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SCREENING OF CELLULASE, PECTINASE AND XYLANASE ACTIVITIES AND OPTIMIZATION OF RADIAL MYCELIAL GROWTH OF TWO THERMOPHILIC FUNGI

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Abstract

The enzymatic activity of *Thermomyces lanuginosus* BPJ-10 and *Rhizomucor pusillus* BPJ-2 were observed through qualitative screening programme which was demonstrated by the hydrolysis of substrate on solid media. Both the fungi exhibited potential xylanolytic and pectinolytic activities whereas no cellulase activity was observed in *T. lanuginosus* BPJ-10 and very low cellulase activity was found in *R. pusillus* BPJ-2. The optimum temperature for mycelial growth of *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 were found to be 50 and 45°C, respectively, whereas the optimum pH for both of them were 6.5 and 5, respectively. Out of five culture media used, both the fungi showed maximum radial mycelia growth on Potato Dextrose Agar.

Introduction

A few species of fungi have the ability to thrive at temperatures between 45 and 55°C. Such fungi comprise of thermophilic and thermotolerant forms, which are arbitrarily distinguished on the basis of their minimum and maximum temperature of growth. The thermophilic fungi have a growth temperature minimum at or above 20°C and maximum at or above 50°C. The growth of thermotolerant forms have a temperature range from 20 - 55°C (Ghatora *et al*. 2006, Maheshwari *et al*. 2000). Thermophilic fungi have wide applications in production of industrially important thermostable enzymes, antibiotics and other biomolecules, predigestion of animal feed to improve the quality or digestibility of fodder, production of fuels and chemical feedstocks from wastes biomass polymers, enzymatic hydrolysis of biopolymers and single cell protein production from lignocellulosic substrates (Yang *et al*. 2006, Senthilkumar *et al*. 2005, Akhtar *et al*. 2003, Kristjansson 1989, Durand *et al*. 1984).

Interest in thermophilic fungi with thermostable enzymes mainly is due to the fact that most of the existing industrial enzyme processes run at high temperatures using enzymes from mesophilic sources (Ghatora *et al*. 2006, Bruce *et al*. 1991). There are many advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes (Yang *et al*. 2006, Kristjansson 1989).

Because of their important applications, two thermophilic fungi *Thermomyces lanuginosus* BPJ-10 and *Rhizomucor pusillus* BPJ-2 were used in this experiment with an objective to study enzymatic activity and radial mycelial growth. Hence the screening for enzymatic activity as well as some of the cultural parameters such as temperature, pH and different culture media were examined to determine the optimum radial mycelial growth of these fungi.

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Materials and Methods

Saw dust, piles of hay and soil were used for isolation of thermophilic fungi. Samples were inoculated on PDA medium (pH 6.5) and incubated at 50ºC temperature. After 4 days of incubation, different colonies with different colours and shapes were found on the culture plates. Then distinct colonies were isolated on PDA medium as pure culture. Pure fungal mycelium was transferred to agar slant for stock culture and the culture was maintained at 4°C and subcultured at 14 days intervals. Then the colonies were observed under microscope.

Enzyme activity to be demonstrated by the hydrolysis of substrate incorporated, generally as the main carbon source such as carboxymethyle cellulose (CMC) for carboxymethyle cellulase (CMCase) activity, xylan for xylanase activity and polygalacturonic acid (PG) for polygalacturonase (PGase) activity, respectively on a solid agar medium following the method of Mohiuddin (1992).

For screening of CMCase activity the fungi were grown on CMC-agar. Culture plates of 4 days were flooded with 10 ml Congo red (0.1%) solution. After 20 minutes, the dye was replaced by 5 mol/l NaCl solution and CMCase activity was revealed by a pale orange zone around the colonies.

For the screening of PGase activity, the fungi were grown on PG-agar. After 5 days of growth the plates were flooded with 1% (w/v) cetyltrimethyl ammonium bromide solution. PGase activity was observed by the formation of hydrolytic zone around the colonies after 5 - 10 min of incubation. For screening of xylanase activity fungal colonies were grown on xylan-agar media. After 4 days the plates were flooded with 96% ethanol. Xylanase activity exhibited by the formation of hydrolytic zone around the colonies after 3 - 4 hrs of incubation.

To determine the maximum vegetative growth, the fungal isolates were allowed to grow on PDA medium at different temperatures such as 40, 45, 50, 55 and 60°C. To determine the optimum pH for maximum mycelial growth, the fungal isolates were cultured on PDA mediun at pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 and 9. To investigate the effect of different culture media on radial mycelial growth, the isolated fungi were allowed to grow on different solid media such as carboxy methyl cellulose (CMC), Czapek's Dox Agar, Malt Extract Agar (MEA), Mendel's and PDA media (Hossain *et al.*1999 and Mohiuddin 1992). For each case radial mycelial growth of fungi was recorded at every 24 hrs up to 7 days.

Data obtained from radial mycelial growth diameters were analysed statistically. Means were compared by least significant difference (LSD) at 5% level of significance through one way ANOVA and DMRT by using SPSS program 11.

Results and Discussion

During this study two fungal isolates were identified, namely *Thermomyces lanuginosus* BPJ-10 (according to laboratory specimen) and *Rhizomucor pusillus* BPJ-2 (after Schipper 1978).

To isolate potential cellulase free xylanolytic and pectinolytic thermophilic fungi, a qualitative screening programme was performed by the hydrolysis of substrate in a solid agar medium. The activity can be detected around the colonies by the appearance of zones revealed either by substrate clearances or decolouration. The isolated *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 were used for determining CMCase, PGase and xylanase activity.

During screening for cellulolytic activity *T. lanuginosus* BPJ-10 did not exhibit any decolouration or hydrolytic zone around their colonies on CMC-agar plate, whereas *R. pusillus* BPJ-2 exhibited a thin pale orange zone around their colonies on CMC-agar plate (Table 1). It

indicates that these fungi do not have the ability to degrade cellulose. In case of *T. lanuginosus*, similar result was found by Akhtar *et al*. (2003).

The fungi *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 exhibited clear zones around their colonies on PG-agar plate of 3 days old culture and xylan-agar plate of 5 days old culture. It was found that the ratio of radial mycelial growth and hydrolytic zone on PG-agar was 5 : 1 and 8 : 1 for *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2, respectively.

Table 1. Enzyme activity measured by the ratio of radial mycelial growth and hydrolytic zone.

Enzymes for screening	Name of the fungi					
	T. lanuginosus BPJ-10			*R. pusillus* BPJ-2		
	Radial mycelial growth (mm)	Hydrolytic zone (mm)	Radial mycelial growth: Hydrolytic zone	Radial mycelial growth (mm)	Hydrolytic zone (mm)	Radial mycelial growth: Hydrolytic zone
CMCase	25	0	25:0	30	3	10:1
PGase	30	6	5:1	40	5	8:1
Xylanase	45	9	5:1	42	7	6:1

The ratio of radial mycelial growth and hydrolytic zone on xylan-agar was 5 : 1 and 6 : 1 for *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2, respectively (Table 1). It indicates that *T. lanuginosus* BPJ-10 exhibited higher xylanolytic activity than that of *R. pusillus* BPJ-2. All the xylanolytic fungi grown on xylan-agar medium can hydrolise the xylan (substrate) from white to transparent (Akhtar *et al*. 2003). Therefore, the result of screening programme indicated that *T. lanuginosus* BPJ-10 have higher xylanolytic and pectinolytic activities than that of *R. pusillus* BPJ-2. The result also showed that both the fungi exhibited higher xylanolytic activity than other two enzymes.

As *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 showed cellulase free xylanase and PGase producing capability, it is therefore imperative to study their radial mycelial growth on solid media for optimizing the cultural parameters. Isolated fungi were grown at different temperature, pH and culture media which lead to determine the optimum cultural condition for mycelial growth of these fungi.

Thermomyces lanuginosus BPJ-10 was found to grow well in the temperature range 45 - 55°C and the optimum temperature for the growth was found to be 50°C (Table 2). Similar results were also reported by Shing *et al.* (2003). It was observed that *R. pusillus* BPJ-2 grows well at a temperature range of 40 - 50°C and the optimum temperature for its maximum growth was 45°C (Table 2). Radial mycelial growth was increased gradually during the incubation period. Very poor growth was observed at 40 and 60°C for *T. lanuginosus* BPJ-10, while *R. pusillus* BPJ-2 showed poor growth at 55 and 60ºC. Wide range of pH (4.5 to 8.0) was observed for the growth of *T. lanuginosus* BPJ-10 and the optimum pH of its growth was found to be 6.5 (Table 3). In the meantime, *R. pusillus* BPJ-2 can grow well at pH range of 4.0 to 6.5 and the optimum pH for its growth was 5 (Table 3). Similar results were also reported for *T. lanuginosus* by Akhtar *et al.* (2003) and Shing *et al.* (2003). Gomes *et al.* (1993) and Purkarthofer *et al.* (1993) also observed optimum pH 6.5 for the maximum growth of *T. lanuginosus.*

Radial mycelial growth of the fungi followed a definite pattern in different media having different carbon sources in the present study (Table 4). In PDA and in Czapeck's Dox agar media *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2 grew very well and their culture plates were covered

Table 2. Effect of temperatures on radial mycelial growth (mm) of *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2.

Temp. (ºC)	Incubation period (days)									
	1st		2nd		3rd		4th		5th	
	Average radial mycelial growth diameter (mm)**									
	T. lanuginosus	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*
40	3.76 ± 0.14d	17.03 ± 0.26b	12.16 ± 0.44c	61.16 ± 0.60b	25.50 ± 0.28d	87.93 ± 0.06a	30.53 ± 0.29d	88.00 ± 00a	41.20 ± 0.61d	88.00 ± 00a
45	9.86 ± 0.46c	20.16 ± 0.60a	23.00 ± 0.57b	66.50 ± 1.04a	38.13 ± 0.24b	88.00 ± 0.03a	41.86 ± .46c	88.00 ± 00a	55.50 ± 0.28c	88.00 ± 00a
50	13.90 ± 0.21a	13.96 ± 0.32c	27.46 ± 0.29a	16.83 ± 0.44c	41.20 ± 0.41a	35.53 ± 0.29b	64.50 ± 0.28a	56.00 ± 0.57b	88.00 ± 0.06a	78.02 ± 0.50b
55	11.50 ± 0.28b	1.33 ± 0.33d	22.83 ± 0.60b	3.40 ± 0.31d	33.86 ± 0.46c	7.83 ± 0.60c	48.50 ± 0.28b	9.00 ± 0.57c	68.60 ± 0.30b	17.03 ± 0.58c
60	4.26 ± 0.26d	0	9.00 ± 0.57d	1.166 ± 0.44e	13.80 ± 0.61e	4.00 ± 1.15d	25.86 ± 0.59e	2.00 ± 0.58d	27.00 ± 0.57e	2.04 ±. 057d
*Mean	8.660	10.50	18.893	29.813	30.50	44.653	42.253	48.60	56.046	54.60
LSD (0.05)	0.931	1.129	1.611	1.956	1.341	1.882	1.048	1.409	1.172	1.409

*Means in a column followed by the same letter do not differ significantly at 5 % level. **Data expressed as mean value of 3 replicates.

Table 3. Effect of different pH on radial mycelial growth (mm) of *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2.

pH	Incubation period (days)									
	1st		2nd		3rd		4th		5th	
	Average radial mycelial growth diameter (mm)**									
	T. lanuginosus	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*
4.0	4.10±0.06f	6.10±0.21e	11.40±0.31g	39.40±0.34e	18.16±0.44i	82.00±0.23b	27.50±0.29h	88.00±00a	35.07±0.52f	88.00±00a
4.5	7.17±0.09d	27.13±0.46b	19.00±0.12d	66.73±0.48a	33.83±0.44g	87.66±0.33a	47.46±1.01f	88.00±00a	61.10±0.58d	88.00±00a
5.0	9.00±0.06c	29.20±0.45a	12.13±0.07f	67.06±0.29a	46.87±0.69e	88.00±0.00a	58.20±0.45d	88.00±00a	73.93±0.52c	88.00±00a
5.5	12.13±0.09b	24.86±0.35c	31.97±0.09b	63.96±0.14b	51.07±0.52d	82.16±0.44b	73.90±0.66b	88.00±00a	87.00±0.52a	88.00±00a
6.0	13.03±0.09a	23.96±0.43c	31.90±0.21b	61.96±0.26c	63.80±0.76b	79.33±0.66c	76.26±0.37a	88.00±00a	87.06±0.00a	88.00±00a
6.5	13.10±0.15a	13.83±0.17d	33.40±0.83a	49.10±0.26d	66.17±0.76a	61.46±0.29d	77.07±0.63a	75.00±.52b	88.00±0.00a	88.00±00a
7.0	9.13±0.09c	3.73±0.37f	28.07±0.52c	30.96±0.26f	53.83±0.60c	56.80±0.41e	68.17±0.52c	69.00±.57c	82.10±0.49b	85.00±.88ab
8.0	5.17±0.09e	1.96±0.09g	15.03±0.55e	21.70±0.45g	36.60±0.29f	46.20±0.91f	46.20±0.45e	62.00±.56d	60.77±0.62d	82.33±0.45b
9.0	3.03±0.09g	1.13±0.09g	10.07±0.58h	11.53±0.78h	25.46±0.29h	36.33±0.88g	37.83±0.44g	59.00±.57e	55.83±0.44e	78.33±.88c
*Mean	8.429	14.659	22.88	45.825	43.97	68.885	56.40	78.33	69.97	85.96
LSD(0.05)	0.277	0.954	1.316	1.212	1.548	1.621	1.687	1.143	1.395	3.317

*Means in a column followed by the same letter do not differ significantly at 5 % level. **Data expressed as mean value of 3 replicates.

Table 4. Effect of different culture media on radial mycelial growth (mm) of *T. lanuginosus* BPJ-10 and *R. pusillus* BPJ-2.

Culture media	Incubation period (days)									
	1st		2nd		3rd		4th		5th	
	Average radial mycelial growth diameter (mm)**									
	T. lanuginosus	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*	*T. lanuginosus*	*R. pusillus*
PDA	13.03 ± 0.14a	20.16 ± 0.60a	31.46 ± 0.74c	71.16 ± 0.60a	63.60 ± 0.30b	88.00 ± 00a	76.46 ± 0.29b	88.00 ± 00a	87.93 ± 0.06a	88.00 ± 00a
MEA	9.60 ± 0.30c	12.50 ± 0.28c	31.86 ± 0.46c	41.86 ± 2.05c	43.16 ± 0.60c	71.16 ± 0.72b	52.50 ± 0.28d	65.00 ± 5.13c	59.66 ± 0.88c	83.33 ± 1.66b
MENDEL	0.00 ± 0.00d	8.20 ± 0.28d	7.60 ± 0.30d	19.20 ± 0.41e	13.16 ± 0.60d	30.86 ± 0.59d	14.20 ± 0.41e	88.00 ± 00a	18.53 ± 0.86d	88.00 ± 00a
CZAPEK'S	9.86 ± 0.46c	14.16 ± 0.44b	52.26 ± 0.371a	55.16 ± 0.44b	71.16 ± 0.60a	80.23 ± 0.23a	80.46 ± 0.29a	82.66 ±1.35b	87.83 ± 0.16a	88.00 ± 00a
CMC	11.53 ± 0.80b	14.20 ± 0.41b	45.40 ± 0.30b	36.80 ± 0.98d	65.20 ± 0.41b	57.20 ± 0.75c	68.20 ± 0.41c	44 ± 2.30d	71.26 ± 0.63b	43.00 ± 1.52c
*Mean	8.807	13.847	33.720	44.840	51.260	67.080	58.367	73.53	65.047	78.06
LSD (0.05)	0.910	1.398	1.473	3.429	1.637	1.733	1.090	4.068	1.975	3.186

*Means in a column followed by the same letter do not differ significantly at 5 % level. **Data expressed as mean value of 3 replicates.

with fungal hyphae on 5 days of incubation. Both CMC-agar and malt-agar medium also served as good source of carbon for these fungi. However, in Mendel's medium very poor colonial growth was recorded for both the fungi.

Thermomyces lanuginosus and *R. pusillus* showed better xylanase and PGase producing capability. *T. lanuginosus* and *R. pusillus* produced maximum hydrolytic or clearing zone indicating that the activity of xylanase and polygalacturonase at 50 and 45°C respectively. The result also revealed that *T. lanuginosus* did not have any cellulase activity whereas *R. pusillus* has negligible amount of cellulase. On the basis of this finding further quantitative enzyme producing experiment has been designed by employing these fungi.

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