The Malaysian Journal of ANALYTICAL SCIENCES

ISOLATION AND SCREENING OF THERMO-STABLE CELLULASE ENZYME FUNGAL PRODUCER AT DIFFERENT TEMPERATURE

(Pengasingan dan Saringan Enzim Selulosa Tahan Haba dari Kulat pada Suhu yang Berbeza)

Noor Ashiqin Jamroo[1*], Noor Azrimi Umor[1], Kamsani [2]

[1]*Faculty of Applied Sciences,*
Universiti Teknologi MARA Negeri Sembilan, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
[2]*Faculty of Applied Sciences,*
Universiti Teknologi MARA, 40450 Shah Alam, Malaysia

**Corresponding author: noorashiqinjamroo@yahoo.com*

Received: 23 November 2014; Accepted: 27 June 2015

Abstract

Thermostable cellulase from fungi has high potential for industrial application. In this study, wild -type of fungal were isolate from different sources such as hot spring water, sea water, soft wood, rice straw and cow dung. The isolates were characterized by cultural and morphological observation. Based on morphological characteristics, the genera of all fungal cultures were identified namely *Aspergillus fumigatus*. The screening for thermostable cellulase were done using 2% carboxymethyl cellulose and congo red as an indicator at temperature 30˚C, 37˚C, 45˚C and 50˚C respectively. Out of 26 fungal isolates, only eight isolates were selected for further screening and showed the abilities to secrete cellulases by forming distinct halo zones on selective agar plate. The maximum halo zone ranging from 32mm to 35mm were obtained after 72 hour incubation at 50˚C by H2, SW1 and C1 isolates. As contrary other isolates showed halo zone range from 22 mm to 29 mm at same temperature. All the isolates showed the abilities to secrete cellulase enzyme at other temperature but lower when compared to 50˚C referred to the halo zone obtained. The SW1 isolates showed highest celluloytic index which was 2.93measured at 37 ˚C and 2.67 at 50˚C respectively.

Keywords: celluloytic index, isolation, screening, thermostable cellulose

Abstrak

Enzim selulosa tahan haba yang diperolehi daripada kulat sangat berpotensi untuk kegunaan industri. Di dalam kajian ini, kulat yang diperolehi adalah dari sumber kolam air panas, air laut, kayu lembut, jerami padi dan juga dari najis lembu. Melalui pemerhatian, didapati kulat yang di kultur adalah dari genus *Aspergillus fumigatus.* Ujian dilakukan untuk memastikan bahawa kulat yang di kultur adalah dari yang tahan haba dengan menggunakan 2% selulosa dan congo red sebagai pewarna dan di kultur pada suhu yang berbeza iaitu 30˚C, 37˚C, 45˚C dan 50˚C. Daripada 26 kulat yang di kultur, hanya lapan kultur kulat yang telah dipilih untuk pemeriksaan lanjut dan menunjukkan kebolehan untuk merembeskan enzim selulosa dengan membentuk zon halo berbeza pada plat agar medium. Zon halo maksimum antara 32 mm hingga 35 mm telah diperolehi selepas 72 jam pengeraman di 50˚C oleh H2, SW1 dan C1 diasingkan. Seperti yang di perolehi menunjukkan pelbagai zon halo dari 22 mm hingga 29 mm pada suhu yang sama. Semua kulat menunjukkan kebolehan untuk merembeskan enzim selulosa pada suhu yang lain tetapi lebih rendah berbanding 50˚C yang disebut zon halo yang diperolehi. The SW1 menunjukkan indeks cellulolitik tertinggi iaitu 2.93 di ukur pada suhu 37˚ C dan 2.67 di 50˚C masing-masing.

Kata kunci: indeks selulolitik, pengasingan, penapisan, selulosa tahan panas

Introduction

Every year, million tonnes of solid lignocellulose biomass wastes were discarded from agricultural, agro-industrial and forestry industries. Without any proper treatment these massive lignocellulose rich materials were not been

utilized satisfactorily and created problems such as causing air pollution and other environmental concerns [1]. Yet due to its heterogeneous compositions that produced from cellulose and hemicellulose could act as raw material for biofuel production.

Microbial enzymes for hydrolysis are preferred rather than others due to their specific biocatalysts such as can operate under milder reaction, not produce undesirable products and environmentally friendly as well [2, 3]. Moreover, in industrial enzymes microbial sources are preferred regarding to the short generation times of the microbes and large volumes of enzymes can be obtained within a short time. Cellulases play an important role in saccharifying cellulosic substrates for bioethanol production [4, 5].

To date, extensive research studies have been done on lowering the cost of enzyme due to production cost of these enzymes is high and accounts for 40-60% of the production cost. Therefore the utilization of abundant renewable lignocellulosic biomas as substrates indirectly reduce cellulase prices [6]. Cellulases are produced by filamentous fungi, bacteria and actinomycetes. However fungi are most preferred as source of enzyme due to higher yield and this feature makes fungal enzymes more attractive for various industrial processes. Moreover, most commercial enzymes are obtained from mesophilic fungi. Since industrial processes employ high temperatures so the thermostable enzymes are in demand [7]. Furthermore, thermophilic fungi are well known to produce thermostable enzymes with activity at high temperature, broad tolerance pH variation and resistance to denaturing agents [8].

Generally, fungi are highly diverse in nature and they have been recognized as a target for screening to find out the appropriate source of enzymes with constructive and novel characteristics [9]. The fungi species such as Trichoderma spp and Aspergillus spp have most widely been used for production of these enzymes. This study reported the isolation and screening of the thermophilic fungi for extracellular cellulase production.

Materials and Methods

Isolation of Cellulolytic Fungi

The fungal strains were isolated from different sources such as fresh cow dung collected from local poultry farm, hot spring water, rice straw, soft wood and sea water. The samples were collected and culture on potato dextrose agar (PDA) for growth and maintenance. These plates were incubate at temperature 30˚C, 37˚C, 45˚C and 50˚C and different colonies found were transferred to new potato dextrose agar (PDA) plate. Further purification was continued till pure cultures obtained. The purified cultures were transferred to potato dextrose agar (PDA) slants and stored at 4˚C for future use. The morphological characteristic of fungal strains including color of mycelia, spore and growth pattern at different temperatures as well as their vegetative and reproductive structures were observed under low power light microscope using lactophenol cotton blue staining.

Inoculum Preparation

The isolates were cultured on potato dextrose agar plates for seven days under temperature of 30˚C, 37˚C, 45˚C and 50˚C. The 1 % (v/v) of sterile Tween-80 solution was used to harvest fungi. The spores were collected by centrifugation at 4000rpm for 20 minutes. The centrifuge pellet was mixed with sterile distilled water and diluted to obtain desired spores concentration.

Qualitative Screening of Cellulase Produces Thermophilic Fungi

Cellulase producing fungi were screened on selective carboxymethyl cellulose (CMC) agar based on modified composition of Mendel basal medium. The basic medium contained 3.0g/L yeast extract, 2.0g/L KH_2PO_4, 3.5g/L peptone, 1.0g/L $MgSO_4.7H_2O$, 1.0g/L $(NH_4)_2SO_4$ and 20g/L agar. The screening medium was supplemented with 2% (w/v) carboxymethyl cellulose (CMC) as the sole carbon source for cellulolytic qualitative screening. 10µl of spore suspension from eight selected fungi of strains were inoculated into precast hole in central of CMC agar plates and incubated at temperature of 30˚C, 37˚C, 45˚C and 50˚C respectively. The hydrolysed zones were examines for every 24 hours by flooding the CMC plates with 1% congo red solution for 15 minutes and destaining with 1M NaCl solution after few minutes. The diameter of zone of decolorization around each colony was measured and the relative enzyme activity of each isolate was determined using the following formula equation 1.

$$\text{Cellulolytic index} = \frac{\text{Diameter of clearing (mm)}}{\text{Diameter of growth (mm)}} \quad (1)$$

Results and Discussion

The Isolation and Morphological of The Cellulase Producing Fungi

The fungal cultures were isolated from different sources and were grown on potato dextrose agar (PDA) at different temperature ranging from 30˚C, to 50˚C for 72 h [10, 11]. Out of 26 isolates, eight fungal isolates were purified for further investigations. The morphological characteristic of fungal isolates was performed by studying their microscopic and macroscopic characters. Lactophenol cotton blue staining was performed for better microscopic observation. The fungal isolates showed diversity in term of colony morphology, spore color as showed in Table 1 and Figure 1. From the morphological characteristics, the genera of all fungal isolates were identified namely *Aspergillus fumigatus.*

Table 1. Morphological features of cellulolytic thermophilic fungal isolates

Fungal Isolates	Configurations	Margins	Elevations	Color of mycelium	Spore color
C 1	Irregular and spreading	Wavy	Raised	Reddish creamy	Dark green
H3	Irregular and spreading	Wavy	Raised	Choc creamy	Dark green
S 2	Irregular and spreading	Wavy	Flat	Creamy	Green
H2	Irregular and spreading	Wavy	Raised	Creamy white	Green
H5	Irregular and spreading	Wavy	Flat	Reddish creamy	Green
SW1	Irregular and spreading	Wavy	Flat	Choc creamy	Dark green
R 1	Round and radiating margin	Ciliate	Hilly	Dark green	Dark green
R4	Round and radiating margin	Ciliate	Hilly	Dark green	Dark green

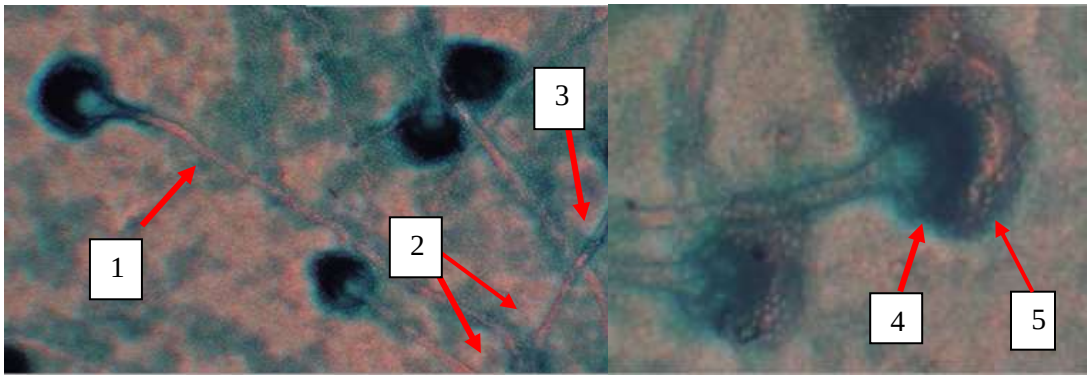

Figure 1. Microscopic feature of cellulolytic thermophilic fungal isolates. 1. Conidiophore 2. Septa 3. hyphae 4. Phialide 5. Conidia

The Screening for Cellulose Producing Fungi

Eight isolates were selected and screening for their ability to degrade cellulose. All strains were designated as H2, S2, H5, H3, R1, R4 and SW1. Table 2 showed the cellulolytic index of each isolate on selective agar .The selection of the cellulolytic fungal producer based on the diameter size of halo zone on the screening media [12, 13]. The SW1, R1 and S2 isolates produced high cellulolytic index ranging from 1.67mm to 2.67mm obtained after 72 hour incubation at 50˚C.The SW1 isolates showed highest cellulolytic index which was 2.93 measured at 37 ˚C and 2.67 at 50˚C respectively. In plate screening method, the diameter of the halo zone is useful for select strains with efficient degradation of polysaccharides such as cellulose, xylan and amylose [14]. The cellulolytic index can be used as a simple and rapid methodology to select strains within the same genus that have potential for the production of enzymes.

From the Table 3 showed that SW1 produced maximum diameter of halo zone in CMC and also presented larger cellulolytic index. The values given represent the average of measurements for 3 experiments performed independently under different conditions. The congo red test was carried out to observe the growth and measurement of the hydrolysis halo that is used for calculation of relative enzyme activity [15, 16, 17]. The halo produced by hydrolysis of cellulase is directly related to the region of action of cellulolytic enzymes which the dye only remains attached to regions where there is β-1, 4-D-glucanohydrolase bonds.

The screening method used, is advantageous because it also showed the thermostability of the isolates due to incubation at different temperature. As contrary to usual screening for cellulose, thermostability is not a main concern. This study highlighted the correlation between thermostability and enzyme activity.

Table 2. The cellulolytic index of fungal isolates

	Cellulolytic index (mm)											
	Day 1				**Day 2**				**Day 3**			
	30°C	**37°C**	**45°C**	**50°C**	**30°C**	**37°C**	**45°C**	**50°C**	**30°C**	**37°C**	**45°C**	**50°C**
H2	1.60	1.11	1.20	1.09	1.60	1.56	1.30	1.11	1.68	1.60	1.34	1.14
H5	1.29	1.17	1.50	1.17	1.54	1.33	1.60	1.20	1.57	1.50	2.00	1.32
S2	1.14	1.40	1.13	1.17	1.20	1.88	1.15	1.33	1.26	2.00	1.18	1.67
R1	1.43	1.14	1.25	1.25	1.47	1.29	1.46	1.46	1.50	1.31	1.67	1.67
R4	1.85	1.48	1.52	1.46	2.00	1.67	1.56	1.50	2.15	1.70	1.60	1.58
H3	1.52	1.14	1.10	1.36	1.52	1.34	1.75	1.36	1.74	1.29	2.00	1.50
SW1	1.60	2.67	2.47	2.47	1.64	2.87	2.53	2.53	1.68	2.93	2.67	2.67
C1	1.00	1.33	1.05	1.05	1.08	1.40	1.13	1.13	1.08	1.50	1.20	1.20

Table 3. Diameter of halos produced by the hydrolysis of CMC

Diameter of halo in CMC (mm)												
	Day 1				**Day 2**				**Day 3**			
	30°C	**37°C**	**45°C**	**50°C**	**30°C**	**37°C**	**45°C**	**50°C**	**30°C**	**37°C**	**45°C**	**50°C**
H2	25	25	30	30	27	30	32	33	28	31	35	35
H5	15	30	20	15	20	33	25	20	22	35	28	22
S2	20	20	20	20	23	22	27	22	24	23	28	23
R1	30	25	25	25	33	28	27	28	33	30	28	29
R4	25	25	20	20	30	28	25	25	32	30	28	26
H3	25	15	25	25	30	22	30	28	30	24	32	29
SW1	25	25	30	30	30	29	32	32	30	30	35	33
C1	25	25	25	25	30	30	30	30	30	30	33	32

Conclusion

It is important to find good producers of lignocellulolytic enzymes, because of its wide application [18]. These enzymes may be used in paper and pulp industries, bioethanol production, animal feed industry and others .In present study, it could be concluded that all fungal cultures possess cellulolytic activity [19, 20]. The maximum cellulolytic indexes are ranging from 1.67mm to 2.67mm. The SW1 isolates showed highest celluloytic index which was 2.93measured at 37 ˚C and 2.67 at 50˚C respectively.

Moreover, in diameter of halos produced by hydrolysis of CMC result showed the maximum clearing zone ranging from 32mm to 35mm were obtained after 72 hour incubation at 50˚C by H2, SW1 and C1 isolates. As contrary other isolates showed clearing zone range from 22mm to 29mm at same temperature. All the isolates showed the abilities to secrete cellulase enzyme at other temperature but lower when compared to 50˚C referred to the clearing zone obtained.

Acknowledgement

The author would like to express their gratitude to Universiti Teknologi Mara due to the financial support, RAGS research grant and Ministry of Higher Education (MOHE).

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