH = 6.8, 5% SDS, 20% glycerol, 10% mercaptoethanol) and disrupted by sonication. Cell preparations were centrifuged to obtain clear supernatants of fungal lysates containing soluble intracellular proteins.

Intracellular and extracellular endoglucanases were assayed using HEC as a substrate (Bailey, 1988). The enzyme assay mixture contained 0.5 ml of 0.5% (w/v) HEC, prepared in 0.1 M citrate buffer, pH 4.8 and 0.5 ml of fungal lysate in case of intracellular enzyme and 0.5 ml of culture filtrate in case of extracellular enzyme. The reaction mixture was maintained at 50°C for 30 min. Reducing–sugar levels achieved as a result of enzyme activity were determined as glucose reducing equivalents by the dinitrosalicylic acid (DNS) method (Miller, 1959). Controls were prepared with 10 min boiled enzyme. One unit of enzyme activity (U) was defined as the production of 1 mg reducing sugar per milliliter per hour. Specific activity was defined as U/ mg biomass.

Electrophoretic analysis of proteins

Intracellular protein profile was assayed by SDS- polyacrylamide gel electrophoresis (SDS-PAGE). Samples of 50 ul containing 5 mg/ml protein were prepared, and electrophoresis was carried out according to Sambrook et al. (1989). The stacking gel was 5% (w/ v) acrylamide and resolving SDS containing gel was 10% (w/v) acrylamide. To visualize the separated protein bands, the gel was stained in Coomassie blue in 50% methanol and 10% acetic acid. The gel was then transferred to 10% acetic acid to make the gel background clearer and was photographed.

Plackett-Burmann design

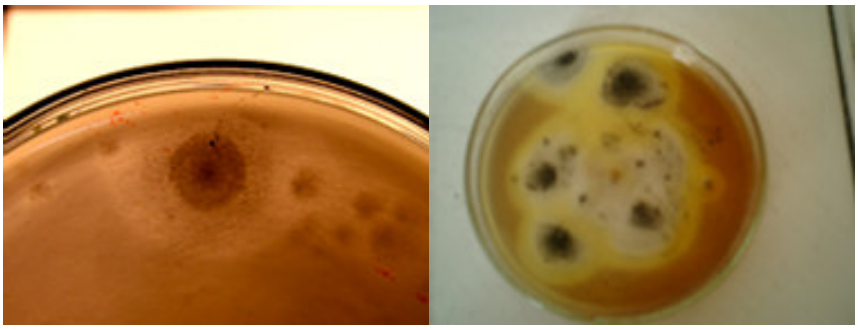
The effect of MM components, including 5 g/l HEC as an inducer on endoglucanase activity was studied by applying the Plackett-Burman experimental design (Plackett and Burman, 1946). In this experiment, seven factors (medium component + HEC) were screened in eight combinations organized according to the Plackett Burman matrix. A 50% increase of the original component level is represented by the "+" sign while 50% decrease of the original component level is represented by the "-" sign. The main effect of each factor was determined using the following equation:

$$Exi = (\sum Mi+ - \sum Mi-)/N$$

Where Exi is the variable main effect, Mi+ and Mi- are the sum of the recorded activity results of endoglucanase by trials which contain + and – levels of independent variables (xi), respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the + level of this variable is nearer to optimum endoglucanase specific activity while a negative sign indicates that the - level of this variable is nearer to optimum. Using Microsoft Excel program, statistical t-values for equal unpaired samples were calculated for the determination of variable significance.

RESULTS AND DISCUSSION

Indoor wall paintings are widely recognised as a favourable environment for microbial biofilms in general and for microscopic fungi in particular (Gorbushina and Peterson, 2001). Drastic changes in appearance, color, and structure occur on indoor wall painting, which has large surface exposed to the atmosphere and prone to biofilm development. High temperature and humidity influence the development of biofilms in this indoor environment (Gorbushina and Peterson, 2001). Microbial induced decay processes as related to the original wall painting has already received much of attention (Guglieminetti et al., 1994). The local strain of *A. niger* AE isolated in this study was capable of hydrolyzing the pseudoplastic paint as a whole and HEC forming clear zones around colonies in agar plates of MM supplemented with either 0.5% pseudoplastic paint or 0.5% HEC thickener (Figure 1A and B). The poly vinly alanine polymer was not hydrolysed by the isolated strain (data not shown). The results indicate that the only component which is hydrolysed in



(A) (B)

Figure 1. Enzymatic hydrolysis of whole plastic paint (A) and HEC thickener (B) by *A. niger* AE as expressed by the clear zones surrounding the colonies.

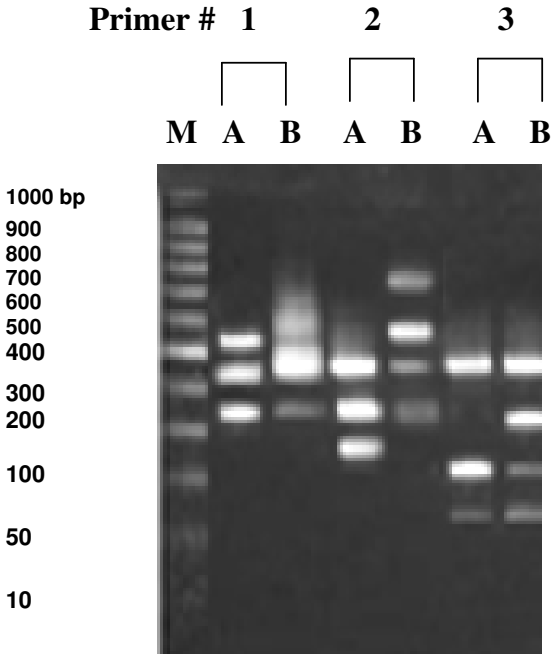


Figure 2. RAPD showing the degree of DNA monomorphism and polymorphism between *A. niger* 16404 (A) and the local strain of *A. niger* AE (B).

the paint was HEC thickener. Zone of clearance was successfully used to detect polymer hydrolysis (Holding and Collee, 1971).

Identification and RAPD analysis

Based on its morphological features, the selected fungal isolate was initially identified in the Fungal Center in Assuit University, Assuit, Egypt as *A. niger* and was designated as *A. niger* AE to be stored among out laboratory stock cultures. RAPD analysis was performed to compare genetically between our isolate and a reference strain *A. niger* ATCC 16404.RAPD analysis is generally used to distinct between members of the same species (Williams et al., 1990).

Three primers were used to examine the DNA sequence relationship between *A. niger* ATCC 16404 (A) and *A. niger* AE (B) by using RAPD (Figure 2). Primer #1 GCGACATTGC used in lanes 2 and 3 show the appearance of 2 polymorphic and 4 monomorphic bands, primer#2 CCTAAGTCCT used in lanes 4 and 5 show the appearance of 3 polymorphic and 4 monomorphic bands, while primer #3 CTAAGTACTA in lanes 6 and 7 show the appearance of 1 polymorphic and 6 monomorphic bands (Table 1). Amplified DNA bands which are the same in all individuals are called monomorphic, others are called polymorphic (Hartl and Jones, 2005). The degree of monomorphism (86%) between *A. niger* 16404 and the experimental strain *A. niger* AE was best described by primer CTAAGTACTA. RAPD analysis demonstrated that there are DNA variations even between members of the same species.

Intracellular protein profile of *A. niger* AE

Microbial endoglucanases have been described in detail (Ooi et al., 1990; Sharma et al., 1991; Gilkes et al., 1991). They catalyze endohydrolysis of 1,4-β-D- glycosidic linkages in cellulose and cellulose derivatives such as CMC and HEC and cut randomly at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends (Lynd et al., 2002).

The initial qualitative paint hydroylsis results indicated that the enzyme was extracellular. Although different Aspergillus species are known for their over expression of extracellular endoglucanases (Onsori et al., 2005), intracellular endoglucanases were isolated from other fungal species (Salohermo et al., 1994).

A. niger AE was grown in MM liquid culture in presence and in absence of HEC. Intracellular proteins were isolated and analysed on SDS PAGE as shown in Figure 3 which show the existence of additional intracellular pro-

Table 1. Analysis of RAPD showing the degree of polymorphism and monomorphism.

Primer	No. of amplified bands	Polymorphic bands	Monomorphic bands	% polymorphism	% of monopmorphism
1-GCGACATTGC	6	2	4	33	66
2-CCTAAGTCCT	7	3	4	43	57
3-CTAAGTACTA	7	1	6	**14**	**86**

Table 2. Plackett Burmann design: Arrangement of the seven factors and the effect of medium components on endoglucanase activity and specific activity in *A. niger* AE.

Trials	HEC	Glucose	K_2HPO_4	KH_2PO_4	Nacitrate	$MgSO_4$	$(NH_4)_2SO_4$	Activity (U)	Specific activity (U/mg biomass)
1	-	-	-	+	+	+	-	0.28	292
2	+	-	-	-	-	+	+	2.24	2435
3	-	+	-	-	+	-	+	2.43	528
4	+	+	-	+	-	-	-	1.21	417
5	-	-	+	+	-	-	+	0.67	2233
6	+	-	+	-	+	-	-	1.31	3453
7	-	+	+	-	-	+	-	1.1	423
8	+	+	+	+	+	+	+	0.43	134

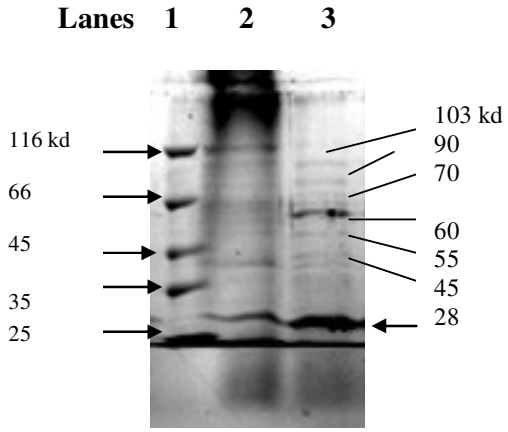


Figure 3. SDS-PAGE analysis showing the intracellular protein profile of *A. niger* AE in absence of HEC (lane 2) and presence (lane 3) of HEC as inducer in the medium. Lane 1 is the molecular weight marker.

teins with different sizes (103, 90, 70, 60, 55, 45 kd) in presence of HEC. A high expression of a protein of 28 kDa was evident in the presence and absence of HEC. Also a 60 kd protein was highly expressed in the presence of HEC only.

Interestingly, although there were expressed inducible proteins in presence of HEC than in its absence, no detectable activity was recorded, revealing that the enzyme was not synthesized intracellular. The additional proteins found in response to HEC, on SDS gel might be stress proteins that have been expressed only in the presence of the inducer. A group of stress proteins are heat shock proteins which are expressed in all cells in all forms of life and in a variety of intracellular locations. They are expressed in vast quantities under normal conditions and their expression can be powerfully induced to much higher levels as a result of heat shock or other forms of stress, including exposure to toxins, oxidative stress, glucose deprivation, etc (Srivastava, 2002).

Another explanation on the additional proteins on SDS gel, is that they might be related to the regulatory proteins which act as a powerful activators of several cellulases or hemicelulases in A. niger, A oryzae and their homologues in other filamentous fungi (Dilip and Arora, 2004).

Plackett-Burmann design

Statistical methods have been applied to bacterial culture (Ahuja et al., 2004) and animal cell culture (Ganne and Mignot, 1991) and recently to fungal cultures (Dakhmouche et al., 2005; Lotfy et al., 2007). The effect of different factors affecting endoglucanase specific activity in *A. niger* AE was determined using Plackett-Burmann design as described in the materials and methods section. The factors included the minimal medium components together with the addition of the thickener HEC. Eight combinations, endoglucanase activity, specific activity records and calculations are shown in Tables 2 and 3. Table 2 shows that trials 2, 5 and 6 yielded the highest endoglucanase specific activity. Table 3 and Figure 4 show that the increase of both K_2HPO_4 and $(NH_4)_2SO_4$ and the decrease of HEC,

Table 3. Statistical analysis of Plackett- Burman design relative to endoglucanase specific activity in *A. niger* AE.

Parameter	HEC		Glucose		K_2HPO_4		KH_2PO_4		Na citrate		$MgSO_4$		$(NH_4)_2SO_4$	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Specific activity (U/mg biomass)	2435	5292	528	292	2233	292	292	2435	292	2435	292	528	2435	292
	417	528	417	2435	3453	2435	417	528	528	417	2435	417	528	417
	3453	2233	423	2233	423	528	2233	3453	3453	2233	423	2233	2233	3453
	134	423	134	3453	134	417	134	423	134	423	134	3453	134	423
Mean	1609	2119	376	2103	1561	918	769	1710	1102	1377	821	1658	1333	1146
Main effect	-510		-1727		643		-941		-275		-837		187	
t-value	-0.37		-2.6		0.68		-1.1		-0.28		-0.92		0.19	

$t_{\alpha\ 0.05} = 2.44$

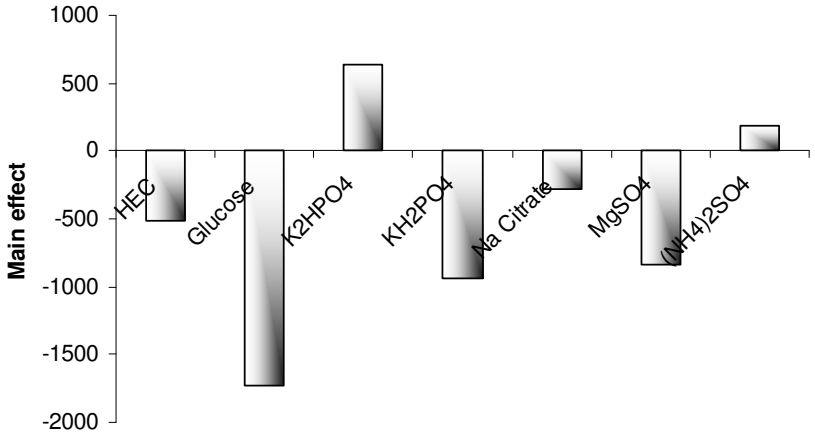


Figure 4. Main effect of the experimental medium components on endoglucanase specific activity.

glucose, MgSO4, Na-citrate and KH_2PO_4 had postive effects on endoglucanase specific activity. According to the calculated t-values, the negative effect of glucose concentration was significant at $\alpha = 0.05$.

Although Nakari-Setala and Penttila (1995) previously showed that glucose acts as good inducers to form endoglucanse, the statistical analysis in this work revealed that the decrease in glucose (- level) was significant for endoglucanase specific activity in *A. niger* AE. Glucose might act as a good growth initiation factor, after which its catabolic repression effect might take place. The extracellular endoglucanase synthesis is cyclic dependent and therefore subject to catabolite repression by soluble sugar accumulation (Chellapandi and Jani, 2008). The decrease in HEC (- level) also exhibited extensive role to enzyme yield but not as much as glucose.

A conclusion derived from this work was that an identified fungal isolate of *A. niger* AE with 86% mono-morphism to *A. niger* 16404, was capable to hydrolyse HEC (paint thickener) by extracellular endoglucanase which is efficiently expressed in the presence of low level of glucose in culture medium. Based on the work presented in this paper, a recommendation is given to paint companies which use HEC as a thickener component among their plastic paints, to use either a polymeric thickener or to add fungal antibiotics to their products to prevent the degradation of thickener by various fungi.

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