اندازه‌گیری میزان فعالیت سلولولیتیک دسته‌ای از آنزیم‌های میکربی و بررسی کیفی محصولات هیدرولیز آنزیماتیک سلولز

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چکیده:

همگام با افزایش مداوم جمعیت جهان و به تبع آن، روند رو به رشد نیاز برای تأمین غذا و انرژی، دستیابی به شیوه‌های نوین استفاده از منابع بالقوه طبیعت، هدف عمده بسیاری از محققین علوم زیستی و بالاخص بیوتکنولوژیستها می‌باشد، سلولز، بعنوان فراوانترین ماده آلی موجود در طبیعت ، در چند دهه اخیر توجه گروه کثیری از پژوهشگران را در راستای حل مشکل فوق به خود معطوف نموده است، ساختمان شیمیایی سلولز متشکل از منومرهای د-گلوکز می‌باشد که با پیوندهای گلیکوزیدی بتا - ۱ و ۴ به یکدیگر متصل شده‌اند، نوع این پیوندها و علاوه بر آن آرایش ساختمانی ویژه سلولز موجب شده‌است که آنزیم‌های گوارشی انسان قادر به اثر هیدرولیتیک بر روی آنها نباشند. لیکن تولید یک سیستم آنزیمی موسوم به «سلولاز» توسط بیوکاتالیستهای مهم طبیعت یعنی میکروارگانیسم‌ها، راه کاربرد این پلی‌ساکارید حیاتی را هموارتر نموده‌است. جداسازی سه جزء مهم این سیستم آنزیمی یعنی «اگزوگلوتاز» «اندوگلوکاتاز» و بتا -گلوکوزیتاز » و به همراه یافته‌های جالب توجه در زمینه مکانیسم عمل کینیتیک آنزیمی تا تنظیم و شرایط مطلوب تولید آنزیم توسط میکروارگانیسم را می‌توان از موفقیتهای شایان ذکر بیوتکنولوژیستها در سالهای اخیر بشمار آورد. علاوه بر این، بهره‌گیری از شیوه‌های برتر مهندسی ژنتیک در زمینه‌های gene cloning و نیز ایجاد موتاسیون در ژنهای کدکننده این آنزیم‌ها در باکتریها و قارچ‌های مولد آنها، افق‌های روشنی را فراسوی آینده این خط تحقیقاتی قرار داده‌است.

مقاله حاضر به بحث و بررسی پیرامون یک کارپژوهشی در زمینه ارزیابی میزان فعالیت هیدرولیز سلولز توسط ۱۸ و عدد از میکروارگانیسم‌های سلولولیتیک جدا شده از میکروفلور ایران به روش آنالیز روی DNSA و همچنین مطالعه بر شناسایی محصولات هیدرولیز سلولز تجارتی توسط این میکروارگانیسم‌ها در مقایسه با میکروارگانیسم شاهد Trichoderma reesie 383.78 می‌پردازد.

کلید واژه‌ها: ۱ـ آنزیم سلولیتیک ۲ـ قارچ هوازی ۳ـ تریکودرما دئیزی ۴ـ کروماتوگرافی لایه نازک

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QUALITATIVE AND QUANTITATIVE INVESTIGATION OF CELLULOLYTIC ENZYMES PRODUCED BY SOME AEROBIC FUNGI ISOLATED FROM MICROFLORA OF IRAN

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ABSTRACT

The cellulolytic activities of eighteen aerobic fungi, isolated from the microflora of Iran, with respect to Avecil were compared with the cellulolytic activity of Trichoderma reesei, CBS 383.78. The data indicated that the majority of the isolated microorganisms were capable of hydrolysing Avicel in the solid agar media to a better extent than T.reesei. However, the hydrolylic activities of the same test microorganisms in the liquid media containing Avecil were less than the relevant activity of T.Reesei. Thin-layer chromatography analysis of the products formed by each fungi from whatman filter paper (no. 1) at 50 °C for two hours of hydrolysis, were mostly consisted of small-size oligosaccharides, while the corresponding products formed by T.reesei have higher molecular weights.

Key Words: *1) Cellulolytic Enzyme 2) Aerobic Fungi 3) Trichoderma reesei 4) Thin-layer chomatography*

INTROUCTION

Degradation of cellulosic materials which constitute the major renewable carbohydrate sources, represent an important aspect of the carbon cycle within the biosphere. In this respect, wide range of research has been conducted to find efficient and economical methods for pretreatment of cellulotic biomass to remove lignin and to loosen up the crystalline structure of cellulose to more amorphous states [1-6]. These various pretreatment approaches make the cellulotic material more amenable to the processes of hydrolysis, in particular, to enzymatic methods of hydrolysis.

Great effort has been devoted to finding cellulolytic microorganism that produce enzymes useful in saccharification of plant materials. Aside from promising aerobic and anaerobic bacteria[7-9], some investigation has been conducted for finding potential aerobic and/or and anareobic fungi with

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different abilities to solubilize a wide variety of cellulosic substances [10-14]. The range of research topics includes: 1) screening the microflora for hyperproducing microbial strains, and/or strain development through mutations and gene cloning [15-17]; 2) optimizing the methods of enzyme production [18-20]; 3) optimizing the enzymatic hydrolysis rates. and finally, 4) developing methods for enzyme recycling to decrease the high cost of enzymatic processes [21-22]. Success in finding powerful cellulolytic enzymes can certainly assist the formulation of some sort of digestive aids. In addition, trasgenic animals may be created with the capability of secreting protease resistant cellulolytic enzymes in their gastrointestinal tract. Some progress has already been reported in this field [23,24]. Obviousely, full success in this respect awaits the discovery of more ideal cellulolytic enzymes.

Our reports deals with the comparison of the cellulolytic activities of some aerobic fungi isolated from the microflora of Iran and the qualitative comparison of their relavent hydrolytic producets, by TLC technique, with the cellulolytic activity of trichoderma reesei.

MATERIALS AND METODS

Materials:

Chemical reagents, the best grade available, were purchased from Aldrich (Gilingham Dorset, UK) and Sigma (St. Louis, Mo., USA) and were used without further purifications. Filter paper no.1 was obtained from whatman Col. (Maidstone, UK). Reagents of microbial culture media such as yeast extract, peptone, Agar, Sabouraud Dextrose agar, were from Difco (Michigan, USA). Distilled water has been used throughout the investigation. Avicel was purchased from Merck (F.R. Germany).

Methods

Substrate preparation-Avicel (10 gram) was gradually added to 75 ml of phosphoric acid (85%). The mixture was kept in ice while stirring slowly for one hour, and then it was added to four liter of cold-distilled water and let to stand in the cold room for 30 min[25]. The top water layer was decanted and one liter of fresh cold water was added. After mixing, the solution was centrifuged at 3000 rpm for 10 min. The water washing was repeated three more times. The cellulose pellet was washed twice using sodium bicarbonate solution (1%) and once more with water. The final cellulose pellet was dissolved in a measured quantity of water and mixed well to get a homogeniouse suspension. The substrate concentration was determined by lyophilizing an aliguot of it and measuring the powder. Sodium azide was added to the substrate stock solution, 0.1% (w/v), to prevent microbial growth while in the refrigerator for long period of

time. aliquots of the substrate solution were dialysed against distilled water befor using them in broth preparations.

Solid state enzyme production-The isolated fungi, were cultured on Sauboraud Dextrose Agar (SDA) slants and kept for five days at 26 °C. A microbial sample of each inoculated SDA tube was transfered to a culture tube (1.8 x 20cm) containing 10 ml of the culture medium with the following composition(g/lig): $(NH_4)_2PO_4$,0.8;KH_2PO_4, 0.6; K_2HPO_4,0.4; $MgSO_4$. $7H_2O$, 0.5; $FeSO_4.5H_2O$, 0.01; $ZnSO_4$. $7H_2O$, 0.04;$MnSO_4$, O.O25; $CaCl_2$, 0.055; $CoCl_2.6H_2O$, 0.001; yeast extract,1; Thiamine. HCl, 0.1; Agar, 1.5, and swollen cellulose (walseth cellulose) 2, dried weight. The pH of the medium was adjusted to 60.0 After boiling the medium, it was divided into tubes, 10 ml per tube; autoclaved and let the tubes to solidify in vertical state. The inoculated tubes were kept at 26 °C for 30 days. the progress of clear zone beneath each microbial mass was under our inspection during the 30 days.

Liquid state enzyme production- For prepara -tion of spore suspension, each microbial isolate was cultivated on a slant of the medium described before. The slants were kept for 7 days at 26 °C. Two ml of autoclaved slaine solution (0.9% NaCl) was then added to each slant, and with the help of an sterilized rod, spores were suspended in the solution. One ml of each spore supension was then added to 20 ml of the preculture medium composed of (g/lit): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.3; $CaCl_2$. $2H_2O$, 0.4; urea, 0.3; peptone, 1; tween 80, 0.2; $FeSO_4$. $7H_2O$, 0.005; $MnSO_4$. H_2O, 0.002; $CoCl_2$, 0.002; $ZnSO_4$. $7H_2O$, 0.014; xylose, 10; pH=5, befor autoclaving the solution [26].

The inoculated media were kept in a shaker incubator at 26 °C with a shaking speed of 130 rpm. After three days of growth, 2ml of each inoculated medium was transferred to 100ml of the enzyme production medium, made of the same components as the preculture medium with the exception of using swollen cellulose (walseth cellulose) at the main carbon source in place of xylose.

Enzyme assay- A stripe of filter paper (1x6 cm, 50mg) was placed in each tube containing 0.5 ml of each centrifuged broth, taken at different time intervals from the inoculated production medium[27]. To each tube 1 ml of the cirate buffer (50 mmole, pH4.8) was added. The ralevant blank tubes were consisted of filter paper, 0.5 ml of the centrifuged uninoculated broth, and 1 ml of the citrate buffer. The tubes were kept at 50°C for 1 hour. Dinitrosalicylic acid solution (dinitroqlicylic acid 4g), phenol. 0.8g, sodium sulfite, 0.2g; and Na/K tararate, 80g

in 400 ml of 1% solution of NaOH) was then added to each tube (3ml). The tubes were boiled in a water bath for 5 min. After cooling, 20ml distilled water was added to each tube. The absorbance of each tube was recorded, againts its relevant blank, at 540 nm [28]. The recorded absorbances were converted to number of µmole of glucose using the calibration graph of glucose prepared under identical conditions to the test samples. The cellulolytic activity of each broth sample was expressed in filter paper units (EPU) which corresponds to the no. of µmole of reducing sugar released from filter paper per min.

Thin-layer chromatography (TLC)-A stripe of filter paper is hydrolysed by each sample broth, as it was described before. After 1 hour of incubation at 50 °C, each centrifuged sample was lyophilized and the resultant residues were dissolved in 300µl of cirtate buffer. Samples were spotted on a silica gel plate along with glucose, cellobiose, and their mixture, as the reference compounds. The plate was developed in the solvent system of Ethanol: Butanol: Water (2:5:3), and after drying at room temperature, the plate was sprayed with a solution of Aniline (4ml), diphenylamine (4g), acetone (200ml), and H_3PO_4 (85%, 30ml) The sprayed plate was baked at 110 °C for 30 min.

RESULTS & DISCUSSION

Enzyme production in solid medium- In comparison to Trichoderma feesei (CBS 383. 78), the reference celluolytic fungus, most of the isolated aerobic fungi showed cellulolytic activities to various degrees (Table 1). As it is evident from table 1, the cellulolytic activity appeared in the medium almost after two weeks of inoculation. These observation may support the view that the specific activities of the cellulolytic enzymes is not high. In contrast, some fungi showed cellulolytic activities almost four days after inoculation, such as fungi identified by the following code numbers: A1B3C1D1[3], A20B2C1D2(12), A20B2C1D1(14), A7B1C1(3), A16B3C1D1(18), A18B2C1(14), A4B2C1D1(1), A16B2C1D1(9), A21B1C1D3(1), A15B1C1D1(2), A24B4C1D1(1), AND A16B2C1D1(9). In addition, it is evident from the data of table 1, that some of the isolated fungi have higher cellulolytic capability compared to the reference fungus, T. reesei, under the experimental conditions used.

Enzyme production in liguid state

By changing the solid media to liguid, the cellulolytic activity of most of the fungi, such as: A7B1(3), A4B2C1D1(1), A18B2C1(14), A3B4C4D2(2), A10B2C1D1(14), A21B1C1D3(1), A16B1C1D1(2), A1B1C1D1(18), A18B1C1D3(11), A20B2C1D2(2), were decreased considerably with respect to T. reesei (Figure 1). On the other hand some

Table 1: Solid state hydrolysis of walseth cellulose by the isolated aerobic fungi. The deepth of clearance (DC) in the Agar layer is expressed in millimeter.

Tube	microorganism	Depth of clearance (mm) at different times (days)					
no.	code	4	7	14	21	28	35
8	A1B3C1D1(3)	2	3	7	7	7	7
1	A2B2C1D2(14)	0	0	8	8	8	8
10(2)	A6B3C1D1(11)	0	1	4	6	8	8
12	A1B1C1D1(18)	0	10	14	20	20	24
11	A18B1C1C3(11)	0	10	14	20	20	24
14(1)	A20B2C1D2	3	6	11	11	12	12
16	A7B1C1(3)	4	6	12	12	14	16
19	A20B2C1C1(14)	0	0	0	0	0	0
24(2)	A16B3C1D1(18)	3	5	10	10	10	10
26(2)	A4B2C1D1(1)	5	8	12	13	17	20
26(1)	A18B2C1(14)	5	7	12	14	17	20
31	A3B4C4D2(2)	0	5	7	7	13	17
35	A10B2C1D1(14)	0	0	12	13	13	17
24(1)	A16B2C1D1(9)	0	3	5	5	6	7
36	A21B1C1D3(1)	4	6	11	13	16	18
43	A15B1C1D1(2)	3	5	9	10	13	13
37	A7B1C1D1(1)	0	3	8	9	10	11
22	A24B4C1D1(1)	0	0	0	0	0	0
23	T.reesei	2	3	7	7	7	8

fungi such as A20B2(14), with no apparent cellulolytic activity in solid state, partially, hydrolysed swollen-cellulose in liquid state (Figure 2a), and some fungi, such as A4B2C1D1(1), showed higher cellulolytic activity compared to T.reesei in both solid and liquid media (Table 1 and Figure 2b). In order to get a more accurate measure of the cellulolytic activity of the isolated fungi, the microorganisms were growth, each broth was centrifuged. the activity of each broth was determined in terms of FPA, and the mass of the centrifuged microbial pellet was recorded in mg, after drying 24 hours at 100 °C. The ratio of EPA to biomass are presented in table 2. These data clearly confirm the validity of data shown in Figure 1. From the data presented in tables 1 and 2,

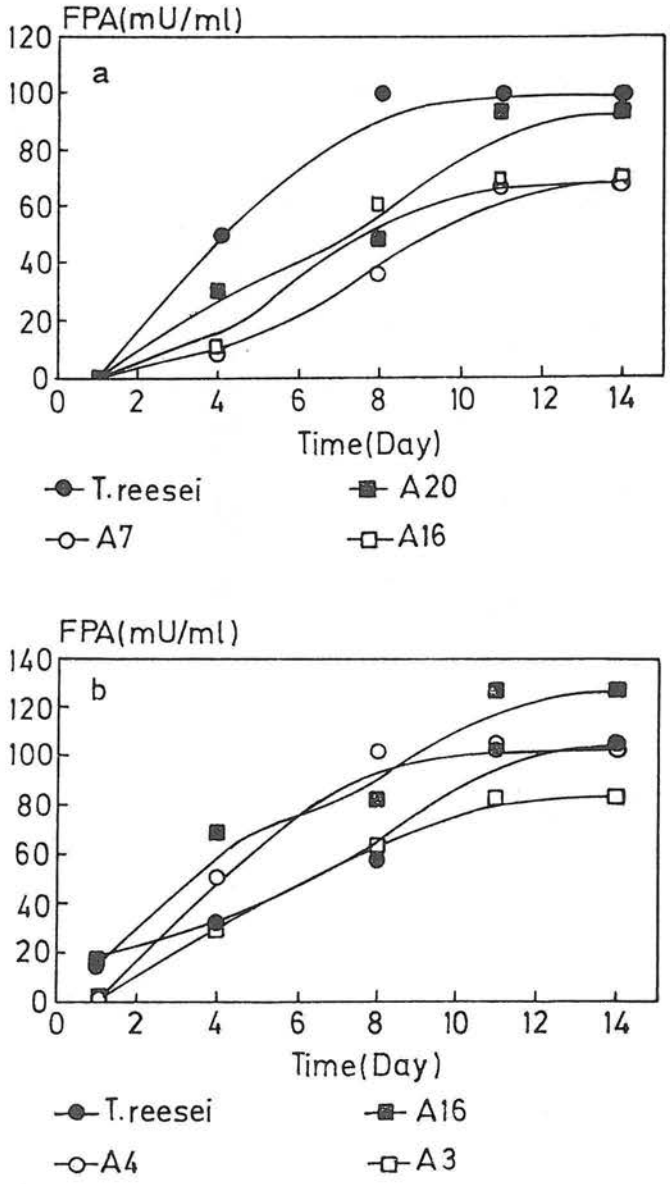

Figure 1. Filter paper activity of the isolated fungi, grown in liquid media, at different time intervals (days) with respect to the activity of T. reesei, for experimental details refer to the experimental section.

it may be concluded that the cellulolytic systems of the fungi reacting differently insolid and liquid media with respect to T.reesei, are different from the cellulolytic system of Trichoderma reesei, In order to clearify this point, the filter paper hydrolysates of each test microorganism were analysed by thin layer chromatography; the results are presented in the next section.

Qualitative comparison of the cellulolytic avtivities- The TLC Chromatogram of the 2 and 24 hours filter paper hydrolysates of each microorganism are shown in figure 2.

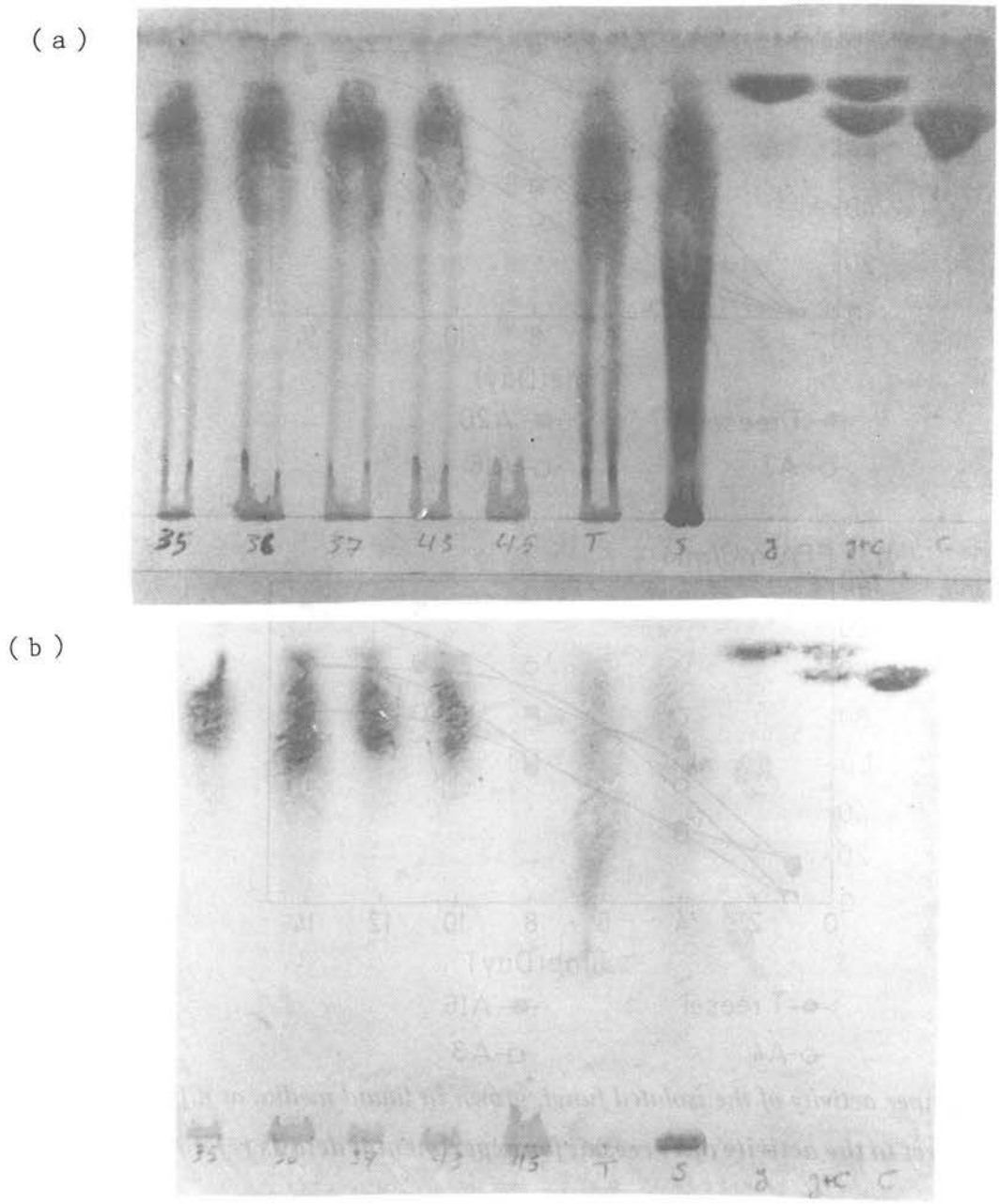

Figure 2: TLC chromatogram of two hours (a) and the twentyfour hours (b) hydrolysates of some of the isolated cellulolytic fungi; the numbers refer to the tube numbers; g, glucose; c, cellobiose; and g + c, the mixture of glucose and cellobiose. For experimental details see material and method section.

the range of R_f values of the products formed in 2 or 24 hours of hydrolysis are presented in table 3. As it is evident from this table and Figure 2a, all microorganisms along with t. reesei, produced mainly large oligosaccharides in the early process of hydrolysis. This observation supports the presence of endoglucanases in each cellulolytic system. However, after 24 hours of hydrolysis, the main products in the

hydrolysates are small size Oligosaccharides, and for some fungi, the main products are cellobiose and glucuse. In contrast to these observation, the 24-hours hydrolysate of T.reesei is composed of large to medium size oligosuccharides (Figure 2b). From these comparisons it may be concluded that cellulolytic system of T.reesei is different from those producing mainly glucose and cellobiose from swollen cellulose; or it may be stated that the ratio of endoglucanase (s)) exoglucanase (s) produced by the isolated fungi are different from the corresponding ratio in T.reesei. Further research is in progress to clearify the differences.

CONCLUSION

The isolated fungi from the microflora of Iran mostly possess cellulolytic activity with respect to filter paper and/or swollen cellulose made from Avecil.

The qualitative analysis of the cellulose hydrolysates of each test microorganism indicated that most fungi showed three enzymatic activities (endoglucanase, exoglucanase, and β- glucosidase) against swollen-cellulose. The high cellobiohydrolase and β-glucosidase activities among some of the isolated fungi, may candidate some of these microorganisms for further research regarding enzyme production for formulating suitable digestive aids. In addition, further extension of the research, particularly in the area of molecular biology may finally lead to generation of transgenic animals capable of feeding on pretreated cellulosic materials.

Table2. Filter paper activity of the isolated fungi per their relevent biomass (mU/mg). The fungi have been grown in liquid media. For experimental details see material and method section.

imocroorganism code no.	*activity cellmass (mU/mg)*
A1B3C1D1(3)	*12.00*
A24B2C1D2(14)	*6.19*
A6B3C1D1(11)	*13.33*
A1B1C1D1(18)	*1.89*
A18B1C1D3(11)	*2.85*
A20B2C1D2(2)	*12.14*
A7B1C1(3)	*37.60*
A20B2C1D1(14)	*16.24*
A16B3C1D1(18)	*34.50*
A4B2C1D1(1)	*49.02*
A18B2C1(14)	*40.80*
A3B4C4D2(2)	*23.14*
A10B2C1D1(14)	*24.29*
A16B2C1D1(19)	*7.54*
A21B1C1D3(1)	*47.33*
A15B1C1D1(2)	*3.90*
A7B1C1D1(1)	*51.50*
A24B4C1D1(1)	*3.25*
T.reesei	*50.00*

Table 3. The range of Rf of the hydrolytic product (s) formed in 2 and/or 24 hours by isolated fungi using filter paper as the sole sub-strate. For experimental details see material and Method section.

microorganis code no.	R_f	
	2 hour	*24-hour*
A1B3C1D1(3)	*0.65-0/87*	*0.60-0.90*
A24B2C1D$_2$(14)	*0.00-0.81*	*0.00-0.88*
A6B3C1D1(11)	*0.55-0.83*	*0.00-0.88*
A1B1C1D1(18)	*0.61-0.87*	*0.60-0.90*
A18B1C1D3(11)	*0.65-0.87*	*0.60-0.90*
A20B2C1D2(2)	*0.59-0.89*	*0.60-0.90*
A7B1C1(3)	*0.55-0.89*	*0.60-0.90*
A20B2C1D1(14)	*0.65-0.89*	*0.63-0.90*
A16B3C1D1(18)	*0.60-0.89*	*0.84-0.90*
A4B2C1D1(1)	*0.59-0.89*	*0.34-0.89*
A18B2C1(14)	*0.55-0.89*	*0.63-0.89*
A3B4C4D(2)	*0.60-0.89*	*0.64-0.89*
A10B2C1D1(14)	*0.60-0.89*	*0.70-0.90*
AA16B2C1D1(9)	*0.61-0.85*	*0.41-0.88*
A21B1C1D3(1)	*0.60-0.89*	*0.63-0.90*
A15B1C1D1(2)	*0.64-0.89*	*0.68-0.90*
A7B1C1D1(1)	*0.60-0.89*	*0.68-0.90*
A24B4C1D1(1)	*not determined*	*Not determined*
T.reesei	*0.53-0.89*	*0.35-0.89*

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